The Effect of Docosahexaenoic Acid in a Mouse Model of Neuroinflammation

by

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A thesis submitted in conformity with the requirements for the degree of Doctorate of Philosophy

Department of Nutritional Sciences
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Abstract

Several studies have shown that dietary omega-3 polyunsaturated fatty acids (n-3 PUFA) are beneficial in neurodegenerative diseases, although the mechanism of action is not agreed upon. Because most neurodegenerative diseases have an inflammatory component, it is possible that docosahexaenoic acid (DHA) is anti-inflammatory in the brain as it is known to be in several non-neural tissues. Specialized pro-resolving mediators (SPM) are metabolized from DHA and are leading candidates to explain the anti-inflammatory effects of DHA. The goal of this work was to investigate the role and potential mechanisms of action of DHA in neuroinflammation.

In our first approach, fat-1 transgenic mice had higher phospholipid and unesterified DHA levels in their hippocampi, and attenuated lipopolysaccharide (LPS)-induced neuroinflammation, compared to wildtype littermates. Feeding wildtype littermates n-3 PUFA mimicked hippocampal DHA levels and LPS-induced neuroinflammatory responses of fat-1 mice, indicating DHA is anti-neuroinflammatory whether derived from the diet or the activity of the fat-1 protein. In an attempt to further augment hippocampal DHA levels, feeding n-3 PUFA adequate mice an n-3 PUFA diet increased phospholipid but not unesterified DHA levels, and
did not attenuate LPS-induced neuroinflammation, highlighting the potential importance of unesterified DHA. Directly infusing unesterified DHA into a cerebral ventricle throughout LPS-induced neuroinflammation mimicked several aspects of the attenuated neuroinflammatory response seen with our chronic dietary and transgenic models, as did infusing its 17S-hydroperoxy-DHA (17S-HpDHA) derivative, a precursor to SPM. The metabolism of DHA to SPM in the brain was found to be distinct from non-neural tissues, characterized by the presence of protectin D1 and maresin 1, and the absence of resolvin D1 or D2. Further, infusing 17S-HpDHA increased protectin D1 concurrent to attenuating neuroinflammation, suggesting protectin D1 is responsible for some of the anti-neuroinflammatory effects of DHA.

In conclusion, DHA is anti-neuroinflammatory in a mouse model of neuroinflammation, in part, via its metabolism to SPM.
Acknowledgments

I would not have made it through the past five years, or have a PhD to show for it, without the support of the many people that surround me.

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Contributions

All experimental designs, procedures, analyses, and writing presented in this thesis are the work of Sarah Orr, with the exception of the following contributions:

**Dr. Richard Bazinet, Primary Supervisor** – oversaw the development of research ideas, provided laboratory resources, and edited manuscripts.

**Marc-Olivier Trépanier** – helped research and edit review paper (Section 2.7).

**Jasmin Tong** – helped prepare samples and execute lipid analysis (Chapter 3).

**Dr. Francesca Bosetti** – provided laboratory resources, oversaw immunohistochemistry measures, and edited the manuscript (Chapter 4).

**Sara Palumbo** – prepared samples for and executed immunohistochemistry measures, and edited the manuscript (Chapter 4).

**Dr. Charles Serhan** – provided laboratory resources, oversaw LC-MS/MS measures, and edited the manuscript (Chapter 4).

**Dr. Fei Gao** – executed LC-MS/MS measures, edited the manuscript (Chapter 4).

**Thad Vickery** – executed LC-MS/MS measures (figure 4.4-7e)
Table of Contents

Acknowledgments .......................................................................................................................... iv
Contributions .................................................................................................................................. vi
Table of Contents .......................................................................................................................... vii
List of Tables ................................................................................................................................ xi
List of Figures ............................................................................................................................... xii
Abbreviations ............................................................................................................................... xiii

CHAPTER 1: Introduction ................................................................................................................. 1

CHAPTER 2: Literature Review ...................................................................................................... 7
  2.1 Neuroinflammation model ....................................................................................................... 8
    2.1.1 Defining neuroinflammation ............................................................................................ 8
    2.1.2 Our model of neuroinflammation ................................................................................... 10
  2.2 Fat-1 mouse model ................................................................................................................. 12
  2.3 Dietary n-3 PUFA modulation ............................................................................................... 15
  2.4 Specialized pro-resolving mediators ..................................................................................... 16
    2.4.1 D-series resolvins ........................................................................................................... 17
    2.4.2 Protectin D1 .................................................................................................................. 19
    2.4.3 Maresins ....................................................................................................................... 20
  2.5 Non-inflammatory mechanisms of DHA in the brain ............................................................. 20
  2.6 The emerging role of docosahexaenoic acid in neuroinflammation ...................................... 21
    2.6.1 Abstract ......................................................................................................................... 22
    2.6.2 Introduction .................................................................................................................... 22
    2.6.3 DHA .............................................................................................................................. 24
    2.6.4 Brain PUFA uptake .......................................................................................................... 27
    2.6.5 Brain ARA metabolism .................................................................................................... 28
2.6.6 Brain DHA metabolism ........................................................................................ 30
2.6.7 Neuroinflammation ............................................................................................... 32
2.6.8 DHA and its derivatives in inflammation ............................................................. 32
2.6.9 Brain DHA metabolism in response to dietary modification .............................. 35
2.6.10 Conclusions ........................................................................................................... 36
2.7 N-3 polyunsaturated fatty acids in animal models with neuroinflammation ........... 37
  2.7.1 Abstract ................................................................................................................. 37
  2.7.2 Introduction ........................................................................................................... 38
  2.7.3 n-3 PUFA and in vitro neuroinflammation ........................................................... 39
  2.7.4 n-3 PUFA and neuroinflammation induced by systemic lipopolysaccharide ...... 41
  2.7.5 n-3 PUFA and neuroinflammation induced by brain ischemia-reperfusion ...... 43
  2.7.6 n-3 PUFA and neuroinflammation in aging .......................................................... 45
  2.7.7 n-3 PUFA and neuroinflammation in other models .............................................. 46
  2.7.8 Conclusion ............................................................................................................ 49
2.8 Hypothesis and Objectives ....................................................................................... 54

CHAPTER 3: The fat-1 mouse has brain docosahexaenoic acid levels achievable
through fish oil feeding ................................................................................................... 55
  3.1 Abstract .................................................................................................................... 56
  3.2 Introduction .............................................................................................................. 56
  3.3 Experimental Procedure .......................................................................................... 58
    3.3.1 Genotyping .......................................................................................................... 60
    3.3.2 Brain Lipid Extraction and Chromatography ...................................................... 60
    3.3.3 Statistics .............................................................................................................. 61
  3.4 Results ...................................................................................................................... 62
    3.4.1 Mouse Body and Brain Region Weights .............................................................. 62
    3.4.2 Brain total lipid and phospholipid fatty acid concentrations ............................ 62
CHAPTER 4: Unesterified DHA is a precursor to specialized pro-resolving mediators in the brain and protective in neuroinflammation .............................................................. 78

4.1 Abstract ............................................................................................................................. 79
4.2 Introduction ....................................................................................................................... 79
4.3 Methods ............................................................................................................................. 82
  4.3.1 Diets .................................................................................................................................. 82
  4.3.2 Animals .......................................................................................................................... 83
  4.3.3 Total phospholipid and unesterified fatty acid analysis ........................................ 83
  4.3.4 Intracerebroventricular administration of LPS ..................................................... 84
  4.3.5 Implantation of osmotic pump .............................................................................. 85
  4.3.6 Radioactive tracer ................................................................................................. 85
  4.3.7 Gene expression analysis ....................................................................................... 86
  4.3.8 Immunohistochemistry ......................................................................................... 87
  4.3.9 Genotyping ............................................................................................................ 87
  4.3.10 Lipid Mediator Metabolo-lipidomics .................................................................... 87
  4.3.11 Statistics ................................................................................................................ 88
4.4 Results ............................................................................................................................... 89
  4.4.1 Fat-1 mice have higher hippocampal DHA levels ................................................ 89
  4.4.2 Fat-1 mice display attenuated neuroinflammatory responses ............................... 93
  4.4.3 FO diet restores hippocampal DHA of wildtype littermates ................................. 96
  4.4.4 FO diet restores protection in wildtype littermates ............................................... 96
  4.4.5 FO diet increases phospholipid DHA in n-3 PUFA adequate mice ...................... 99
  4.4.6 Neuroinflammation not altered by increased phospholipid DHA ....................... 99
  4.4.7 icv DHA or 17S-HpDHA attenuates neuroinflammation ................................ 102
  4.4.8 DHA and 17S-HpDHA selective in attenuating neuroinflammation ................. 107
List of Tables

**Table 2.7-1:** Summary of neuroinflammatory outcomes in animal models of brain injury ........ 50

**Table 3.3-1:** Fatty acid percent composition of total fat in 10% safflower oil (SO) and 2% fish oil, 8% safflower oil (FO) chows................................................................. 59

**Table 3.4-1:** Brain choline glycerophospholipid fatty acid concentrations (nmol/g) of wildtype and fat-1 mice consuming either the 10% safflower oil or 2% fish oil and 8% safflower oil chow. ....................................................................................................................................................... 64

**Table 3.4-2:** Brain ethanolamine glycerophospholipid fatty acid concentrations (nmol/g) of wildtype and fat-1 mice consuming either the 10% safflower oil or 2% fish oil and 8% safflower oil chow........................................................................................................................................ 66

**Table 3.4-3:** Brain phosphatidylserine fatty acid concentrations (nmol/g) of wildtype and fat-1 mice consuming either the 10% safflower oil or 2% fish oil and 8% safflower oil chow........ 68

**Table 3.4-4:** Brain phosphatidylinositol fatty acid concentrations (nmol/g) of wildtype and fat-1 mice consuming either the 10% safflower oil or 2% fish oil and 8% safflower oil chow........... 70

**Table 3.4-5:** Brain total lipid fatty acid concentrations (nmol/g) of wildtype and fat-1 mice consuming either the 10% safflower oil or 2% fish oil and 8% safflower oil chow. ................. 73

**Table 4.4-1:** Fatty acid (FA) percent composition of total fat in 10% safflower oil (SO), 2% fish oil, 8% safflower oil (FO), and standard chow diets. ................................................................. 90

**Table 4.4-2:** Hippocampal fatty acid composition of n-3 PUFA adequate mice ....................... 105
List of Figures

Figure 2.6-1: The structures of DHA and ARA................................................................. 25

Figure 2.6-2: The conversion of ALA to DHA ................................................................. 26

Figure 2.6-3: ARA (AA) and DHA in neuroinflammation .............................................. 29

Figure 3.4-1: Brain total lipid DHA concentrations of wildtype and fat-1 mice consuming either the 10% safflower oil or 2% fish oil and 8% safflower oil chow .............................................. 63

Figure 4.4-1: Fatty acid and neuroinflammatory profile of the fat-1 mouse .................. 91

Figure 4.4-2: Effect of fat-1 genotype on hippocampal neuroinflammatory and neurodegenerative markers by immunohistochemistry (IHC).................................................. 95

Figure 4.4-3: Fatty acid and neuroinflammatory profile of wildtype littermates fed FO compared to fat-1 mice ............................................................................................... 97

Figure 4.4-4: Fatty acid and neuroinflammatory profile of C57BL/6 mice on SO and FO diets .......................................................................................................................... 100

Figure 4.4-5: Neuroinflammatory profile of mice infused with DHA or 17-HpDHA ........ 103

Figure 4.4-6: Brain distribution of $^{14}$C-DHA from osmotic pump................................. 106

Figure 4.4-7: Specialized pro-resolving mediators (SPM) derived from DHA in mouse hippocampus: LC-MS/MS based lipid mediator metabo-lipidomics. ......................... 109

Figure 4.4-8: LC-MS/MS lipidomics of lipid mediators derived from arachidonic acid ARA in mouse hippocampus.......................................................... 111
Abbreviations

Aβ  amyloid-β
aCSF  artificial cerebrospinal fluid
ALA  α-linolenic acid, 18:3n-3
ARA  arachidonic acid, 18:2n-6
AT-Rv  aspirin-triggered resolvins
BDNF  brain derived neurotrophic factor
CD  cluster of differentiation
CCL  chemokine (c-c motif) ligand
ChoGpl  choline glycerophospholipids
CNS  central nervous system
COX  cyclooxygenase
cPLA2  calcium-dependent cytosolic phospholipase A2
CREB  cyclic AMP-dependent response element binding protein
CYBB  cytochrome B beta
DHA  docosahexaenoic acid, 22:6n-3
DPAn-6  docosapentaenoic acid, 22:5n-6
EtnGpl  ethanolamine glycerophospholipids
EPA  eicosapentaenoic acid, 20:5n-3
FJB  fluoro-jade B
FO  fish oil diet (2% fish oil, 8% safflower oil)
GFAP  glial fibrillary acidic protein
GLA  gamma linolenic acid, 18:3n-6
HpDHA  hydroperoxy-docosahexaenoic acid
HDHA  hydroxy-docosahexaenoic acid
HEPE  hydroxy-eicosapentaenoic acid
HETE  hydroxy-eicosatetraenoic acid
Iba1  ionized calcium binding adaptor molecule 1
IFN-γ  interferon-γ
IHC  immunohistochemistry
IL-1β  interleukin-1β
IL-6  interleukin-6
iNOS  inducible nitric oxide synthase
iPLA2  calcium-independent phospholipase A2
LC-MS/MS  liquid chromatography-tandem mass spectrometry
LO(X)  lipoxygenase
LPS  lipopolysaccharide
LT  leukotriene
LX  lipoxin
MAPK  p38 mitogen-activated protein
MaR1  maresin 1
mPGES  microsomal prostaglandin E synthase
MPTP  1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRM  multiple reaction monitoring
n-3 PUFA  omega-3 polyunsaturated fatty acid
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFkB</td>
<td>nuclear factor κ B</td>
</tr>
<tr>
<td>PD1/NPD1</td>
<td>protectin D1/neuroprotectin D1</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>PGK1</td>
<td>phosphoglycerate kinase 1</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PtdIns</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PtdSer</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>Rv</td>
<td>resolvin</td>
</tr>
<tr>
<td>SN</td>
<td>substantia nigra</td>
</tr>
<tr>
<td>SO</td>
<td>safflower oil diet (10% safflower oil)</td>
</tr>
<tr>
<td>sPLA2</td>
<td>calcium-dependent secretory phospholipase A2</td>
</tr>
<tr>
<td>SPM</td>
<td>specialized pro-resolving mediator</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>TLR4</td>
<td>toll-like receptor 4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor α</td>
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CHAPTER 1:

Introduction
Neurological diseases and injuries pose heavy individual, societal, and economic burdens in Canada and worldwide, and include Alzheimer’s disease, Parkinson’s disease, Amyotrophic Lateral Sclerosis, Multiple Sclerosis, traumatic brain injury, and stroke (World Health Organization. 2006; Canadian Institute for Health Information. et al 2007). In Canada, Alzheimer’s disease and stroke account for 3.5% and 4.7% of total disability-adjusted years of life, respectively, and over $1 billion in direct health care costs (Canadian Institute for Health Information. et al 2007). Further, chronic neurological disease prevalence and stroke incidence is projected to increase dramatically with the aging population (Canadian Institute for Health Information. et al 2007). In the case of chronic neurological diseases, there are no public health prevention strategies, and current treatment options, at best, only delay disease progress. Effective treatments for acute brain injuries are also lacking. Stroke, for instance, has thrombolysis as its only approved treatment, but only 5% of patients are eligible for this procedure (Iadecola et al 2011). Therefore, research that helps to identify prevention strategies and therapeutic targets for neurological diseases and injuries is of the utmost importance.

Neurodegenerative diseases show diverse pathologies and consequent symptoms, but they share neuroinflammation as a common component. Neuroinflammation also plays a detrimental role in the secondary damage of traumatic brain injury and stroke. Neuroinflammation, therefore, provides a promising target for the prevention and treatment of a range of neurological disorders and injuries.

The goal of this thesis is to elucidate the role of brain fatty acids, particularly the omega-3 polyunsaturated fatty acid (n-3 PUFA) docosahexaenoic acid (DHA; 22:6n-3), as a potential attenuator of neuroinflammation. While the research presented herein is in fundamental
biological pathways and uses an animal model, it helps lay the foundation of our understanding of inflammation in the human brain.

Long chain n-3 PUFA, eicosapentaenoic acid (EPA; 20:5n-3) and DHA, were first cited as anti-thrombotic and anti-inflammatory in the late 1970s (Dyerberg et al 1978). EPA and DHA are found almost exclusively in marine oils, and their proposed anti-thrombotic and anti-inflammatory properties provided a mechanism to explain the low rates of cardiovascular disease in the Inuit of Greenland, who consume large amounts of marine animals (Dyerberg et al 1978). More recently, epidemiological studies have linked dietary n-3 PUFA intake with a decreased incidence of several neurodegenerative diseases including Alzheimer’s and Parkinson’s disease (Kalmijn et al 1997; Morris et al 2003; Barberger-Gateau et al 2007; Gao et al 2007; Okubo et al 2012). While anti-apoptotic, neurotrophic, and synaptogenic pathways have been suggested as potential mechanisms for the actions of DHA in neurological disorders, it is possible that DHA also acts directly in brain inflammatory pathways.

In tissues peripheral to the brain, DHA has potent anti-inflammatory effects through the action of its hydroxylated metabolites. In the brain, increased DHA levels are protective in animal models of injury, attenuating pathologies associated with stroke, Alzheimer’s disease, and Parkinson’s disease (reviewed in Orr et al 2012). What is not known, however, is if DHA and its metabolites act directly in neuroinflammatory pathways (Orr et al 2012). If DHA and its metabolites are anti-neuroinflammatory, then they may provide candidate dietary and drug targets in the prevention and treatment of neurological disorders with a neuroinflammatory component.
Chapter 2 of this thesis is a literature review containing five subsections and two published review papers that outline the rationale for my research. The first three sections (2.1-2.3) provide a rationale for the techniques used in my research, including the neuroinflammatory model, fat-1 mouse model, and dietary model. Section 2.4 introduces specialized pro-resolving mediators (SPM). DHA-derived SPM are investigated in this thesis as a candidate mechanism for the anti-inflammatory effects of DHA. Section 2.5 describes neuroprotective pathways in the brain regulated by DHA. The first review paper (section 2.6), *The emerging role of docosahexaenoic acid in neuroinflammation*, discusses PUFA uptake and metabolism in the brain with a focus on DHA, and proposes the hypothesis that higher brain DHA levels decrease neuroinflammation, which may be relevant to the prevention and treatment of neurological disorders, particularly those with a neuroinflammatory component. The second paper (section 2.7), *N-3 polyunsaturated fatty acids in animal models with neuroinflammation*, is a comprehensive review of the animal literature that shows protective effects of n-3 PUFA against neuroinflammation in models of sickness behaviour, stroke, aging, depression, Parkinson’s disease, and cytokine-, irradiation-, and diabetes-induced cognitive impairments. It highlights that the literature lacks *in vivo* studies that test the direct effects of n-3 PUFA in a model of neuroinflammation. These two published papers provide the broader context for my research. This chapter ends with the formal hypotheses and objectives of my thesis.

There were sufficient pilot data on the fat-1 mouse, including its higher hippocampal DHA levels, to choose it as the first model to test the effects of brain DHA on neuroinflammation (section 2.2). What was unknown, however, was the physiological relevance of the fat-1 mouse model. Since n-3 PUFA levels are increased in the fat-1 mouse via a protein product of a transgene that had not been fully described, it was important to determine if the n-3 PUFA levels
present in fat-1 mice were achievable through diet. The diet is where mammals normally obtain their essential fatty acids. Chapter 3 is adapted from a published study titled *The fat-1 mouse has brain docosahexaenoic acid levels achievable through fish oil feeding*, and tests the hypothesis that fat-1 mice have brain DHA levels attainable through fish oil feeding. Fat-1 mice and their wildtype littermates are typically kept on an n-3 PUFA deprived diet (10% safflower oil), with the difference in tissue n-3 PUFA levels due to conversion of n-6 PUFA to n-3 PUFA. This study finds that fish oil feeding (2% fish oil, 8% safflower oil) produces similar brain DHA levels as the fat-1 gene, demonstrating that the fat-1 mouse is a physiologically relevant model of study.

Chapter 4 contains the bulk of my experimental work, presented in a paper titled *Unesterified DHA is a precursor to specialized pro-resolving mediators in the brain and protective in neuroinflammation*. Having described the relevance of fat-1 mice (Chapter 3), we determined how their increased brain DHA levels affect neuroinflammation (Chapter 4). Fat-1 mice were protected against neuroinflammation and the resultant neuronal degeneration. A dietary model was used to confirm that it was the increase in brain DHA and not an anomaly of the fat-1 transgenic mouse that caused the attenuated neuroinflammatory responses. The ideal model for this test was the n-3 PUFA-fed wildtype littermate of the fat-1 mouse, since previous work (Chapter 3) showed that n-3 PUFA-fed wildtype littermates have comparable brain DHA levels to fat-1 mice. Wildtype littermates proved to have similar neuroinflammatory responses as fat-1 mice (Chapter 4), further supporting the hypothesis that brain DHA, whether derived from the diet or the conversion of n-6 PUFA, attenuates neuroinflammation. The low brain DHA levels in wildtype littermates of fat-1 mice reflect animals that have undergone multi-generational dietary n-3 PUFA deprivation. A less-extreme model of n-3 PUFA modulation was sought. C57BL/6 mice that were born to n-3 PUFA adequate mothers were randomized to the fish oil or safflower
oil diet described in Chapter 3. Hallmarks of neuroinflammation were examined following 9 weeks of dietary intervention. Unexpectedly, no difference in neuroinflammation was found between fish oil- and safflower oil-fed mice. While this study calls into question the benefits of fish oil supplementation in n-3 PUFA adequate animals (discussed in Chapter 5), it highlighted the unesterified DHA pool as potentially crucial for the modulation of neuroinflammation since fish oil-fed mice had increased levels of brain phospholipid DHA but not of unesterified DHA. The next study investigated the importance of the unesterified pool, and supported the hypothesis that increases in unesterified DHA are necessary to attenuate neuroinflammation (Chapter 4). Acutely increasing the DHA derivative and precursor to resolvins and protectins, 17S-hydroperoxy-DHA (17S-HpDHA), mimicked the effects of unesterified DHA. This finding suggests that the anti-neuroinflammatory effects of DHA are achieved, at least in part, via SPM derivatives. This is the first such finding in the brain, but is in agreement with the literature showing SPM to be mediators of the anti-inflammatory effects of DHA in peripheral tissues. DHA-derived SPM have been primarily identified and characterized from peritoneal inflammatory exudates, and systematic measures in the brain have not been reported. Previously, only protectin D1 and a stable marker of its precursor 17S-HpDHA, 17-hydroxy-DHA (17-HDHA), had been identified in the brain. To address this gap in the literature, the final portion of my thesis explored the identification of SPM in the brain (Chapter 4). DHA metabolism to SPM in the brain is distinct from all previously reported non-neural tissues. Further, there is an increased production of protectin D1 during an attenuated neuroinflammatory response.

Chapter 5 summarizes my thesis, while avoiding redundancies with the chapter-specific discussions. I also review the limitations of my research, future directions, and implications.
CHAPTER 2:

Literature Review
2.1 Neuroinflammation model

2.1.1 Defining neuroinflammation

The cardinal signs of inflammation were first described by the Roman Aulus Cornelius Celsus (ca 25 BC—ca 50) as *calor* (warmth), *dolor* (pain), *tumor* (swelling), and *rubor* (redness). *Functio laesa* (loss of function) was added as the fifth cardinal sign in the late 1800s by Rudolf Virchow, a German physician-scientist (Rocha e Silva 1978). The cardinal signs are symptoms of underlying cellular events (vasodilation, leukocyte influx), which themselves are dictated by temporally organized fluxes of signaling molecules and cascades, i.e. molecular phenomena (Scott et al 2004). The measurement of cellular events and molecular phenomena has been particularly important for the identification and study of inflammation within the central nervous system (CNS), where cardinal signs of inflammation do not manifest.

The definition of neuroinflammation is debated, with some arguing that it is restricted to responses involving the influx of innate immune cells from the periphery (Graeber et al 2011), while others use a more broad definition that also includes responses that are limited to the activation and proliferation of resident microglia (Glass et al 2010). The more restricted definition of neuroinflammation put forward by Graeber et al. includes both microorganism-stimulated and sterile (ischemia-reperfusion, trauma) responses (2011). Notably excluded from this definition are the microglial responses observed in Alzheimer’s and Parkinson’s diseases, which lack migratory cells from the periphery. Graeber et al. suggest that these be referred to as “microglial activation” or “CNS pseudo-inflammation” reactions (2011). Graeber et al. argue that this separation is necessary since it is not known if microglial activation in Alzheimer’s and Parkinson’s diseases is beneficial or detrimental, and that anti-inflammatory treatments may not be beneficial as they are in more classically defined inflammatory reactions such as Multiple...
Sclerosis (2011). Graeber et al.’s argument is tempting for its simple separation of local and migratory (inflammatory) reactions; however, it fails to recognize the similarities between them in the brain. Microglia and macrophages mediate similar pathways in immune reactions. Resident microglia have the capacity for extensive proliferation, unlike macrophages, and thus an influx of macrophages from the blood is not necessary like it is in tissues with only resident macrophages. Dismissing locally-mediated reactions as non-inflammatory is to dismiss their similarities with processes that include migratory cells, and it is premature given our current knowledge, or lack thereof. Further, use of the term neuroinflammation already implies that it is a distinct reaction from peripheral inflammation, in addition to the widely recognized fact that neuroinflammation is unique since it is mediated by microglia. Those who adopt the more broadly defined use of neuroinflammation recognize that its causal nature in neurological disorders has not been established (Glass et al 2010). For these reasons, in this thesis any form of inflammation in the brain, local or migratory, is referred to as neuroinflammation.

A second controversy surrounding neuroinflammation is whether it is beneficial or detrimental in neurological disorders and injuries. In acute brain injuries, it is well recognized that neuroinflammation can be detrimental (Gosselin et al 2007). However, complete blockage of molecules vital for the initiation of neuroinflammation, for instance in tumor necrosis factor-α (TNF-α)-null mice, will reduce initial microglial activation in acute injury but exacerbate eventual neuronal death (Turrin et al 2006). This result demonstrates the idea that the beginning programs the end, or said differently, that properly organized pro-inflammatory signaling is necessary to program resolution and repair mechanisms (Serhan et al 2005; Gosselin et al 2007). Therefore, therapeutic targets should not be completely blocking inflammation, but instead re-programming it to achieve better inflammation resolution and tissue repair. More controversial
than the role of neuroinflammation in acute brain injury is its role in chronic brain diseases like Alzheimer’s disease. Robust neuroinflammatory signaling measured by cytokines and activated microglia has been found by a large number of studies to be detrimental in animal models of Alzheimer’s disease, but beneficial effects have also been seen by stimulating microglia or migrating monocytes that clear pathogenic amyloid-β (Aβ) (Rivest 2009). Whether considering acute or chronic brain injuries, the role of neuroinflammation and its usefulness as a therapeutic target remains highly controversial. However, there is only one study that has found a detrimental effect of DHA treatment in the brain, in ischemia-reperfusion, whereas the rest have consistently found a beneficial effect (see section 2.7). If DHA is acting directly on neuroinflammatory pathways in these models, it suggests that it is beneficially re-programming neuroinflammation rather than blocking or disorganizing it in a way that impairs tissue resolution. Supporting this idea is evidence that DHA-derived SPM derivatives are anti-inflammatory and pro-resolving, by reducing overall inflammatory signals and stimulating phagocytosis (Bannenberg et al 2010).

2.1.2 Our model of neuroinflammation

We sought a model that, to the highest degree possible, isolated the inflammatory response of the brain from that of the periphery. To achieve this, we wanted to administer the inflammatory treatment through an icv injection, insuring that neuroinflammation stimulation was local and any influx of cells was a consequence of brain immune signaling. It is common in models of icv LPS-induced neuroinflammation that leukocytes and monocytes from the periphery infiltrate brain tissue (Gurney et al 2006), as is the case in our model (Aid et al 2010b). Second, we wanted to use an established initiator of an innate neuroinflammatory response. To achieve this, we considered using lipopolysaccharide (LPS), an endotoxin and a major component of the outer
membrane of Gram-negative bacteria. LPS is well characterized and known to bind to the toll-like receptor 4 on microglial cells, thus activating the nuclear factor κB (NFκB) pathway (Mayeux 1997; Block et al 2007; Miyake 2007). Third, we wanted to induce a global inflammatory reaction in the brain. We elected to use mice, rather than rats, because they have significantly smaller brains (~475 mg vs. ~2,000 mg) (Paxinos et al 2001; Mirfazaelian et al 2007). Thus, when injected into a central ventricle, LPS can penetrate a greater proportion of the smaller brain.

The neuroinflammatory model we chose to use satisfied the three criteria described above, and provided a dose, a time-point, and outcome measures. It was developed in Dr. Francesca Bosetti’s lab by Dr. Sang-Ho Choi, who used it to study the role of cyclooxygenase enzymes in neuroinflammation (Choi et al 2008; Aid et al 2010a; Choi et al 2010; Russo et al 2011). Through a pilot study, Drs. Choi and Bosetti established that a single icv injection of 5 μg of LPS induced a neuroinflammatory response that peaked 24 hours later. LPS was injected into the left lateral ventricle, where it joins the flow of cerebrospinal fluid that bathes the brain. The bulk of cerebrospinal fluid flows from the lateral ventricles into the third then fourth ventricle before entering the spinal cord canal; and cerebral ventricles are in open communication with the surrounding brain intercellular fluids (Perez-Figares et al 2001). Therefore, when LPS is injected into the left lateral ventricle, it flows through the entire brain and reaches brain tissue through intracellular fluids. Drs. Choi and Bosetti characterized the hippocampal response to LPS by measuring gene and protein expression. They found increased NFκB activity; increased levels of cytokines (TNF-α; interleukin-1β, IL-1β; and interleukin-6, IL-6), chemokines (chemokine (c-c motif) ligands 2 and 3, CCL2 and CCL3), enzymes of the arachidonic acid (ARA; 20:4n-6) cascade (calcium-dependent cytosolic phospholipase A₂, cPLA₂; calcium-dependent secretory
phospholipase A2, sPLA2; and cyclooxygenase-2, COX-2), and markers of glial activation (cluster of differentiation 11b and 45, CD11b and CD45; glial fibrillary acidic protein, GFAP; and ionized calcium binding adaptor molecule 1, Iba1); and increased levels of prostaglandin E2 (PGE2) (Aid et al 2008; Choi et al 2008). Therefore, this model induced a complement of genes that could be measured to assess neuroinflammation. Further, this model was developed in C57BL/6 mice, which is the background of the fat-1 transgenic mice used in our first experiments.

2.2 Fat-1 mouse model

The fat-1 transgenic mouse was developed by Dr. Jing Kang at Harvard Medical School (Kang et al 2004). The fat-1 gene comes from Caenorhabditis elegans and codes for an n-3 fatty acid desaturase that converts n-6 PUFA to n-3 PUFA through the addition of a double bond at the third carbon from the methyl end. One advantage to using the fat-1 mouse model is use of a single diet for both fat-1 mice and their wildtype littermates, since it has been argued that other constituents of fish oil, for instance vitamin D, are responsible for effects attributed to n-3 PUFA (Annweiler et al 2010). Mice are fed a diet rich in n-6 PUFA and devoid of n-3 PUFA, thus differences in tissue n-3 PUFA between fat-1 mice and their wildtype littermates are attributable to the action of the fat-1 gene product.

We chose the fat-1 mouse model as our first experimental approach because of pilot data showing that fat-1 mice had markers in the brain of chronic DHA administration. Modulation of brain DHA has been best-described in rats. Of particular interest, rats fed an n-3 PUFA adequate diet for 15 weeks were found to have increased brain DHA levels and a downregulated brain ARA cascade, including COX-2 and cPLA2 protein and gene expression, compared to rats fed an n-3 PUFA deprived diet (Rao et al 2007a). This study suggests that DHA could potentially
attenuate neuroinflammation through two mechanisms, i) indirectly through a downregulation of the ARA cascade, and ii) directly, possibly through metabolism to bioactive derivatives. In 2007, unpublished data from Cynthia Boudrault and Dr. David Ma showed that fat-1 mice had a significantly higher cortical phospholipid DHA (% composition) and decreased cortical COX-2 protein compared to their wildtype littermates [later published (Boudrault et al 2010)]. Thus, fat-1 mice and their wildtype littermates were a mouse model of Rao and coworkers 15-week rat feeding model (Rao et al 2007a), and ideal to test the effects of chronically elevated brain DHA levels on neuroinflammation. Other advantages included the use of a single dietary treatment as described above, and the promising neuroprotective and peripheral anti-inflammatory responses in fat-1 mice that had been reported in the literature (Hudert et al 2006; Schmocker et al 2007; Taha et al 2008).

Since its development in 2004, the fat-1 mouse has been used to test the effects of increased n-3 PUFA on a variety of processes. Brain DHA levels of fat-1 mice and their wildtype littermates correlate to seizure resistance (Taha et al 2008). Fat-1 mice also have increased neurogenesis, neuritogenesis, and spatial learning compared to their wildtype littermates (He et al 2009). When the fat-1 mouse was crossed with the 3×Tg-AD mouse, which models Alzheimer’s disease neuropathology, mice with the fat-1 transgene showed reduced neuropathology including decreased tau protein, soluble Aβ42, and GFAP (Lebbadi et al 2011). Fat-1 mice are moderately protected against pathologies in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson’s disease (Bousquet et al 2011). The fat-1 mouse has higher levels of EPA and DHA in its spinal cord, and improved functional recovery after sciatic nerve crush (Gladman et al 2012). In several inflammatory disorder models, fat-1 mice experience a less robust inflammatory response and have less tissue damage compared to their wildtype littermates. For
example, fat-1 mice have attenuated inflammation and tissue damage in experimental colitis (Hudert et al 2006; Gravaghi et al 2011), d-galactosamine/LPS-induced hepatitis (Schmocker et al 2007), cerulean-induced pancreatitis (Weylandt et al 2008), acute lung injury (Mayer et al 2009), age-related systemic inflammation (Rahman et al 2009), high-fat diet-exacerbated dorsal air pouch inflammation (White et al 2010), atherosclerosis (Wan et al 2010), allergic airway response (Bilal et al 2011), and a streptozotocin (STZ)-induced model of diabetes (Bellenger et al 2011). In summary, the fat-1 mouse is protected in several neuro-pathologies and against systemic inflammation resulting from various insults. Importantly, wildtype littermates of fat-1 mice are n-3 PUFA deprived and thus another interpretation of these studies is that wildtype littermates are susceptible to neuro-pathologies and systemic inflammation.

One study has investigated brain global gene expression in fat-1 mice and their wildtype littermates (Menesi et al 2009). Of the 25,000 genes measured, 2.2% were upregulated and 2% were downregulated in fat-1 mice. It is important to note that gene regulation differences were determined by t-tests at p<0.05. Therefore, the genes that were significantly different (~5%) could be entirely accounted for by type I errors (false positives). To strengthen their data, the authors performed quantitative real-time polymerase chain reaction (qRT-PCR) on several genes of interest that showed differential gene expression by micro-array. Of the 24 qRT-PCR results reported, 17 confirmed micro-array results. Prostaglandin D₂ synthase, cPLA₂-epsilon, and chemokine (C-Cmotif) receptor 5 were all downregulated in the brains of fat-1 mice (Menesi et al 2009). The mice used in this study were fed a normal chow diet rather than the n-6 PUFA enriched, n-3 PUFA deprived diet typical of fat-1 mouse studies. As a result, brain DHA levels were biologically similar between fat-1 mice (18.67 ± 0.53 % total fatty acid) and wildtype
littermates (18.43 ± 0.42 % total fatty acids), though statistically different. The similar baseline DHA levels make this study difficult to compare to other fat-1 studies, including our own.

2.3 Dietary n-3 PUFA modulation

In addition to the use of a transgenic model, we wanted to investigate how n-3 PUFA derived from the diet impact brain DHA levels and, in turn, affects neuroinflammation. Our first experiment was to determine the physiological relevance of the fat-1 mouse model (Chapter 2). The fat-1 colony is fed a 10% safflower oil diet, making it the “baseline” in designing a fish oil-containing diet. We chose to substitute 2% of the safflower oil with menhaden fish oil, to provide a diet containing a level of EPA and DHA reasonably attainable in the diet. The calculation was based on quantitative food intake data from Canadian pregnant women, using lipid extraction on 3 d duplicate food collections and thus providing the most accurate assessment of specific fatty acid consumption in a Canadian population (Denomme et al 2005). Women consumed an average of 35 mg/d of EPA and 85mg/d or DHA (117 mg/d combined), which accounted for 0.23% of their total fatty acid intake. To find a reasonable level of fish oil intake, we used the equivalent of taking two 1 g fish oil pills per day, based on common commercial formulations, or eating a standard serving of dark meat fish three times per week. These would provide 1800-2000 mg/d of EPA and DHA, and would make up around 3.5-4% of total fatty acid intake. Therefore, to achieve 3.5-4% of total fatty acid intake as EPA and DHA, we needed to replace 20% of the safflower oil with menhaden oil, which itself contains around 20% of fatty acids as EPA and DHA. Triplicate analysis of total lipids on the 2% fish oil, 8% safflower oil diet confirmed these estimates, finding that EPA and DHA provide a total of 3.58% of dietary fatty acids, or 1.97% and 1.61%, respectively (Orr et al 2010).
The level of EPA and DHA in our 2% fish oil, 8% safflower oil diet is on the lower end of commonly used values in the n-3 PUFA literature. The laboratory of Dr. Normal Salem (National Institutes of Health, Bethesda, MD), used a comparable fish oil diet in mouse studies and found brain DHA concentrations to be significantly different between mice consuming and deprived of dietary n-3 PUFA (Fedorova et al 2009; Tuo et al 2009). Several other laboratories use diets that contain 3-8 times more EPA and DHA per g diet in order to alter brain DHA levels, including those of Dr. Gregory Cole and Dr. Philip Calder (Miles et al 2000; Wallace et al 2000; Calon et al 2004; Ma et al 2009; Arsenault et al 2011). Still other laboratories have fed rodent diets with 50-500 times the dose provided by our 2% fish oil diet (Anderson et al 1996; Lonergan et al 2004; Lynch et al 2007).

2.4 Specialized pro-resolving mediators

N-3 PUFA have long been hypothesized as anti-inflammatory, although their proposed mechanism of action has evolved considerably over time. In the 1980s, n-3 PUFA were thought to be anti-inflammatory by competitive inhibition with the n-6 PUFA pathway. Inhibition of the n-6 PUFA ARA was most significant, since ARA was known to be a precursor for pro-inflammatory prostaglandins and thromboxanes. The widely accepted mechanism of anti-inflammatory effects was through i) EPA and DHA displacing ARA at the sn-2 position in cell membrane phospholipids, and ii) competition of EPA with ARA for metabolism through COX enzymes, producing its own less bioactive prostaglandin and thromboxane analogues (Kelley et al 1985; Palmblad et al 1988). DHA was considered anti-inflammatory through its ability to retro-convert to EPA (Palmblad et al 1988). Researchers even hypothesized that DHA might simply act as a storage form of EPA (Leaf et al 1988).
The original mechanism of action of EPA and DHA rests on the idea that inflammation is a self-limited process, beginning with an increase in pro-inflammatory signaling molecules to recruit tissue-defending but also damaging polymorphonuclear leukocytes (PMN), followed by an influx of phagocytic monocytes and macrophages, finally ending when the production of pro-inflammatory mediators has ceased and the cell debris and mediators have been cleared from the tissue. In this model of inflammation, EPA and DHA limit the production of pro-inflammatory mediators. It is now recognized that the arrest and resolution of inflammation is an active process directed by its own set of mediators. Dr. Charles Serhan and his research group have led in the discovery and characterization of these mediators, coining them SPM (Bannenberg et al 2010). SPM are enzymatically oxygenated derivatives of ARA, EPA, and DHA, which are actively biosynthesized during an inflammatory response and have potent anti-inflammatory and pro-resolving properties. Four distinct families of SPM exist: lipoxins (LX; ARA-derived), resolvins (Rv; EPA- and DHA-derived), protectins (PD; DHA-derived) and maresins (MaR; DHA-derived). They were identified using a systems approach with liquid chromatography-tandem mass spectrometry (LC-MS/MS) lipidomics and informatics, followed by complete structural elucidation (Serhan et al 2011). While pro-inflammatory prostaglandins and leukotrienes are produced in the initial phase of an inflammatory response, SPM production increases later, in the resolution phase of inflammation. It has been hypothesized that a deficiency in the resolution of inflammation contributes to several chronic inflammatory disorders including cardiovascular diseases and arthritis (Serhan et al 2011). DHA-derived SPM are the focus of this subsection.

2.4.1 D-series resolvins

17S-HpDHA (17S-hydroperoxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid) is produced by the addition of a hydroperoxy group to carbon 17 of DHA by 15-lipoxygenase-1 (15-LO). D-
series resolvins are produced following the action of a 5-LO on 17S-HpDHA that forms either a 7S(8)- or 4S(5)-epoxide, which are precursors to RvD1/RvD2 or RvD3/RvD4/RvD5, respectively (Bannenberg et al 2010). The main action attributed to resolvins is their ability to stop PMN recruitment and infiltration to sites of inflammation.

RvD1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid) was first identified in mice exudates from the dorsal air pouch model (Serhan et al 2002). RvD1 has been shown to inhibit PMN infiltration in models of peritonitis (Hong et al 2003; Sun et al 2007; Spite et al 2009b; Recchiuti et al 2011), dorsal skin air pouch injury (Serhan et al 2002; Hong et al 2003), and corneal injury (Jin et al 2009). RvD1 also protects against neovascularization in the retina and cornea (Connor et al 2007; Jin et al 2009), and reduces kidney damage following ischemia-reperfusion (Duffield et al 2006). Focusing in the CNS, RvD1 was shown to inhibit TNF-α-stimulated IL-1β gene expression in human microglial cell culture (Serhan et al 2002). In human glial and murine brain cell cultures, systematic lipidomics analyses show a predominant production of 17S-dihydroxy derivatives of DHA, and not 17S-trihydroxy derivatives such as resolvins (Serhan et al 2002; Hong et al 2003). It is therefore not known whether RvD1 is endogenously produced in the CNS, or if it plays a role in neuroinflammation.

Considerably less research has been done on RvD2 (7S,16R,17S-trihydroxy-4Z,8E,10Z, 12E,14E,19Z-docosahexaenoic acid), compared to RvD1. In peritonitis, RvD2 reduces PMN infiltration at doses of 10 pg per mouse, by reducing PMN-endothelial interaction (Spite et al 2009a). In a murine model of sepsis, RvD2 reduced the amount of live bacteria in the blood and peritoneum, likely through increasing the ratio of macrophages/PMN thereby increasing phagocytic-dependent bacterial clearance (Spite et al 2009a). Concurrent to the reduction in bacteria and increased phagocytic activity, septic mice treated with RvD2 had lower levels of
pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) both locally in the peritonium and systemically in the plasma (Spite et al 2009a). RvD2 also protects mice against colitis through similar mechanisms (Bento et al 2011). RvD2 has not been reported in *in vitro* or *in vivo* studies relating to the brain. Furthermore, studies on the biological actions of RvD3, RvD4, and RvD5 have not been reported.

### 2.4.2 Protectin D1

Like resolvins, protectin D1 (PD1; 10R,17S-dihydroxy-4Z,7Z,11E,13E,15Z,19Z-docosahexaenoic acid) is a product of 17S-HpDHA. 17S-HpDHA undergoes a second enzymatic epoxygenation by 15-LO followed by hydrolysis to produce PD1 (Bannenberg et al 2010). PD1 is also coined neuroprotectin D1 (NPD1) when referring to its production in the brain and retina. PD1/NPD1 has protective effects in models of disease and inflammation peripheral to the brain and retina, including in peritonitis (Ariel et al 2005; Bannenberg et al 2005; Schwab et al 2007), asthma (Levy et al 2007), and kidney injury (Duffield et al 2006). In mouse retina, PD1/NPD1 protects against vaso-obliteration and neovascularization in oxygen-induced retinopathy (Connor et al 2007). *In vitro*, PD1/NPD1 is detected in human retinal cells and protects against retinal apoptosis (Mukherjee et al 2004). Unlike resolvins, PD1/NPD1 has consistently been detected *in vitro* and *in vivo*, in retinal and brain cells and tissues, making it a more likely mechanism of endogenous neuroinflammatory regulation by DHA.

In the brain, PD1/NPD1 has been detected in the hippocampus during the first 24 h of reperfusion following transient middle cerebral artery occlusion (Marcheselli et al 2003). When PD1/NPD1 was administered icv during ischemia-reperfusion, it significantly reduced leukocyte infiltration into the hippocampus and neocortex, along with a reduction in infarct volume, and hippocampal NFκB protein and COX-2 gene expression (Marcheselli et al 2003). In human
neural cell culture, PD1/NPD1 reduces the age-related release of Aβ₄₀ and Aβ₄₂ peptides (Lukiw et al 2005), and Aβ₄₂-induced pro-inflammatory gene expression (Lukiw et al 2005; Zhao et al 2011). The triple-transgenic mouse model of Alzheimer’s disease has lower levels of hippocampal unesterified DHA and PD1/NPD1 compared to non-transgenic, age-matched controls of a similar genetic background (Zhao et al 2011). Post-mortem hippocampi from patients with moderate Alzheimer’s disease contain two-fold less unesterified DHA and twenty-fold less PD1/NPD1 compared to age-matched neurologically normal controls (Lukiw et al 2005), indicating that a deficiency in DHA, or its derivatives, may be a relevant therapeutic target. PD1/NPD1 is protective in animal models of epilepsy (Musto et al 2011) and pain (Park et al 2011).

2.4.3 Maresins

Maresins are the newest identified family of SPM, and are not derived from 17S-HpDHA like resolvins and PD1/NPD1. Maresin 1 (MaR1; 7,14S-dihydroxy-4Z,8,10,12,16Z,19-docosahexaenoic acid) is formed via a 14S-HpDHA intermediate by human 12-LO, an enzyme present in macrophages and platelets (Serhan et al 2009). Like D-series resolvins and PD1/NPD1, MaR1 potently (0.1 ng/mouse) reduces PMN infiltration in murine peritonitis and increased phagocytosis of apoptotic cells (Serhan et al 2012). MaR1 also seems to modulate pain similarly to PD1/NPD1. MaR1 has not yet been reported in brain cells either in vitro or in vivo.

2.5 Non-inflammatory mechanisms of DHA in the brain

While it is not known if DHA targets inflammatory pathways in the brain, DHA is known to be neurotrophic and neuroprotective. DHA is particularly enriched in the phosphatidylserine (PtdSer) fraction of neuronal membranes, and increasing DHA levels increases the accumulation of PtdSer in neural cell membranes (Akbar et al 2005). In in vitro serum starvation-induced
apoptosis, the accumulation of PtdSer is anti-apoptotic and facilitates a more rapid translocation and phosphorylation of Akt, which is critical for cell survival signaling via phosphatidylinositol 3-kinase (Akbar et al 2005). In Aβ42-induced stress in neuronal cell cultures, DHA and PD1/NPD1 increased gene expression of anti-apoptotic signaling factors Bcl-2, Bfl-1, and Bcl-xl (Lukiw et al 2005). Enriched brain DHA levels have also been associated with increased brain derived neurotrophic factor (BDNF), a factor important in neuronal survival and plasticity (Rao et al 2007b). Cyclic AMP-dependent response element binding protein (CREB) is a transcription factor that regulates BDNF expression, and is itself regulated by several kinases, including p38 mitogen-activated protein kinase (MAPK). DHA was found to increase BDNF production via a MAPK –dependent mechanism (Rao et al 2007b).

In addition to neuronal cell survival, n-3 PUFA are necessary for endocannabinoid-mediated synaptic plasticity (Lafourcade et al 2011). N-3 PUFA deprived mice have disrupted endocannabinoid signaling resulting from a desensitization of cannabinoid CB1 receptors and their uncoupling from effector proteins (Lafourcade et al 2011).

Docosahexaenoic acid is another derivative of DHA that is thought to play a role in synaptogenesis (Kim et al 2011b). Docosahexaenoic acid is produced in fetal hippocampal neuronal cell cultures, and promotes neurite growth and synaptogenesis as measured by an increase in synapsin and glutamate receptor expression (Kim et al 2011a).

2.6 The emerging role of docosahexaenoic acid in neuroinflammation

Adapted from:

2.6.1 Abstract

Epidemiological studies have linked fish consumption to lower rates of neurological diseases. Fish contain high levels of n-3 PUFA, and several lines of evidence suggest that the n-3 PUFA DHA acts in the brain via anti-apoptotic and neurotrophic pathways. In addition, DHA may act through anti-neuroinflammatory pathways, as DHA possesses anti-inflammatory properties in the periphery. Evidence from animal models has indicated that DHA and its derivatives (resolvin D1 and protectin D1) attenuate colitis, peritonitis and ischemic stroke. n-3 PUFA deprivation in rats decreases brain levels of DHA and increases markers of the brain ARA cascade, a proinflammatory pathway. Thus, chronic low intake of n-3 PUFA may predispose the brain to weak anti-inflammatory, as well as strong proinflammatory signals. Neurological disorders, including Alzheimer’s disease, Parkinson’s disease and major depression, display a neuroinflammatory component. n-3 PUFA supplementation, as well as drugs targeting brain PUFA metabolism, are promising candidates in the prevention and treatment of neurological disorders.

2.6.2 Introduction

Fish consumption has been linked to lower rates of neurological diseases, including Alzheimer's disease (Kalmijn et al 1997; Barberger-Gateau et al 2002; Morris et al 2003), Parkinson's disease (de Lau et al 2005), multiple sclerosis (Swank et al 1952; Agranoff et al 1974), and major and postpartum depression (Hibbeln et al 1995; Hibbeln 2002). Clinical trials investigating the effects of fish oil or n-3 PUFA supplementation on disease progression have been inconsistent, with beneficial effects demonstrated in some (Nemets et al 2002; Peet et al 2002; Su et al 2003; Hallahan et al 2007), but not all (Silvers et al 2005; Grenyer et al 2007), studies of major depression, and some benefits in very mild Alzheimer's disease, but not in mild to moderate
Alzheimer's disease (Freund-Levi et al 2006). The reason for these inconsistencies is difficult to
determine, as studies differ in n-3 PUFA compositions used, dosages, dosage durations, disease
stages and other parameters (Ross et al 2007; Stahl et al 2008). Furthermore, protective effects of
n-3 PUFA have been reported in animal models of Alzheimer's and Parkinson's diseases (Calon
et al 2004; Calon et al 2007; Bousquet et al 2008). Because there are several strong
epidemiological correlations, some promising clinical trials, and benefits observed in animal
models and, given that the aforementioned diseases affect 3% of the world's population (World
Health Organization 2004), it is important to investigate the possible mechanisms of n-3 PUFA
in neurological processes.

Several lines of evidence have suggested that n-3 PUFAs function in the brain through anti-
apoptotic and neurotrophic pathways (section 2.5). In addition, n-3 PUFA may also act through
anti-neuroinflammatory pathways. Studies in non-neural tissues have demonstrated that EPA and
DHA are anti-inflammatory PUFAs, in contrast to ARA, which acts predominantly, but not
exclusively, in proinflammatory pathways (Prickett et al 1982; Austen 1987; Yoshino et al 1987;
Stenson et al 1992; Calder 1997). Originally, the anti-inflammatory effects of EPA and DHA
were largely attributed to the inhibition of ARA metabolism by COX and thus the decreased
production of ARA-derived proinflammatory eicosanoids. However, more recently, EPA and
DHA derivatives have been identified that demonstrate potent anti-inflammatory properties
(Serhan et al 2002; Hong et al 2003; Marcheselli et al 2003); these include the aspirin-triggered
EPA resolvins E1 and E2 (RvE1 and RvE2), aspirin-triggered DHA resolvins D1 to D6 (AT-
RvD1 to AT-RvD6), DHA resolvins D1 to D6 (RvD1 to RvD6) and protectin D1 (PD1) (Serhan
et al 2004). Although EPA possesses anti-inflammatory properties and, after chronic feeding,
EPA attenuates IL-1β-induced anxiety-like behavior in rats (Song et al 2008), EPA and its
precursor α-linolenic acid (ALA; 18:3n-3) are present in the brain in low to non-detectable amounts (Igarashi et al. 2007; Chen et al. 2008a). Therefore this review focuses on DHA, the most abundant n-3 PUFA in the brain, and its role in neuroinflammation.

In an ischemia-reperfusion model of stroke, DHA and its derivative PD1 reduced the expression of neuroinflammatory markers (Marcheselli et al. 2003); however, ischemia-reperfusion affects many interconnected pathways, including signal transduction, metabolism, apoptosis, oxidative stress and inflammation (Hossmann 2006). It has not been determined whether DHA acts directly via neuroinflammatory pathways in vivo. As DHA has been established as an anti-inflammatory compound in the periphery and produces potent anti-inflammatory derivatives, it is possible that DHA also exerts these anti-inflammatory effects in the milieu of the CNS.

This review discusses DHA, how its levels are regulated in the brain, potential mechanisms of DHA in neuroinflammation, and how changes in brain DHA levels may impact neuroinflammation. Evidence has indicated that PUFA metabolism and its effects on neuroinflammation should be further investigated as potential targets in neurological disease prevention and treatment.

2.6.3 DHA

DHA is a 22-carbon carboxylic acid containing six cis methylene-interrupted double bonds that begin at the third carbon from the methyl end of the molecule, hence its nomenclature 22:6n-3 (Figure 2.6-1). DHA can be obtained directly from an individual's diet where it is found predominantly in fish oil. In general, fatty and cold water fish contain the highest levels of DHA. DHA can also be synthesized endogenously from ALA, a plant n-3 PUFA, through a series of desaturation and elongation reactions that occur primarily in the liver (Figure 2.6-2). Seed oils
There are two common systems for naming fatty acids. The first is used in chemistry and labels double bonds in the fatty acid by numbering from the carboxylic acid end. Using this system, DHA is 4\textit{cis}, 7\textit{cis}, 10\textit{cis}, 13\textit{cis}, 16\textit{cis}, 19\textit{cis} docosahexaenoic acid (DHA) and arachidonic acid (ARA) is 5\textit{cis}, 8\textit{cis}, 11\textit{cis}, 14\textit{cis} eicosatetraenoic acid. The second system is commonly used in nutritional sciences. Fatty acids are numbered from their methyl terminal end, referred to as the omega (n) end. In DHA, the first double bond begins three carbons from the methyl end, thus DHA is an n-3 polyunsaturated fatty acid, also commonly written as 22:6n-3 (ie, 22 carbons, 6 double bonds, n-3). ARA is an n-6 polyunsaturated fatty acid because its double bonds begin at the sixth carbon from the methyl terminal end, thus ARA is referred to as 20:4n-6. For detailed information on fatty acid nomenclature, see reference (Fahy et al 2005).
The conversion of $\alpha$-linolenic acid (ALA; 18:3n-3) to docosahexaenoic acid (DHA; 22:6n-3) occurs through a series of desaturation and elongation reactions (Voss et al 1991). At each stage, the rate of metabolism is decreased, as represented by arrow size. Most conversions of ALA to DHA is believed to occur in the liver (Rapoport et al 2007). A similar pathway exists for the conversion of linoleic acid (18:2n-6) to arachidonic acid (20:4n-6).
are the richest sources of ALA, particularly flaxseed, rapeseed and chia seed oil (U.S. Department of Agriculture 2007). Aside from their availability in whole foods, there are various commercially available n-3 PUFA supplements that contain DHA, EPA, ALA or a combination of PUFAs. These supplements are available in liquid and gel capsule formulations, and contain purified oils from plants, fish or algae (Arterburn et al 2007).

2.6.4 Brain PUFA uptake

There are currently two major theories for the process of PUFA uptake in the brain: passive diffusion of the unesterified fatty acid and receptor-mediated uptake of lipoproteins (Edmond 2001; Hamilton et al 2007). The latter proposes the involvement of lipoproteins, probably LDLs, and their receptor (Edmond 2001). It has been demonstrated, however, that LDL receptor-mediated uptake is not necessary for maintaining brain PUFA concentrations, suggesting that other mechanisms must exist and are sufficient (Chen et al 2008a).

Once in the neural intracellular compartment, unesterified PUFAs are activated by an acyl-coenzyme A (acyl-CoA) synthetase (Mashek et al 2006; Jia et al 2007). A small portion of activated PUFAs can be β-oxidized, while most PUFAs are esterified via acyl-CoA transferase into 2-lysophospholipids (Robinson et al 1992). In the body, the majority of PUFAs are esterified in the stereospecifically numbered (sn)-2 position of membrane phospholipids. Brain phospholipids are unique from most other tissues because they are abundant in ARA and DHA (Brenna et al 2007). The importance of their metabolism in normal and pathological brain function is discussed in the following sections.
2.6.5 Brain ARA metabolism

ARA is an omega-6 (n-6) PUFA (**Figure 2.6-1**) that is preferentially cleaved from brain phospholipids via group IV calcium-dependent cytosolic phospholipase A2 (cPLA2) and group IIA and V calcium-dependent secretory phospholipase A2 (sPLA2) (Schaloske et al 2006). A portion of the released, unesterified ARA is oxygenated via COX and lipoxygenase (LO) to form eicosanoids (eg, PGs, leukotrienes and lipoxins) (Funk 2001); however, the majority of unesterified ARA is activated by acyl-CoA synthetase and re-esterified into 2-lysophospholipids by acyl-CoA transferase (**Figure 2.6-3**) (Robinson et al 1992; Lee et al 2007b).

There are two COX isoforms: COX-1, which is expressed in most tissues, and COX-2, which is inducible. Relative to the periphery, COX-2 has a high basal expression in the brain (Breder et al 1992; Yamagata et al 1993). In non-pathological conditions, ARA and its derivatives act as neural signaling molecules (Axelrod 1990), for example, in the modulation of memory and learning (Dumuis et al 1988; Patel et al 1990; Nishizaki et al 1999). ARA is released as a secondary messenger in response to 5-HT2A/2C (Felder et al 1990; Basselin et al 2005), D2-like (Piomelli et al 1991; Bhattacharjee et al 2005), muscarinic (Felder et al 1991; Basselin et al 2003) and NMDA (Dumuis et al 1988; Basselin et al 2006) receptor stimulation either via G-protein coupling to phospholipase A2 (PLA2) or indirectly via calcium-stimulated PLA2 activity.

Cell membrane damage and large increases in intracellular free calcium, as observed in ischemia and excitotoxicity, cause increased activity of ARA-selective phospholipases and COX-2 (Bazan et al 1981; Strauss 2008). Increased ARA metabolism by COX-2 following brain injury leads to increases in brain PG levels, particularly PGE2 (Dewitt et al 1988; de Vries et al 1995). PGs can have divergent and sometimes opposing effects on cell signaling and inflammation; however, an
Arachidonic acid (AA; 20:4n-6) is preferentially cleaved from phospholipids (PLs) by calcium-dependent cytosolic phospholipase A2 (cPLA2) or calcium-dependent secretory phospholipase A2 (sPLA2). Once in its unesterified form, AA is available for metabolism by COX-2, among other enzymes. AA is metabolized via COX-2 into largely proinflammatory oxygenated derivatives, including PGs and leukotrienes (LTs). The transcription of enzymes facilitating the production of proinflammatory AA derivatives (cPLA2, sPLA2 and COX-2) is upregulated through the activity of NFκB. NFκB also increases the transcription of proinflammatory cytokines, including TNFα, IL-1β and IL-6. Docosahexaenoic acid (DHA; 22:6n-3) is preferentially cleaved from PLs by calcium-independent phospholipase A2 (iPLA2). In its unesterified form, a putative 15-lipoxygenase (15-LOX) metabolizes DHA into its oxygenated derivative protectin D1 (PD1), which is anti-inflammatory in non-neural tissues and may also be anti-neuroinflammatory. DHA is also metabolized to D-series resolvins (RvDs). Both DHA and PD1 can decrease the activity of NFκB, and levels and activity of COX-2, mechanisms by which they may be anti-neuroinflammatory. + increase, - decrease, acyl-CoA acyl-coenzyme A, IκB IκB protein, IκK IκB kinase, NFκB-RE NFκB response element.

Figure 2.6-3: ARA (AA) and DHA in neuroinflammation
upregulated ARA-COX-2-PG cascade is present in acute neurological disorders, including cerebral ischemia (Miettinen et al 1997) and head trauma (Dash et al 2000), as well as in chronic neurological disorders, including Alzheimer's disease (Pasinetti et al 1998) and Parkinson's disease (Teismann et al 2003) (for a review, see references Bosetti 2007 and Strauss 2008). The ARA-COX-2-PG cascade is also upregulated in mouse brains following the intracerebroventricular injection of LPS, a neuroinflammatory agent (Choi et al 2008). The inhibition of COX-2 with NSAIDs and specific COX-2 inhibitors confers neuroprotection in animal models of cerebral ischemia (Whitehead et al 2007), head trauma (Gopez et al 2005), Alzheimer's disease (Melnikova et al 2006) and Parkinson's disease (Teismann et al 2003); however, neuroprotection by COX-2 inhibitors has not translated well to clinical studies (Aisen et al 2003; Reines et al 2004; Thal et al 2005; Andersohn et al 2006). Regardless, these studies strongly suggest that the proinflammatory ARA-COX-2-PG cascade is deleterious to neurons during brain injury. Although not designed as COX-2 inhibitors, drugs used to treat bipolar disorder downregulate the brain ARA cascade (Lee et al 2007b; Rao et al 2008). Of particular interest is lithium, which blocks LPS-induced ARA signaling (Basselin et al 2007). In support of a role for PG in brain damage, knocking out or pharmacologically inhibiting COX-1 decreased neuroinflammatory markers in a mouse model of neuroinflammation (Choi et al 2008). There is also an ARA shunting hypothesis that argues that while inhibiting COX results in reduced PG formation, it also importantly directs ARA metabolism through alternate metabolic pathways to produce beneficial eicosanoids (Strauss 2008).

2.6.6 Brain DHA metabolism

DHA, like ARA, is released from the sn-2 position of brain phospholipids by PLA2. Although not fully agreed upon, evidence suggests that group VI calcium-independent PLA2 (iPLA2) is a
DHA-selective phospholipase (Green et al 2008). The first evidence for iPLA₂ as a DHA-selective phospholipase was reported in 2003 from an *in vitro* study by Strokin et al, wherein bromoenolactone, an iPLA₂ inhibitor, inhibited DHA, but not ARA release from ATP-stimulated astrocyte membrane phospholipids (Strokin et al 2003). In 2006, Strokin et al confirmed these results, demonstrating that bromoenolactone inhibited oxygen/glucose deprivation-induced DHA release in hippocampal cells (Strokin et al 2006). In 2007, the researchers addressed the issue of bromoenolactone specificity by reproducing the selective inhibition of DHA release with small interfering RNA of group VIB iPLA₂ (Strokin et al 2007).

Once DHA is cleaved from brain phospholipids, it is predominantly activated by an acyl-CoA synthetase and re-esterified into a 2-lysophospholipid by an acyl-CoA transferase. A portion of unesterified DHA is available to function in other pathways. For example, DHA binds to peroxisome proliferator-activated receptors (Keller et al 1993) and inhibits protein kinase C activity (Seung Kim et al 2001). Unesterified DHA can also become oxygenated via COX and LOX, producing derivatives that can act as lipid messengers (*Figure 2.6-3*). There is also evidence that these derivatives have roles in neural cell-cycle regulation and survival (for a review see Bazan 2005 and Calon et al 2007).

*In vitro* studies have suggested that n-3 PUFAs act via anti-apoptotic pathways. The addition of DHA or its oxygenated derivative PD1 to retinal or neural cell cultures under stress increases the expression of anti-apoptotic factors Bcl-2 and Bcl-xL while decreasing the expression of pro-apoptotic factors Bax and Bad (Mukherjee et al 2004; Lukiw et al 2005). Depleting DHA in neural membranes decreases the accumulation of phosphatidylserine, a membrane phospholipid that facilitates the translocation and activation of the anti-apoptotic protein Akt, thus compromising cell survival (Akbar et al 2005). An *in vivo* model in which brain DHA levels are
reduced in rats by dietary means results in decreased expression of BDNF, CREB and p38 MAPK (Rao et al 2007b). The decrease in BDNF by DHA was mediated by p38 MAPK in vitro (Rao et al 2007b). In addition to its role in neural cell-cycle regulation and cell survival (for a review, see references Bazan 2005 and Calon et al 2007), DHA may also play a role in neuroinflammation.

2.6.7 Neuroinflammation

Microglial cells are the main mediators of inflammation in the CNS. They account for 12% of the total brain cell population (Del-Rio Hortega 1932), and are often described as the macrophages of the CNS. When activated by CNS injury or immunological stimuli, microglia transform from their inactive, ramified morphology to an activated, amoeboid morphology. Microglia can be activated by a number of ligands through a number of receptors, all of which lead to an increased activity of NFκB (Block et al 2007). NFκB is an inducible transcription factor that mediates non-pathological processes, but once induced above basal levels, NFκB also plays a critical role in the inflammatory processes of glial and other immune cells (Camandola et al 2007). NFκB activation leads to an increase in the transcription of proinflammatory genes, including TNFα, IL-1β, IL-6, cPLA2 and COX-2 (Block et al 2007), propagating the neuroinflammatory response (Figure 2.6-3).

2.6.8 DHA and its derivatives in inflammation

Many DHA derivatives (DHA docosanoids) have anti-inflammatory properties. For example, the colons of transgenic fat-1 mice, which endogenously produce n-3 PUFA (Kang et al 2004), contain increased levels of EPA and DHA. Compared with the colons of wild-type animals on an n-3 PUFA-deprived diet, the colons of fat-1 mice had increased levels of EPA- and DHA-derived resolvins, and decreased colonic injury in response to an inflammatory insult (Hudert et
al 2006). n-3 PUFA enriched colons had decreased NFκB activity, and decreased levels of TNFα, IL-1β and inducible nitric oxide synthase.

In an acute inflammatory model of peritonitis, PD1 and a mixture of DHA resolvins (also known as 17S series resolvins) reduced TNF-α-induced leukocyte infiltration (Hong et al 2003). Another peritonitis study by the same research group demonstrated that PD1 promotes macrophage phagocytosis of polymorphonuclear neutrophils in vitro and in vivo (Schwab et al 2007).

In a model of oxygen-induced retinopathy, mice with increased n-3 PUFA levels via dietary means or the fat-1 transgene had decreased retinal damage (defined by vaso-obliteration and neovascular area) when compared with wild-type mice fed an n-3 PUFA-deprived diet (Connor et al 2007). The protective effects of n-3 PUFA enrichment were replicated in chow-fed mice by an injection (ip) of RvE1, RvD1 or PD1, but not vehicle, suggesting that docosanoid derivatives are, at least in part, responsible for the attenuation in retinal damage (Connor et al 2007). Furthermore, TNF-α expression was lower in the n-3 PUFA diet-fed and fat-1 transgenic mice (Connor et al 2007), suggesting that the protective effect was mediated in part by anti-inflammation.

There is a lack of studies investigating the effects of DHA derivatives on neuroinflammation (Farooqui et al 2007). The one study examining DHA derivatives in the brain used a model of stroke. In this study, chronic infusions of DHA or PD1 into the third cerebral ventricle prior to middle cerebral artery occlusion significantly decreased inflammatory markers, including leukocyte infiltration, NFκB activity and COX-2 expression (Marcheselli et al 2003). PD1 had a more pronounced effect compared with DHA on each inflammatory mediator, leading the
researchers to suggest that the effects on markers of neuroinflammation in the DHA-infused group may be due to DHA metabolism to PD1. Furthermore, when directly compared in vivo or in cell culture, PD1 was more potent than DHA (Marcheselli et al 2003; Lukiw et al 2005).

Collectively, animal and in vitro studies suggest that DHA derivatives are potent agonists of inflammation resolution in the periphery. There is evidence that n-3 PUFAs are also anti-inflammatory in humans (Calder 2007). Studies in healthy individuals have correlated dietary fish oil or n-3 PUFA supplementation with attenuated cytokine production and T-lymphocyte proliferation (Meydani et al 1991; Endres et al 1993; Thies et al 2001). Most studies in patients with rheumatoid arthritis have also demonstrated beneficial effects of n-3 PUFA supplementation (Volker et al 2000; Adam et al 2003; Remans et al 2004; Berbert et al 2005; Calder 2006), while studies in patients with Crohn's disease have produced inconsistent results (Belluzzi et al 1996; Lorenz-Meyer et al 1996; Romano et al 2005). Despite these inconsistencies, n-3 PUFAs demonstrate promise as a safe adjunctive therapy for inflammation-driven disease.

Dietary and supplemental studies, although valuable, cannot differentiate the effects of DHA and docosanoids. As importantly, they also cannot distinguish between the effects of EPA and DHA, which is of particular interest in peripheral inflammation. Three studies already discussed that used separate n-3 PUFA and n-3 PUFA derivative treatments provide some insight, although their experimental groups were not designed to differentiate the effects of the PUFAs and their derivatives (Hong et al 2003; Marcheselli et al 2003; Connor et al 2007). For example, the contribution of non-oxygenated DHA to anti-inflammation is not clear. Future studies are needed in this area to investigate the effects of each compound in neuroinflammation, and from this
information optimal supplementation compositions can be designed and drug targets can be identified.

2.6.9 Brain DHA metabolism in response to dietary modification

In healthy humans, the brain half-life of DHA is estimated to be 773 days or 2.1 years (Rapoport et al 2007). The length of time n-3 PUFA supplements must be administered to achieve an increase in brain DHA levels is unknown, but the long half-life of DHA indicates that the human brain is resistant to changes in DHA levels. Rapoport et al calculated that with no DHA present in the plasma, it would take 41 days to reduce brain DHA levels by 5% (Rapoport et al 2007). While the clinical effects of a 5% change in DHA levels in the brain are unknown, certain populations are suggested to be chronically n-3 PUFA deprived (Denomme et al 2005). Importantly, the length of time required to replete brain DHA levels, or at what time point following repletion clinical benefits will be observed in humans are unknown.

Animal studies have established that chronic dietary n-3 PUFA deprivation decreases brain levels of DHA both in phospholipids and in its unesterified form (Contreras et al 2000; DeMar et al 2004; DeMar et al 2006b; Igarashi et al 2007). These studies also demonstrated that brain DHA is conserved in times of n-3 PUFA deprivation. Dietary n-3 PUFA deprivation in rats for 15 weeks decreased brain DHA concentration while dramatically increasing brain DHA half-life from 33 days (in n-3 PUFA adequate rats) to 90 days (DeMar et al 2004). A succeeding study offered insight into mechanisms of DHA preservation, as iPLA₂ activity, protein and mRNA expression were significantly decreased in n-3 PUFA-deprived rats (Rao et al 2007a). If iPLA₂ is downregulated, less DHA could be available for, and lost to, oxygenation and β-oxidation. Decreased levels of DHA, as well as docosanoids have been detected in the retina in response to low n-3 PUFA intake (Connor et al 2007). While RvD1 and PD1 have been identified in murine
and human neural tissue (Hong et al 2003; Mukherjee et al 2007), evidence for dietary modulation of brain docosanoid levels has yet to be reported; however, levels of DHA and PD1 are decreased in postmortem hippocampi samples from patients with Alzheimer's disease (Lukiw et al 2005). If DHA and the docosanoids regulate neuroinflammation as potently as peripheral inflammation, then a decrease in their levels could lead to injury-susceptible brain tissue.

One intriguing result of the 15-week rat feeding model is that n-3 PUFA-deprived rats have increased expression of enzymes of the ARA cascade, including cPLA$_2$, sPLA$_2$ and COX-2 (Rao et al 2007a). Two of three studies using an n-3 PUFA-deprived dietary model reported no change in phospholipid ARA concentrations, although DHA levels were reduced by 37% on average (DeMar et al 2004; DeMar et al 2006b; Igarashi et al 2007); however, elevated cPLA$_2$, sPLA$_2$ and COX-2 suggest that the turnover of ARA within brain phospholipids increases during n-3 PUFA deprivation, although kinetic studies are needed to directly confirm this (Lee et al 2007a; Lee et al 2008). If low levels of brain DHA result in upregulated ARA-COX-2-PG metabolism, then DHA deprived brains could be prone to strong proinflammatory signals in addition to weak anti-inflammatory signals that result from low levels of DHA and its docosanoid derivatives.

2.6.10 Conclusions

In rodents, low dietary n-3 PUFA intake downregulates brain DHA metabolism and upregulates markers of the brain ARA-COX-2-PG cascade. Based on these changes in PUFA metabolism, and the growing knowledge of DHA-derived docosanoids and their role in inflammation, it is hypothesized that brains low in DHA are more susceptible to neuroinflammation. This is important, as neuroinflammation is a common underlying pathology in many neurological disorders (Combs et al 2000; Kim et al 2006; Aktas et al 2007). The modulation of DHA
metabolism and its effects on neuroinflammation require further investigation, as it is a promising target pathway for the prevention and treatment of neurological disorders.

2.7 N-3 polyunsaturated fatty acids in animal models with neuroinflammation

Adapted from:


2.7.1 Abstract

Neuroinflammation is present in the majority of acute and chronic neurological disorders. Excess or prolonged inflammation in the brain is thought to exacerbate neuronal damage and loss. Identifying modulators of neuroinflammation is an active area of study since it may lead to novel therapies. n-3 PUFA are anti-inflammatory in many non-neural tissues; their role in neuroinflammation is less studied. This review summarizes the relationship between n-3 PUFA and brain inflammation in animal models of brain injury and aging. Evidence by and large shows protective effects of n-3 PUFA in models of sickness behaviour, stroke, aging, depression, Parkinson’s disease, and diabetes, cytokine- and irradiation-induced cognitive impairments. However, rigorous studies that test the direct effects of n-3 PUFA in neuroinflammation *in vivo* are lacking. Future research in this area is necessary to determine if, and if so which, n-3 PUFA directly target brain inflammatory pathways. N-3 PUFA bioactive metabolites may provide novel therapeutic targets for neurological disorders with a neuroinflammatory component.
2.7.2 Introduction

Inflammation is a beneficial innate response to insults and injuries; however, an overly robust or chronic inflammatory response can contribute significantly to tissue damage. Excessive inflammation in the brain is thought to exacerbate acute injuries including stroke (Iadecola et al 2011), and chronic diseases including multiple sclerosis and Alzheimer’s disease (Akiyama et al 2000; Stadelmann et al 2011). Elucidating neuroinflammatory pathways and identifying modulators is a growing area in brain research, since it may provide novel targets in disease prevention and treatment.

The resolution of inflammation was originally considered a passive process that occurs through the dissipation of pro-inflammatory signals. More recently has it become clear that inflammation resolution is an active process, driven by its own mediators (Serhan et al 2008). The most studied of these signalling molecules are SPM, which are enzymatically derived from n-3 PUFA. SPM include EPA-derived resolvins, and DHA-derived resolvins, protectins, and maresins (Bannenberg et al 2010). Through targeting inflammation resolution, SPM provide a potential mechanism to explain the benefits of n-3 PUFA seen in the prevention and treatment of neurodegenerative diseases (Kalmijn et al 1997; Barberger-Gateau et al 2002; Morris et al 2003; Barberger-Gateau et al 2007; Gao et al 2007).

DHA is essential for neural and retinal development (Hoffman et al 2009), and is highly enriched in brain compared to most other tissues, making up approximately 0.33% brain wet weight in adult human cortex (10-12% of total fatty acids) (Martinez 1992; Igarashi et al 2010). DHA is similarly enriched in the brains of other animals, including non-human primates (Diaz et al 2005), rats (DeMar et al 2006a; Chen et al 2011), and mice (Ort et al 2010; Lalancette-Hebert et al 2011). EPA and its shorter chain n-3 PUFA precursor, ALA, are present at very low levels in
the brain (Orr et al. 2010; Chen et al. 2011; Lin et al. 2011). Rapid metabolism via β-oxidation appears to be a mechanism by which EPA and ALA are maintained at concentrations 200-500 fold lower than DHA (Demar et al. 2005; Chen et al. 2011), but what is unclear is the functional reason for their low levels in the brain. To date, mechanistic evidence from animal models shows that DHA is anti-apoptotic (Akbar et al. 2005; Lukiw et al. 2005), neurotrophic (Rao et al. 2007b), and important for synaptic plasticity (Lafourcade et al. 2011). There are, however, no studies testing the direct effects of DHA on the innate brain inflammatory response. The next section of this thesis reviews the effect of n-3 PUFA modulation in animal models of brain injury and disease where inflammation is also measured (Table 2.7-1). In vitro research in this area is also briefly reviewed (for an in-depth review see Hjorth et al. 2012). Together, these models provide persuasive but indirect evidence of anti-inflammatory actions of n-3 PUFA in the brain.

2.7.3 n-3 PUFA and in vitro neuroinflammation

Microglial cells are the main immune cell mediators of the brain (Block et al. 2007). BV-2 cell cultures are immortalized microglial cells. In BV-2 cells, 3-30 μM DHA or EPA attenuates interferon-γ (IFN-γ)-induced up-regulation of pro-inflammatory marker gene expression, including iNOS, COX-2, IL-6, and TNF-α (Lu et al. 2010). BV-2 microglial cells stimulated with LPS have increased mRNA and protein levels of iNOS, COX-2, IL-1β, and TNF-α, all of which are dose-dependently attenuated by the addition of 10, 20, or 30 μM of EPA (Moon et al. 2007). Insight into the mechanism of action of DHA was provided by De Smedt-Peyrusse et al. who demonstrated that 30 μM DHA significantly downregulated the cell-surface expression of CD14 and toll-like receptor 4 (TLR4) in LPS-stimulated BV-2 microglial cells concomitant to reducing TNF-α protein expression, mature IL-1β protein expression, and activation of the pro-inflammatory gene transcription factor, NFκB (De Smedt-Peyrusse et al. 2008). CD14 and TLR4
are co-receptors that bind LPS to initiate an innate immune response. Interestingly, EPA did not reduce CD14 or TLR4 cell-surface expression (De Smedt-Peyrusse et al 2008).

In C6 glioma cells, an astrocyte cancer cell line, EPA concentrations of 25-100 μM dose-dependently attenuated IL-1β-induced IL-6 gene expression, an effect also observed with 50 μM ARA or DHA (Kawashima et al 2008). The effect of EPA on IL-6 was blocked by peroxisome proliferator-activated receptor-γ (PPARγ) and PPARα antagonists, suggesting PPAR signalling is necessary for the anti-inflammatory effects of EPA (Kawashima et al 2008). Similar effects of EPA were reported in primary hippocampal glial cell cultures from 1-day old rats, where 50 μM EPA increased IL-4 protein and mRNA, and attenuated subsequent LPS-induced increases in IL-1β protein (Lynch et al 2007). In the same cell model, 75 μM EPA reduced LPS- and Aβ-induced increases in IL-1β protein (Minogue et al 2007). This effect was partially blocked by a PPARγ antagonist (Minogue et al 2007). Also using glial cell cultures from 1-day old rats, Pan et al. observed that 75 but not 10 μM DHA co-treatment with LPS/IFN-γ-induced attenuated increases in IL-6 protein (Pan et al 2009). Pre-treating with 75 μM DHA was even more effective at inhibiting LPS/IFN-γ-induced IL-6 protein expression (Pan et al 2009), suggesting incorporation of DHA into phospholipid membranes may optimize immune modulation.

We identified three studies that measured the neuroinflammatory effects of SPM in vitro. In human neuronal-glial primary cell cultures, 10,17S-dihydroxy-DHA, a 15-lipoxygenase metabolite of DHA, downregulated COX-2, TNF-α, and IL-1β expression while increasing PPARγ activity in a Aβ42-induced cell culture model of Aβ-deposition, which is a major pathological feature of Alzheimer’s disease (Lukiw et al 2005; Zhao et al 2011). In the same model of Aβ42-induced Aβ-deposition, DHA downregulated COX-2, TNF-α, and IL-1β
expression (Lukiw et al 2005). In IL-1β-stimulated human neural progenitor cells, 10,17S-dihydroxy-DHA downregulated NFκB activation and COX-2 gene expression; however, there was no effect of 0.03-3000 nM DHA on NFκB activation in response to IL-1β (Marcheselli et al 2003).

*In vitro* studies support the hypothesis that n-3 PUFA and their derivatives are anti-inflammatory in the brain, and suggest that they target TLR4 signalling, and NFκB and PPAR pathways. Importantly, however, unesterified DHA occurs at concentrations of around 1-6 μM in brain and 1-40 μM in plasma; and phospholipid DHA occurs at concentrations of 10-150 μM in plasma and 7,000-14,000 μM in the brain of rats and mice (DeMar et al 2006a; Igarashi et al 2007; Igarashi et al 2011; Ramadan et al 2012; Trepanier et al 2012). EPA occurs at even lower concentrations, particularly in the brain where unesterified EPA occurs below detection limits, and phospholipid EPA concentrations range from being undetectable to 115 μM (Igarashi et al 2007; Chen et al 2011; Igarashi et al 2011). Plasma unesterified and phospholipid EPA concentrations both range from undetected to 9 μM (Igarashi et al 2007; Chen et al 2011; Igarashi et al 2011). Whether these pathways are affected by DHA or EPA at fatty acid concentrations seen in brain tissue is not clear, and warrants *in vivo* study.

### 2.7.4 n-3 PUFA and neuroinflammation induced by systemic lipopolysaccharide

We identified three studies that used intraperitoneally (i.p.) injected LPS to test the effects of n-3 PUFA on sickness behaviour and/or brain inflammation. In the first, rats consumed one of four diets: standard chow, standard chow plus 50 mg/day of ethyl-EPA, standard chow plus 50 mg/day of ethyl-gamma linolenic acid (GLA), or standard chow plus 50 mg/day total of ethyl-
EPA and ethyl-GLA for 4 weeks prior to 100 μg/kg i.p. LPS-induced hippocampal inflammation (Kavanagh et al 2004). No dietary treatment protected against i.p. LPS-induced hippocampal IL-1β protein expression; however, all treatment diets prevented the reduction in hippocampal protein of anti-inflammatory cytokines IL-4 and IL-10 observed in rats fed standard chow. Compared to standard chow, ethyl-EPA and ethyl-EPA + ethyl-GLA supplemented rats significantly increased i.p. LPS-induced hippocampal protein expression of the anti-inflammatory transcription factor PPARγ (Kavanagh et al 2004). In a similar study, the consumption of 500 mg/day ethyl-EPA for 4 weeks did attenuate hippocampal increases in IL-1β protein induced by 100 μg/kg i.p. LPS (Lonergan et al 2004).

While examining i.p. LPS-induced sickness behaviour, Mingam et al. measured IL-6 in mice consuming isocaloric diets containing or deprived of n-3 PUFA (2008). One diet included African peanut oil, rich in linoleic acid (18:2n-6; n-6 PUFA diet), and the second diet included African peanut oil plus rapeseed oil, rich in ALA (n-3 PUFA + n-6 PUFA diet). Mice consumed treatment diets from gestation to 8 weeks of age and sickness behaviour was induced by 30 μg/kg i.p. LPS administration (Mingam et al 2008). Mice consuming n-3 + n-6 PUFA had 2 to 3 fold higher cortical phospholipid DHA compared to mice fed n-6 PUFA alone. There was no consistent effect of diet on LPS-induced hippocampal IL-6 mRNA expression (Mingam et al 2008). Overall, there is little evidence that increased DHA attenuates neuroinflammation following systemic LPS, bearing in mind there is a lack of studies. Ethyl-EPA and ethyl-GLA treatments appear to confer protection against neuroinflammation during systemic inflammation.
2.7.5 n-3 PUFA and neuroinflammation induced by brain ischemia-reperfusion

Inflammation is a major and detrimental component of stroke (Iadecola et al 2011). Not surprisingly, then, inflammation is a common outcome in brain ischemia-reperfusion animal models. Seven studies have measured the effect of increased n-3 PUFA or n-3 PUFA derivatives on brain inflammation resulting from brain ischemia-reperfusion. Only one study tested chronic dietary modulation of n-3 PUFA. In this paper, Lalancette-Hébert et al. used a 1-hour middle cerebral arterial occlusion and found mice with higher brain DHA levels had attenuated infarct areas, decreased \textit{in vivo} innate immune responses measured by TLR2-linked luciferase, and downregulated protein expression of IL-1\textbeta, IL-6, COX-2 (Lalancette-Hebert et al 2011). Similarly, Pan et al. reported 6 weeks of daily i.p. injection of DHA (500 nmol/kg body weight) significantly attenuated infarct volume, leukocyte infiltration, and protein levels of IL-6 in rats (Pan et al 2009). Two other treatment groups received single i.p. injection treatments (500 nmol/kg body weight), either 1 hour or 3 days prior to ischemia. Single injections at either time-point mimicked the effect of the 6-week chronic treatment group (Pan et al 2009). There was no effect of 100 nmol DHA/kg body weight in any of the three treatments (Pan et al 2009). No DHA measures were reported in this study, making it difficult to determine if brain or plasma accretion of DHA were similar between treatments, which might explain the similar responses.

Four studies have infused DHA post-injury, by i.c.v. (Marcheselli et al 2003), i.v. (Belayev et al 2009; Belayev et al 2011), or i.p. (Yang et al 2007) routes. Yang et al. reported that DHA exacerbated infarct volume, loss in motor activity, leukocyte infiltration, and COX-2 expression (Yang et al 2007). A similar exacerbation of pathology occurred in rats treated with ARA, but not stearic acid (18:0). Oxidative damage increased in the ARA and DHA groups, but not the saturated fat-treated group; suggesting that PUFA contribute to oxidative stress (Yang et al
In contrast, the three other studies administering DHA post-ischemia found protective effects of DHA on infarct volume, behavioural deficits, and neurological scores (Marcheselli et al 2003; Belayev et al 2009; Belayev et al 2011). Interestingly, the therapeutic window of post-ischemic DHA administration was identified as 3-5h; by 6h there was no longer an effect of treatment (Belayev et al 2011). One study reported a decrease in leukocyte/neutrophil infiltration, NFκB activation, and COX-2 expression in DHA-treated animals (Marcheselli et al 2003), while the others found an increase in GFAP in DHA-treated animals (Belayev et al 2009; Belayev et al 2011) concurrent to fewer activated microglia (Belayev et al 2011). Whether GFAP expression is beneficial or detrimental in studies of brain injury is not agreed upon. GFAP is often reported as a detrimental marker of neuroinflammation (Piermartiri et al 2010; Bousquet et al 2011; Raza et al 2011; Javed et al 2012), but was reported as beneficial in the work by Belayev et al (Belayev et al 2009; Belayev et al 2011).

While DHA can be protective in animal models of ischemia, the mechanism is unclear. One hypothesis is that DHA-derived SPM beneficially modulate immune responses to brain injury. Evidence for this comes from three studies. Marcheselli et al. reported that i.c.v. infusion of PD1/NPD1 following brain ischemia reduced infarct volume, leukocyte infiltration, NFκB activation, and COX-2 expression (Marcheselli et al 2003). Similar, but less robust effects were seen when infusing DHA at much higher doses, demonstrating the potency of PD1/NPD1 compared to DHA itself (Marcheselli et al 2003). Belayev et al. report increased levels of PD1/NPD1 and a stable marker of its 17S-hydroperoxy-DHA precursor, 17-HDHA, in the penumbra of rats treated with DHA post-ischemia (Belayev et al 2011). PD1/NPD1 and 17-HDHA production is inversely associated with stroke damage, neurological impairments, and cellular markers of neuroinflammation (Belayev et al 2011). In a third study, i.v. infusion of the
aspirin triggered (AT) epimer of PD1/NPD1, AT-NPD1 (10,17R-dihydroxy-DHA), in both sodium salt and methyl ester forms, is similarly protective against stroke damage as NPD1, and similarly reduces cellular markers of neuroinflammation (Bazan et al 2012).

Taking all these studies into consideration, DHA is protective in animal models of stroke where it also attenuates neuroinflammation. However, there is some controversy around the benefit of post-ischemic treatment. In these models DHA acts, at least in part, through its 10,17-dihydroxy-DHA derivatives.

2.7.6 n-3 PUFA and neuroinflammation in aging

Cognitive decline with aging is associated with neuroinflammation and an increase in pro-inflammatory cytokines, including IL-1β (Martin et al 2002; Ownby 2010). In rats, consumption of a 10 then 20mg/day ethyl-EPA-enriched diet over 8 weeks prevents age-induced increases in cortical and hippocampal IL-1β protein (Martin et al 2002; Maher et al 2004), and the age-induced decrease in cortical IL-4 protein (Maher et al 2004). Consumption of 125 mg/day ethyl-EPA for 4 weeks attenuated age-induced increases in hippocampal IL-1β mRNA and protein, IFN-γ protein, and age-induced decreases in IL-4 mRNA and protein (Lynch et al 2007). The same EPA treatment protected aged rats (22 months old) from further increases in hippocampal IL-1β protein induced by Aβ (Lynch et al 2007). Giving mechanistic insight, 4 weeks of 125 mg/day EPA consumption attenuated age-induced decreases in PPARγ protein and Aβ-induced decreases in PPARγ in adult (3 months old) rats (Minogue et al 2007).

Lifelong consumption of the plant n-3 PUFA, ALA, increased cortical DHA levels in adult (3-5 month old) and aged (19-23 month old) mice compared to mice that consumed an n-3 PUFA deprived diet (Moranis et al 2011). Increased cortical DHA protected against depressive-like
symptoms, but did not prevent age-related memory deficits, and did not dampen age-related increases in cortical IL-6 or decreases in IL-10 (Moranis et al 2011). Another aging study by the same researchers fed 1 month old or 20 month old mice an EPA and DHA enriched diet for 2 months and compared them to mice fed the ALA-containing diet used previously (Moranis et al 2011). All mice consumed the ALA-containing diet until 1 or 20 months of age (Labrousse et al 2012). Mice fed the EPA+DHA diet were protected against age-induced spatial memory deficits, and reduced neuronal activity as measured by c-Fos (Labrousse et al 2012). EPA+DHA-fed mice also had less TNF-α, IL-6, and CD11b mRNA expression in the hippocampus compared to ALA-fed mice (Labrousse et al 2012). Collectively, these two studies show that feeding ALA and EPA+DHA can protect against age-induced spatial memory deficits, with EPA+DHA providing more protection than ALA. EPA+DHA feeding is also more effective at reducing age-induced neuroinflammation. Since brain n-3 PUFA levels were reported in different brain regions and fractions between these two studies it cannot be determined if brain n-3 PUFA levels help explain these phenomena.

2.7.7 n-3 PUFA and neuroinflammation in other models

The effect of n-3 PUFA supplementation on inflammation has also been studied in the olfactory bulbectomized rat model of depression (Song et al 2009). The majority of inflammatory markers were assayed in serum; however, cPLA2 mRNA and protein activity were measured in the hypothalamus. cPLA2 is an enzyme of the ARA cascade that increases during neuroinflammation and thus can be used as neuroinflammatory marker (Aid et al 2011). A 1% EPA diet for seven weeks protected olfactory bulbectomized rats from the increased cPLA2 mRNA and activity that was observed in palm oil-fed olfactory bulbectomized rats (Song et al 2009). There was also an
attenuation of systemic inflammatory measures and depressive-like behavioural measures in EPA-fed mice.

Neuroinflammation is present in Parkinson’s disease (Whitton 2007), and in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) animal model of Parkinson’s disease (Barnum et al 2010). Compared to a palm oil-supplemented chow, consumption of a 0.8% ethyl-EPA-supplemented chow for 6 weeks protected mice against MPTP-induced hypokinesia and other behavioural deficits (Luchtman et al 2012). Mice that consumed ethyl-EPA also had no increase in striatal TNF-α, IFN-γ, or midbrain IL-10, while mice consuming palm oil had increases in all three (Luchtman et al 2012). Conversely, fat-1 transgenic mice, which endogenously convert n-6 to n-3 PUFA, are not protected against MPTP-induced dopaminergic injury compared to their wildtype littermates (Bousquet et al 2011). However, secondary analysis that grouped fat-1 and wildtype mice found a significant inverse correlation between brain DHA levels and nigral dopaminergic damage (Bousquet et al 2011). In the substantia nigra (SN) LPS injection model of Parkinson’s disease, rats fed a 15% fish oil diet for 2 weeks prior to SN-LPS were protected against SN dopaminergic injury, microglial activation, and TNF-α and IL-1β protein expression (Ji et al 2012). Overall, dietary or brain DHA levels appear to be protective against dopaminergic damage and neuroinflammation in models of Parkinson’s disease.

Cytokines are endogenously produced molecules that act as autacoids and paracoids to coordinate immune responses. IL-1β is a pro-inflammatory cytokine produced by astrocytes, microglia, and neurons in response to injury or infection. Two studies have examined the modulatory effects of n-3 PUFA on memory impairment induced by an i.c.v. injection of IL-1β, and also measured brain inflammation. In the first, rats consumed diets containing 5% coconut oil (n-3 PUFA deprived, n-6 PUFA low), 5% soybean oil (n-3 PUFA low, n-6 PUFA rich), 4.8%
coconut oil + 0.2% ethyl-EPA, or 4% coconut oil + 1% ethyl-EPA. After 7 weeks of dietary treatment, 15 ng IL-1β was injected i.c.v. on 3 different days throughout 7 days of behavioural testing (Song et al 2004). One hour following the final IL-1β treatment, brains were removed and hippocampal PGE2 levels were measured. Rats consuming the 1% ethyl-EPA diet, but not the 0.2% diet, had decreased IL-1β-induced PGE2 compared to rats on the coconut oil diet. In a subsequent study by the same group, 0.5% ethyl-EPA diet consumption was found to protect rats against i.c.v. IL-1β-induced increases in PGE2 concentrations and decreases in IL-10 protein in several brain regions including the hippocampus (Song et al 2008). In a different locally-induced rat model of neuroinflammation, four-week consumption of 125 mg/day ethyl-EPA attenuated i.c.v. Aβ-induced increases in IFN-γ and IL-1β protein (Minogue et al 2007).

Irradiation as a therapy for cancers, particularly brain tumors, can lead to cognitive deficits that animal studies suggest to be the result of neuronal apoptosis (Soussain et al 2009). Inflammation has been investigated as a mechanism contributing to brain damage following irradiation, and one study has looked specifically at the effects of n-3 PUFA supplementation on brain inflammation pathways following irradiation (Lynch et al 2003). Four weeks on a 1% (250 mg/d) or 2% (500 mg/d) EPA diet significantly attenuated hippocampal inflammation in rats following gamma irradiation (Lynch et al 2003). EPA-fed rats had attenuated irradiation-induced increases in IL-1β and IL-1 receptor proteins, along with attenuated irradiation-induced decreases in IL-10 compared to n-3 PUFA deprived controls (Lynch et al 2003).

Cognitive deficits are associated with diabetes (Stewart et al 1999; Reijmer et al 2010). In a streptozotocin-induced model of diabetes, daily gavaging 13.3 mg/kg/d of DHA for 12 weeks protected rats from spatial learning and memory deficits, though DHA did not protect rats from diabetes-associated weight loss or glycemia (Alvarez-Nolting et al 2012). DHA treatment
increased neurogenesis, and decreased neuronal loss and oxidative stress in the hippocampus. NFκB activation is also attenuated in the hippocampi of DHA treated rats, suggesting that they have less neuroinflammation (Alvarez-Nolting et al 2012).

2.7.8 Conclusion

This review has summarized data on the effects of n-3 PUFA on brain inflammation outcomes in animal brain injury models of sickness behaviour, stroke, cognitive impairment, Parkinson’s disease, depression, aging, and irradiation. N-3 PUFA are anti-neuroinflammatory in all of these brain disease models. However, we cannot conclude that n-3 PUFA are acting directly in neuroinflammatory pathways, since there was also a consistent attenuation of the primary injury (i.e. systemic inflammation, ischemic area, dopaminergic system injury, behavioural and cognitive deficits). While these results are promising because disease models are likely more relevant to human pathologies, studies on the effects of n-3 PUFA in models of direct and focused neuroinflammation are necessary. Further, there is a paucity of studies on the mechanisms of DHA or EPA on neuroinflammation pathways in vivo. It is hypothesized that n-3 PUFA are anti-inflammatory via their enzymatically-derived metabolites, however comprehensive lipidomics profiling during neuroinflammation has yet to be reported in the literature. SPM, SPM receptors, and the pathways that they mediate should be investigated as they may provide novel targets for the modulation of neuroinflammation.
<table>
<thead>
<tr>
<th>Authors (year)</th>
<th>Injury Model</th>
<th>Species</th>
<th>Comparison Treatment</th>
<th>PUFA Treatment(s)</th>
<th>Treatment Duration/Time point</th>
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Table 2.7-1: Summary of neuroinflammatory outcomes in animal models of brain injury
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<th>Treatment</th>
<th>Control</th>
<th>Intervention</th>
<th>Time Post-Infarction</th>
<th>Outcomes</th>
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<td>Dawley rats</td>
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<td>reperfusion</td>
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<td>reperfusion</td>
<td>GFP transgenic mice (C57BL/6)</td>
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<td>3 wks (10 mg/day) + 5 wks (20 mg/day)</td>
<td>↑ LTP ↓ apoptotic markers</td>
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<td>↑ CX DHA</td>
</tr>
<tr>
<td>Study</td>
<td>Treatment/Model</td>
<td>Diet/Condition</td>
<td>Time</td>
<td>Outcome/Effect</td>
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<tr>
<td>Labrousse et al. (2012)</td>
<td>Aging (3* vs 22 mo)</td>
<td>C57BL/6 mice n-3 adequate diet (10.7% LA, 0.9% ALA, 1.6% ALA) n-3 enriched diet (15.2% LA, 0.9% ALA, 10.9% EPA, 7.2% DHA)</td>
<td>2 mo</td>
<td>↑ spatial memory loss, ↑ DG neuronal activity (c-Fos) ↓ brain DHA</td>
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<tr>
<td>Song et al. (2009)</td>
<td>Olfactory bulbectomy</td>
<td>Sprague-Dawley rats 1% palm oil supplemented diet 1% eEPA supplemented diet</td>
<td>7 wks</td>
<td>not reported ↓ depressive-like symptoms (MWM and OF) ↓ hypothalamus cPLA2 mRNA and activity</td>
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<tr>
<td>Bousquet et al. (2011)</td>
<td>MPTP (i.p.)</td>
<td>Fat-1 transgenic mice (C57BL6/C3H) wildtype littermates on high n-6/low n-3 PUFA diet fat-1 transgenic mice on high n-6/low n-3 PUFA diet</td>
<td>life</td>
<td>↑ CX DHA ↔ striatal or nigral dopaminergic injury *↓ hypokinesia and anxiety (RT, PT) ↑ learning and memory (MWM)</td>
<td></td>
</tr>
<tr>
<td>Luchtman et al. (2012)</td>
<td>MPTP-P (s.c.)</td>
<td>C57BL/6 mice chow supplemented with 0.8% eEPA chow supplemented with 0.8% palm oil</td>
<td>6 wks</td>
<td>↑ CX EPA, DPAn-3 ↔ CX DHA ↓ hypokinesia and anxiety (RT, PT) ↑ learning and memory (MWM)</td>
<td></td>
</tr>
<tr>
<td>Ji et al. (2012)</td>
<td>SN LPS (i.c.v.)</td>
<td>Sprague-Dawley rats 15% (wt) corn oil diet 15% (wt) fish oil diet (30% fish oil as EPA and DHA)</td>
<td>2 wks</td>
<td>not reported ↓ dopaminergic injury ↓ nigral dopaminergic neuron degeneration ↓ SN OX42 protein (microglial activation) ↓ SN TNF-α and IL-1β protein ↓ SN p65 (NFκB subunit) protein</td>
<td></td>
</tr>
<tr>
<td>Song and Horrobin (2004)</td>
<td>IL-1β (i.c.v.)</td>
<td>Wistar rats 5% coconut oil diet 5% soybean, 4.8% coconut oil + 0.2% eEPA, or 4% coconut oil + 1% eEPA* diet</td>
<td>7 wks</td>
<td>not reported *↓ memory loss (MWM) *↓ brain PGE2</td>
<td></td>
</tr>
<tr>
<td>Song et al. (2008)</td>
<td>IL-1β (i.c.v.)</td>
<td>Wistar rats 5% palm oil diet 4.5% palm oil + 0.5% eEPA*, 4.5% palm oil + 0.5% eGLA®, or 4% palm oil + 1% AA-rich oil diet</td>
<td>7 wks</td>
<td>not reported *↓ memory loss (MWM) *↓ anxiety (EPM) *↓ HIP PGE2 *↓ amygdala PGE2 *↓ amygdala, hypothalamus IL-10 protein</td>
<td></td>
</tr>
<tr>
<td>Lynch et al. (2003)</td>
<td>Whole body irradiation</td>
<td>Wistar rats chow chow supplemented with 250 mg/d* or 500 mg/d† eEPA</td>
<td>4 wks</td>
<td>not reported *#↓ apoptotic markers #↓ HIP CD11b, TNF-α, IL-6 mRNA ↔ HIP GFAP and IL1-β mRNA</td>
<td></td>
</tr>
<tr>
<td>Alvarez-Nölting et al. (2012)</td>
<td>STZ (i.p.)</td>
<td>Wistar rats</td>
<td>chow*</td>
<td>chow plus 13.3 mg/kg/d DHA by gavage</td>
<td>12 wks</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>---------</td>
<td>-------------</td>
<td>------</td>
<td>---------------------------------</td>
<td>-------</td>
</tr>
</tbody>
</table>

17-HDHA, 17-hydroxy-DHA (DHA derivative); Aβ, amyloid-β; ALA, α-linolenic acid (18:3n-3); ARA, arachidonic acid (20:4n-6); AT-NPD1, aspirin triggered NPD1; COX-2, cyclooxygenase-2; cPLA2, cytosolic phospholipase A2; CX, cortex; DG, dentate gyrus; DHA, docosahexaenoic acid (22:6n-3); DPAn-3, docosapentaenoic acid (22:5n-3); EPA, eicosapentaenoic acid (20:5n-3); eEPA, ethyl-EPA; eGLA, ethyl-gamma-linolenic acid (18:3n-6); EPM, elevated plus maze; FST, forced swim test; GFAP, glial fibrillary acidic protein; HIP, hippocampus; i.c.v., intracerebroventricular; IFN-γ, interferon-γ; IL, interleukin; IL-1RI, IL-1 receptor protein type I; IL-1RAcP, IL-1 receptor accessory protein; IRAK, IL-1 receptor-associated kinase; LA, linoleic acid (18:2n-6); LPS, lipopolysaccharide; i.p., intraperitoneal; LTP, long term potentiation; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPTP-P, MPTP and probenecid; MWM, Morris water maze; MUFA, monounsaturated fatty acid; NFκB, nuclear factor-κB; NPD1, neuroprotectin D1 (DHA derivative); OF, open field; PGE2, prostaglandin E2; PN, penumbra; PPARγ, peroxisome proliferator activated protein γ; PT, pole test; RT, rotorod test; SA, stearic acid (18:0); s.c., subcutaneous; SCX, subcortex; SN, substantia nigra; ST, striatum; TLR2, toll-like receptor 2; TNF-α, tumor necrosis factor-α; wt, weight

*#Δπ indicates treatment group represented in outcome columns (brain n-3 PUFA, behavioural outcome, inflammatory outcome)

EPA lowered COX-2 mRNA compared to palm oil in saline (control) injected animals

# inflammation was primary outcome

Δ main effect of n-3 adequate vs. n-3 deprived diet (no effect of age)

Ψ protection from nigral dopaminergic injury was correlated to brain DHA levels (secondary analysis)

* insulin-treated rats were also included in the study and were similar to DHA-only treated rats in all measures excluding weight, glycemic control, and oxidative stress
2.8 Hypothesis and Objectives

Overall Hypothesis

DHA has anti-inflammatory effects in the brain, mediated, at least in part, by its SPM derivatives.

Specific Objectives:

1. To determine if the fat-1 mouse has a physiologically relevant brain fatty acid profile.

2. To determine if chronic increases in hippocampal DHA, both in the phospholipid and unesterified pools, attenuate neuroinflammation.

3. To determine if acute increases in unesterified DHA or 17S-HpDHA attenuate neuroinflammation.

4. To determine the metabolomics-lipidomic profile of the hippocampus, and if infusing DHA or 17S-HpDHA augments the production of SPM.
CHAPTER 3:
The fat-1 mouse has brain docosahexaenoic acid levels achievable through fish oil feeding
Adapted from:


### 3.1 Abstract

Fat-1 transgenic mice endogenously convert n-6 to n-3 polyunsaturated fatty acids (PUFA). The aims of this study were to test whether a) fish oil feeding can attain similar brain n-3 PUFA levels as the fat-1 mouse, and b) fat-1 mouse brain docosahexaenoic acid (22:6n-3; DHA) levels can be potentiated by fish oil feeding. Fat-1 mice and their wildtype littermates consumed either a 10% safflower oil (SO) or a 2% fish oil and 8% safflower oil chow (FO). Brain total lipid and phospholipid fraction fatty acids were analyzed using GC-FID. Wildtype mice fed FO chow had similar brain levels of DHA as fat-1 mice fed SO chow. Fat-1 mice fed FO chow had similar brain n-3 PUFA levels as fat-1 mice fed SO chow. In conclusion, brain levels of DHA in the fat-1 mouse can be obtained by, and were not further augmented with, fish oil feeding.

### 3.2 Introduction

Docosahexaenoic acid (22:6n-3; DHA) is the most abundant n-3 PUFA in the brain, and is required for normal neuronal development and function (Alessandri et al 2004). Currently, DHA is being investigated for its potential role in the prevention and treatment of neurological disorders (Lukiw et al 2008; Stahl et al 2008) and inflammation driven diseases (Orr et al 2008; Calder 2009). Some but not all studies on human port-mortem brains show lower levels of DHA in Alzheimer’s Disease (Soderberg et al 1991; Skinner et al 1993; Corrigan et al 1998; Prasad et al 1998; Lukiw et al 2005; Fraser et al 2010), schizophrenia (Yao et al 2000; McNamara et al 2000; ...
and major depressive disorder (Lalovic et al 2007; McNamara et al 2007a; McNamara et al 2009).

Although still a matter of debate, evidence suggests that DHA entry into the brain occurs via passive diffusion of the unesterified fatty acid (Hamilton et al 2007; Rapoport et al 2007; Chen et al 2008a). Once DHA has entered the brain, it is acylated via an acyl-CoA synthetase and then esterified into a 2-lysophospholipid by an acyl transferase. The majority of DHA in the brain is found in the sn-2 position of plasma membrane phospholipids. From there it is released via a phospholipase A2 to its unesterified form (Green et al 2008). Studies have shown that unesterified DHA regulates neuronal development (Darios et al 2006), excitability (Nishikawa et al 1994; Poling et al 1995; Poling et al 1996; Vreugdenhil et al 1996), and survival (Lukiw et al 2005). With the ability of DHA to influence brain cell function, it is important to understand how the brain regulates DHA levels.

One aspect of regulation is a proposed plateau, or upper limit, to the amount of DHA in the brain, which has been suggested by previous n-3 PUFA feeding studies (Bourre et al 1989; Hsieh et al 2007). However, other studies have found no plateau in brain DHA levels with increasing dietary n-3 PUFA (Huang et al 2007a). Here we take a novel approach to the question by combining the fat-1 transgenic mouse model with a dietary n-3 PUFA feeding model. Fat-1 mice carry an n-3 fatty acid desaturase gene from Caenorhabditis elegans that allows them to convert n-6 fatty acids to n-3 fatty acids endogenously (Kang et al 2004).

The fat-1 mouse is currently being used to study the role of DHA in the brain (Taha et al 2008; He et al 2009; Boudrault et al 2010). However, it is not known if the fat-1 mouse has supranormal brain levels of n-3 PUFA, or if these levels can be achieved through diet. In order to
determine the relevance of the fat-1 mouse model in brain studies, we compared absolute quantities of n-3 PUFA in fat-1 mice brains to those of wildtype littermates fed an n-3 PUFA containing chow. We found that fat-1 mice had DHA levels attainable by diet alone, and that fish oil feeding did not potentiate brain DHA levels in the fat-1 mouse.

3.3 Experimental Procedure

All procedures were carried out in accordance to the policies set out by the Canadian Council on Animal Care (Canadian Council on Animal Care. 1993) and were approved by the Animal Ethics Committee at the University of Toronto, Toronto, Canada (protocol #20007376). Fat-1 breeders on a C57BL/6 x C3H background were obtained from Dr. Jing X. Kang (Kang et al 2004). Experimental mice were obtained by mating male fat-1 mice with female C57BL/6 wildtype mice from Charles River Laboratories (CRL; Saint-Constant, Quebec, Canada). Female F1 progeny were used in this study. Mice were housed 3–4 per cage in a facility in which temperature (21°C), humidity and light cycle (12 h light/dark) were controlled, and had ad libitum access to food and water.

Wildtype and fat-1 F1 progeny were weaned at 3 weeks of age and randomized to one of two chows that differed only in their fat composition. Both chows consisted of casein 200 g/kg, L-cystine 3 g/kg, corn starch 337 g/kg, maltodextrin-10 132 g/kg, sucrose 100 g/kg, cellulose 50 g/kg, t-butylhydroquinone 0.019 g/kg, mineral mix (S10022G) 35 g/kg, vitamin mix (V10037) 10 g/kg and choline bitartrate at 2.5 g/kg. Fat was composed of 100.4 g/kg safflower oil (10% safflower oil chow; SO) or 20.1 g/kg menhaden oil and 80.3 g/kg safflower oil (2% fish oil, 8% safflower oil chow; FO). SO (D04092701) and FO (D04092702) chows were purchased from Research Diets Inc (New Brunswick, NJ, USA). Dams were fed the SO chow to reduce transfer
of n-3 PUFA to offspring via the placenta or milk. As measured by gas chromatography, the SO chow contained 0.03 and 0.09% of eicosapentaenoic acid (20:5n-3) and DHA, respectively, while the FO chow contained 1.97 and 1.61% of 20:5n-3 and DHA, respectively (Table 3.3-1).

The low amounts of longer chain PUFA in the SO chow are expected given the animal-source of other components of the chow, for instance casein. At 8 weeks of age, mice were euthanized with CO2, their brains excised, halved, flash frozen with liquid nitrogen and then stored at -80°C for further analysis. By 8 weeks, the mouse brain has reached its adult size (Dobbing et al 1979) and brain PUFA concentrations have normalized (Ward et al 1996). Brain half-lives of PUFA in rodents are approximately 1 month (Lee et al 2007a; Lee et al 2007b).

**Table 3.3-1:** Fatty acid percent composition of total fat in 10% safflower oil (SO) and 2% fish oil, 8% safflower oil (FO) chows.

<table>
<thead>
<tr>
<th>Fat composition (g/kg chow)</th>
<th>10% Safflower Oil</th>
<th>2% Fish Oil, 8% Safflower Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menhaden oil</td>
<td>0</td>
<td>20.1</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>100.4</td>
<td>80.3</td>
</tr>
<tr>
<td>FA profile (% of total FA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>0.43</td>
<td>2.88</td>
</tr>
<tr>
<td>16:0</td>
<td>9.34</td>
<td>12.14</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>0.18</td>
<td>3.14</td>
</tr>
<tr>
<td>18:0</td>
<td>2.77</td>
<td>2.97</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>0.51</td>
<td>1.17</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>14.20</td>
<td>12.75</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>71.29</td>
<td>58.70</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.65</td>
<td>0.97</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.17</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>20:5n-3</strong></td>
<td><strong>0.03</strong></td>
<td><strong>1.97</strong></td>
</tr>
<tr>
<td>22:5n-6</td>
<td>0.06</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>22:6n-3</strong></td>
<td><strong>0.09</strong></td>
<td><strong>1.61</strong></td>
</tr>
</tbody>
</table>

Data are % of total fatty acids and the means of triplicate analysis. Fatty acids that are not shown are <1.0% total in both chows. FA; fatty acid.
3.3.1 Genotyping

DNA was extracted from approximately 2-3 mm of mouse tail. Tissue was digested overnight at 55°C with proteinase K and cell lysis buffer (Cell Signaling Technology, Danvers, MA). DNA was then extracted using phenol followed by ethanol precipitation and purification. DNA was resuspended in 25 µl of Tris-EDTA buffer, and DNA extracts were used for genotyping by PCR. The primers used for the fat-1 gene were 5’CTGCACCACGCTTCACCAACC3’ and 5’ACACAGCAGCGATTCCAGAGATT3’. The 25 µl PCR reaction also contained 0.2mM of deoxyribonucleotide mix (Fermentas, R0192), 1.5mM magnesium chloride, and 1.0 units Platinum Taq DNA Polymerase with its buffer (Invitrogen, 10966-018). The thermocycler program consisted of a period of 2 minutes at 94°C followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 1 minute at 72°C. The run was completed with 10 minutes at 72°C, and then cooled to 4°C.

Amplified PCR products were run through a 2% agarose gel containing SYBR Safe DNA Gel Stain (Invitrogen, S33102), and visualized using the Fluorochem image system (model 8000, Alpha Innotech Corp., San Leandro, CA).

3.3.2 Brain Lipid Extraction and Chromatography

Total lipids were extracted from the left brain hemisphere according to the method of Folch (Folch et al 1957). Thin layer chromatography plates (Cat. #10011; Analtech, Newark, DE) were activated by heating at 100°C for 1 h. Phospholipid fractions were separated along with authentic standards in chloroform/MeOH/2-propanol/KCl (0.25% wt/vol)/triethylamine (30:9:25:6:18, by vol). Bands corresponding to choline glycerophospholipids (ChoGpl), ethanolamine glycerophospholipids (EtnGpl), PtdSer and phosphatidylinositol (PtdIns) were
visualized under UV light after lightly spraying with 8-anilino-1-naphthalene sulfonic acid (0.1% wt/vol). A portion of the total lipid extract or phospholipid bands was added into a test tube containing a known amount of heptadecanoic acid (17:0) and converted to fatty acid methyl esters with 14% boron trifluoride/methanol at 100°C for 1 h. Fatty acid methyl esters were quantified on an Agilent 6890N gas chromatograph equipped with flame ionization detection and separated on an Agilent J&W fused-silica capillary column (DB-23; 30 m, 0.25 μm film thickness, 0.25 mm i.d.; Agilent, Palo Alto, CA). Samples were injected in splitless mode. The injector and detector ports were set at 250°C. Fatty acid methyl esters were eluted using a temperature program set initially at 50°C and held for 2 min, increased at 20°C/min and held at 170°C for 1 min, increased at 3°C/min, and held at 212°C for 10 min to complete the run. The carrier gas was helium, set to a 0.7 mL/min constant flow rate. Peaks were identified by retention times of fatty acid methyl ester standards (Nu-Chek-Prep, Elysian, MN). Fatty acid concentrations (nmol/g brain) were calculated by proportional comparison of gas chromatography peak areas to that of the 17:0 internal standard. The chows were also measured according to the method described above.

3.3.3 Statistics

Results are expressed as means ± SD. Fatty acid concentration means were compared by two-way ANOVA with genotype and diet as main factors. Upon finding a significant genotype by diet interaction, one-way ANOVA with Tukey’s post-hoc test was performed. Body and brain left hemisphere weights were analyzed by two-way ANOVA when stratifying by genotype and diet, or Student’s t-test when stratifying by genotype or diet alone. Statistical significance was set as p < 0.05 for all analyses.
3.4 Results

3.4.1 Mouse Body and Brain Region Weights

We found that wildtype and fat-1 females did not have significantly different body weights when stratified by genotype (17.0 ± 2.3 g vs. 17.3 ± 1.6 g, respectively), diet (SO 17.0 ± 2.1 g vs. FO 17.3 ± 1.8 g, respectively), or both. There were no statistically significant differences in brain left hemisphere weight when stratified by genotype (wildtype 241 ± 34 mg vs. fat-1 219 ± 32 mg), diet (SO 222 ± 32 mg vs. FO 239 ± 36 mg), or both.

3.4.2 Brain total lipid and phospholipid fatty acid concentrations

There was a significant interaction of genotype and diet on brain total lipid DHA concentrations (Figure 3.4-1). Wildtype mice fed the SO chow had significantly lower DHA levels in brain total lipids compared to wildtype mice fed the FO chow and fat-1 mice fed either chow. There was no statistical difference in brain total lipid DHA between wildtype mice fed the FO chow and fat-1 mice fed either the SO or FO chows (Figure 3.4-1).

Similar results were found in brain ChoGpl (Table 3.4-1), EthGpl (Table 3.4-2), and PtdSer (Table 3.4-3), where there was a significant interaction of genotype and diet on DHA concentration within each phospholipid fraction. In these three fractions, wildtype mice fed a FO chow did not have significantly different DHA levels compared to fat-1 mice fed either a SO or FO chow. Brain PtdIns showed a significant main effect of genotype and diet on DHA concentrations (Table 3.4-4).
**Figure 3.4-1:** Brain total lipid DHA concentrations of wildtype and fat-1 mice consuming either the 10% safflower oil or 2% fish oil and 8% safflower oil chow.

There was a significant interaction between genotype (wildtype or fat-1) and diet (10% safflower oil or 8% safflower and 2% fish oil) as determined by 2-way ANOVA. Means were compare by 1-way ANOVA, with Tukey’s post-hoc test. Significantly different means (p<0.05) are indicated by different letters.
Table 3.4-1: Brain choline glycerophospholipid fatty acid concentrations (nmol/g) of wildtype and fat-1 mice consuming either the 10% safflower oil or 2% fish oil and 8% safflower oil chow.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Wildtype 10% Safflower Oil</th>
<th>Wildtype 8% Safflower Oil</th>
<th>Fat-1 10% Safflower Oil</th>
<th>Fat-1 8% Safflower Oil</th>
<th>Int. Effect (p-value)</th>
<th>Gene Effect (p-value)</th>
<th>Diet Effect (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>169 ± 51</td>
<td>186 ± 28</td>
<td>145 ± 78</td>
<td>148 ± 40</td>
<td>0.031</td>
<td>0.072</td>
<td>0.021</td>
</tr>
<tr>
<td>16:0</td>
<td>26651 ± 2233</td>
<td>28190 ± 1500</td>
<td>25561 ± 3427</td>
<td>25394 ± 2399</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>7655 ± 795</td>
<td>7897 ± 508</td>
<td>7820 ± 1120</td>
<td>7607 ± 876</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:0</td>
<td>181 ± 67</td>
<td>131 ± 34</td>
<td>263 ± 317</td>
<td>129 ± 32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Σ of Saturates*</td>
<td>34657 ± 3101</td>
<td>36404 ± 1901</td>
<td>33789 ± 4383</td>
<td>33278 ± 3165</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:1n-7</td>
<td>234 ± 72 a</td>
<td>348 ± 18 b</td>
<td>293 ± 39 a,b</td>
<td>314 ± 36 b</td>
<td>0.021</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1n-9</td>
<td>9338 ± 2400</td>
<td>11635 ± 757</td>
<td>11053 ± 1407</td>
<td>11231 ± 861</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:1n-9</td>
<td>195 ± 112</td>
<td>136 ± 140</td>
<td>134 ± 44</td>
<td>171 ± 100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24:1</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Σ of Monounsaturates*</td>
<td>9767 ± 2469</td>
<td>12118 ± 823</td>
<td>11480 ± 1439</td>
<td>11716 ± 885</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2n-6</td>
<td>304 ± 99 a</td>
<td>538 ± 54 b</td>
<td>463 ± 105 b</td>
<td>500 ± 69 b</td>
<td>0.0097</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:2n-6</td>
<td>133 ± 38</td>
<td>161 ± 20</td>
<td>159 ± 36</td>
<td>156 ± 27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:3n-6</td>
<td>35 ± 39 a</td>
<td>177 ± 32 b</td>
<td>157 ± 70 b</td>
<td>144 ± 31 b</td>
<td>0.0005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:4n-6</td>
<td>2242 ± 533</td>
<td>2376 ± 283</td>
<td>2652 ± 656</td>
<td>2252 ± 220</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:4n-6</td>
<td>286 ± 36 a</td>
<td>174 ± 22 b</td>
<td>146 ± 76 b</td>
<td>159 ± 21 b</td>
<td>0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:5n-6</td>
<td>767 ± 259 a</td>
<td>73 ± 79 b</td>
<td>154 ± 376 b</td>
<td>40 ± 63 b</td>
<td>0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Σ of n-6 PUFA*</td>
<td>3767 ± 947</td>
<td>3498 ± 323</td>
<td>3730 ± 1035</td>
<td>3251 ± 260</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3n-3</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:5n-3</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:5n-3</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:6n-3</td>
<td>692 ± 188 a</td>
<td>1864 ± 189 b</td>
<td>1739 ± 239 b</td>
<td>1835 ± 202 b</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Σ of n-3 PUFA*</td>
<td>692 ± 188 a</td>
<td>1864 ± 189 b</td>
<td>1739 ± 239 b</td>
<td>1835 ± 202 b</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mice consumed chows from weaning until 8 weeks of age. nd = not detected. Data are mean ± SD, n= 6 independent samples per group. Main effects and interactions between genotype (wildtype or fat-1) and diet (10% safflower oil or 2% fish oil and 8% safflower) were determined with
2-way ANOVA. When a significant interaction was detected, means were compared with 1-way ANOVA, with Tukey’s post-hoc test. Means with different superscripts within a row are statistically significant, p<0.05. Int; interaction. *Subtotal values may include fatty acids not shown.
Table 3.4-2: Brain ethanolamine glycerophospholipid fatty acid concentrations (nmol/g) of wildtype and fat-1 mice consuming either the 10% safflower oil or 2% fish oil and 8% safflower oil chow.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Wildtype 10% Safflower Oil</th>
<th>2% Fish Oil, 8% Safflower Oil</th>
<th>Fat-1 10% Safflower Oil</th>
<th>2% Fish Oil, 8% Safflower Oil</th>
<th>Int. Effect (p-value)</th>
<th>Gene Effect (p-value)</th>
<th>Diet Effect (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>52 ± 8</td>
<td>45 ± 12</td>
<td>48 ± 14</td>
<td>53 ± 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>2988 ± 279</td>
<td>3035 ± 228</td>
<td>2880 ± 404</td>
<td>3145 ± 531</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>18:0</td>
<td>9073 ± 1031</td>
<td>9080 ± 883</td>
<td>9188 ± 1421</td>
<td>9924 ± 1713</td>
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</tr>
<tr>
<td>20:0</td>
<td>256 ± 76</td>
<td>226 ± 64</td>
<td>280 ± 85</td>
<td>271 ± 83</td>
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<tr>
<td>Σ of Saturates*</td>
<td>12369 ± 1369</td>
<td>12437 ± 1101</td>
<td>12431 ± 1897</td>
<td>13451 ± 2335</td>
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<tr>
<td>16:1n-7</td>
<td>97 ± 20</td>
<td>117 ± 22</td>
<td>113 ± 31</td>
<td>127 ± 33</td>
<td></td>
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</tr>
<tr>
<td>18:1n-9</td>
<td>7506 ± 1878</td>
<td>7716 ± 1124</td>
<td>7474 ± 1402</td>
<td>8840 ± 2611</td>
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<td></td>
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</tr>
<tr>
<td>22:1n-9</td>
<td>143 ± 48</td>
<td>111 ± 36</td>
<td>127 ± 11</td>
<td>125 ± 29</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>24:1</td>
<td>45 ± 24</td>
<td>78 ± 63</td>
<td>58 ± 46</td>
<td>78 ± 18</td>
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<tr>
<td>Σ of Monounsaturates*</td>
<td>7790 ± 1934</td>
<td>7752 ± 1389</td>
<td>8021 ± 1123</td>
<td>9170 ± 2673</td>
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<td></td>
<td></td>
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<tr>
<td>18:2n-6</td>
<td>212 ± 51</td>
<td>283 ± 46</td>
<td>270 ± 76</td>
<td>316 ± 94</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:2n-6</td>
<td>168 ± 42</td>
<td>172 ± 36</td>
<td>204 ± 54</td>
<td>217 ± 59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:3n-6</td>
<td>169 ± 33</td>
<td>281 ± 51</td>
<td>265 ± 53</td>
<td>346 ± 115</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:4n-6</td>
<td>5264 ± 517</td>
<td>4469 ± 440</td>
<td>4992 ± 883</td>
<td>4680 ± 629</td>
<td>0.011</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>22:4n-6</td>
<td>2226 ± 328 ^a</td>
<td>1520 ± 180 ^b</td>
<td>1747 ± 349 ^b</td>
<td>1687 ± 287 ^b</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
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<tr>
<td>22:5n-6</td>
<td>3385 ± 801 ^a</td>
<td>459 ± 339 ^b</td>
<td>228 ± 60 ^b</td>
<td>367 ± 299 ^b</td>
<td>&lt;0.0001</td>
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<td></td>
</tr>
<tr>
<td>Σ of n-6 PUFA *</td>
<td>11445 ± 1651 ^a</td>
<td>7225 ± 698 ^b</td>
<td>7754 ± 1439 ^b</td>
<td>7646 ± 1045 ^b</td>
<td>0.0007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3n-3</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:5n-3</td>
<td>26 ± 5</td>
<td>24 ± 13</td>
<td>26 ± 33</td>
<td>29 ± 16</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>22:5n-3</td>
<td>57 ± 8</td>
<td>188 ± 62</td>
<td>143 ± 40</td>
<td>224 ± 67</td>
<td>0.0073</td>
<td>&lt;0.0001</td>
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</tr>
<tr>
<td>22:6n-3</td>
<td>3941 ± 886 ^a</td>
<td>7721 ± 1234 ^b</td>
<td>7853 ± 1441 ^b</td>
<td>8421 ± 1551 ^b</td>
<td>0.0068</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Σ of n-3 PUFA *</td>
<td>4099 ± 925 ^a</td>
<td>7975 ± 1296 ^b</td>
<td>8106 ± 1513 ^b</td>
<td>8756 ± 1628 ^b</td>
<td>0.009</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mice consumed chows from weaning until 8 weeks of age. nd = not detected. Data are mean ± SD, n= 6 independent samples per group. Main effects and interactions between genotype (wildtype or fat-1) and diet (10% safflower oil or 2% fish oil and 8% safflower) were determined with
2-way ANOVA. When a significant interaction was detected, means were compared with 1-way ANOVA, with Tukey’s post-hoc test. Means with different superscripts within a row are statistically significant, p<0.05. Int; interaction. *Subtotal values may include fatty acids not shown.
Table 3.4-3: Brain phosphatidylserine fatty acid concentrations (nmol/g) of wildtype and fat-1 mice consuming either the 10% safflower oil or 2% fish oil and 8% safflower oil chow.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Wildtype 10% Safflower Oil</th>
<th>2% Fish Oil, 8% Safflower Oil</th>
<th>Fat-1 10% Safflower Oil</th>
<th>2% Fish Oil, 8% Safflower Oil</th>
<th>Int. Effect (p-value)</th>
<th>Gene Effect (p-value)</th>
<th>Diet Effect (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>116 ± 54</td>
<td>128 ± 43</td>
<td>135 ± 25</td>
<td>124 ± 75</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>16:0</td>
<td>875 ± 221</td>
<td>1281 ± 775</td>
<td>970 ± 160</td>
<td>842 ± 288</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>6211 ± 362</td>
<td>6683 ± 1456</td>
<td>5878 ± 655</td>
<td>5248 ± 1638</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>102 ± 13 ^a,b ^</td>
<td>151 ± 50 ^a</td>
<td>112 ± 24 ^a,b ^</td>
<td>89 ± 35 ^b</td>
<td>0.018</td>
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</tr>
<tr>
<td>Σ of Saturates*</td>
<td>7649 ± 560</td>
<td>8574 ± 2213</td>
<td>7396 ± 881</td>
<td>6467 ± 1906</td>
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<td></td>
</tr>
<tr>
<td>16:1n-7</td>
<td>56 ± 36</td>
<td>100 ± 64</td>
<td>82 ± 41</td>
<td>98 ± 59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1n-9</td>
<td>2477 ± 399</td>
<td>3032 ± 841</td>
<td>2715 ± 701</td>
<td>2524 ± 682</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:1n-9</td>
<td>158 ± 31</td>
<td>200 ± 27</td>
<td>196 ± 53</td>
<td>153 ± 68</td>
<td>0.041</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24:1</td>
<td>20 ± 17</td>
<td>5 ± 13</td>
<td>nd</td>
<td>nd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Σ of Monounsaturates*</td>
<td>2711 ± 435</td>
<td>3337 ± 910</td>
<td>2993 ± 745</td>
<td>2776 ± 759</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2n-6</td>
<td>61 ± 14 ^a</td>
<td>134 ± 24 ^b</td>
<td>117 ± 35 ^b</td>
<td>88 ± 36 ^a,b</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:2n-6</td>
<td>80 ± 34 ^a,b</td>
<td>119 ± 53 ^a</td>
<td>87 ± 33 ^a,b</td>
<td>45 ± 12 ^b</td>
<td>0.012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:3n-6</td>
<td>86 ± 39</td>
<td>107 ± 27</td>
<td>119 ± 33</td>
<td>67 ± 26</td>
<td>0.016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:4n-6</td>
<td>351 ± 67</td>
<td>323 ± 61</td>
<td>322 ± 53</td>
<td>206 ± 67</td>
<td>0.013</td>
<td>0.014</td>
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<tr>
<td>22:4n-6</td>
<td>467 ± 102</td>
<td>296 ± 78</td>
<td>328 ± 191</td>
<td>180 ± 44</td>
<td>0.014</td>
<td>0.003</td>
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</tr>
<tr>
<td>22:5n-6</td>
<td>1559 ± 351 ^a ^</td>
<td>317 ± 175 ^b</td>
<td>157 ± 97 ^b</td>
<td>91 ± 57 ^b</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Σ of n-6 PUFA *</td>
<td>2651 ± 508 ^a ^</td>
<td>1319 ± 323 ^b ^</td>
<td>1148 ± 353 ^b ^</td>
<td>688 ± 137 ^b ^</td>
<td>0.007</td>
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</tr>
<tr>
<td>18:3n-3</td>
<td>10 ± 15</td>
<td>24 ± 8</td>
<td>43 ± 73</td>
<td>15 ± 11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:5n-3</td>
<td>14 ± 12</td>
<td>7 ± 12</td>
<td>3 ± 8</td>
<td>6 ± 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:5n-3</td>
<td>23 ± 16</td>
<td>41 ± 11</td>
<td>35 ± 17</td>
<td>29 ± 12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:6n-3</td>
<td>1268 ± 207 ^a ^</td>
<td>2359 ± 456 ^b</td>
<td>2073 ± 132 ^b ^</td>
<td>2036 ± 330 ^a,b ^</td>
<td>0.0008</td>
<td></td>
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</tr>
<tr>
<td>Σ of n-3 PUFA *</td>
<td>1329 ± 207 ^a ^</td>
<td>2432 ± 463 ^b ^</td>
<td>2171 ± 130 ^b ^</td>
<td>1890 ± 559 ^a,b ^</td>
<td>0.0002</td>
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<td></td>
</tr>
</tbody>
</table>

Mice consumed chows from weaning until 8 weeks of age. nd = not detected. Data are mean ± SD, n= 6 independent samples per group. Main effects and interactions between genotype (wildtype or fat-1) and diet (10% safflower oil or 2% fish oil and 8% safflower) were determined with
2-way ANOVA. When a significant interaction was detected, means were compared with 1-way ANOVA, with Tukey’s post-hoc test. Means with different superscripts within a row are statistically significant, p<0.05. Int; interaction. *Subtotal values may include fatty acids not shown.
Table 3.4-4: Brain phosphatidylinositol fatty acid concentrations (nmol/g) of wildtype and fat-1 mice consuming either the 10% safflower oil or 2% fish oil and 8% safflower oil chow.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Wildtype 10% Safflower Oil</th>
<th>Wildtype 2% Fish Oil, 8% Safflower Oil</th>
<th>Fat-1 10% Safflower Oil</th>
<th>Fat-1 2% Fish Oil, 8% Safflower Oil</th>
<th>Int. Effect (p-value)</th>
<th>Gene Effect (p-value)</th>
<th>Diet Effect (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>119 ± 39</td>
<td>102 ± 10</td>
<td>112 ± 27</td>
<td>96 ± 29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>1162 ± 172</td>
<td>1109 ± 120</td>
<td>1160 ± 145</td>
<td>1092 ± 107</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>2669 ± 261</td>
<td>2477 ± 189</td>
<td>2760 ± 395</td>
<td>2676 ± 348</td>
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</tr>
<tr>
<td>20:0</td>
<td>68 ± 17</td>
<td>57 ± 7</td>
<td>77 ± 21</td>
<td>56 ± 8</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Σ of Saturates*</td>
<td>4252 ± 505</td>
<td>3944 ± 272</td>
<td>4362 ± 572</td>
<td>4124 ± 452</td>
<td>0.014</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:1n-7</td>
<td>47 ± 39</td>
<td>29 ± 5</td>
<td>27 ± 5</td>
<td>27 ± 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1n-9</td>
<td>691 ± 78</td>
<td>842 ± 145</td>
<td>858 ± 139</td>
<td>856 ± 49</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>22:1n-9</td>
<td>73 ± 44</td>
<td>43 ± 13</td>
<td>71 ± 30</td>
<td>39 ± 2</td>
<td>0.012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24:1</td>
<td>15 ± 5</td>
<td>16 ± 2</td>
<td>22 ± 6</td>
<td>21 ± 4</td>
<td>0.004</td>
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</tr>
<tr>
<td>Σ of Monounsaturates*</td>
<td>826 ± 129</td>
<td>929 ± 137</td>
<td>978 ± 144</td>
<td>943 ± 54</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>18:2n-6</td>
<td>77 ± 26</td>
<td>93 ± 38</td>
<td>110 ± 38</td>
<td>73 ± 11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:2n-6</td>
<td>53 ± 44</td>
<td>37 ± 9</td>
<td>54 ± 30</td>
<td>21 ± 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:3n-6</td>
<td>44 ± 31</td>
<td>52 ± 14</td>
<td>70 ± 34</td>
<td>41 ± 5</td>
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<tr>
<td>20:4n-6</td>
<td>1379 ± 139</td>
<td>1215 ± 28</td>
<td>1326 ± 170</td>
<td>1220 ± 68</td>
<td>0.010</td>
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<tr>
<td>22:4n-6</td>
<td>185 ± 149</td>
<td>88 ± 19</td>
<td>106 ± 26</td>
<td>72 ± 24</td>
<td>0.014</td>
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<td></td>
</tr>
<tr>
<td>22:5n-6</td>
<td>177 ± 55</td>
<td>29 ± 6</td>
<td>63 ± 43</td>
<td>28 ± 32</td>
<td>&lt;0.001</td>
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<tr>
<td>Σ of n-6 PUFA*</td>
<td>1923 ± 346</td>
<td>1514 ± 11</td>
<td>1740 ± 286</td>
<td>1456 ± 65</td>
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</tr>
<tr>
<td>18:3n-3</td>
<td>23 ± 31</td>
<td>20 ± 11</td>
<td>22 ± 19</td>
<td>9 ± 11</td>
<td>0.009</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:5n-3</td>
<td>nd</td>
<td>15 ± 10</td>
<td>21 ± 13</td>
<td>20 ± 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:5n-3</td>
<td>3 ± 7</td>
<td>10 ± 2</td>
<td>14 ± 4</td>
<td>13 ± 6</td>
<td>0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:6n-3</td>
<td>129 ± 15</td>
<td>291 ± 71</td>
<td>310 ± 132</td>
<td>363 ± 133</td>
<td>0.006</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>Σ of n-3 PUFA*</td>
<td>190 ± 98</td>
<td>338 ± 83</td>
<td>393 ± 131</td>
<td>415 ± 131</td>
<td>0.017</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mice consumed chows from weaning until 8 weeks of age. nd = not detected. Data are mean ± SD, n= 6 independent samples per group. Main effects and interactions between genotype (wildtype or fat-1) and diet (10% safflower oil or 2% fish oil and 8% safflower) were determined with
2-way ANOVA. When a significant interaction was detected, means were compared with 1-way ANOVA, with Tukey’s post-hoc test. Means with different superscripts within a row are statistically significant, p<0.05. Int; interaction. *Subtotal values may include fatty acids not shown.
The n-6 PUFA docosapentaenoic acid (22:5n-6; DPAn-6) is a marker of n-3 PUFA deprivation, as it is known to increase in concentration as the levels of DHA decrease (Murthy et al 2002; Stark et al 2007; Hussein et al 2009). In ChoGpl, EthGpl, and PtdSer fractions there was a significant interaction of genotype and diet on DPAn-6 concentrations (Tables 3.4-1 to 3.4-3). Within these three fractions, wildtype mice fed the SO chow had significantly higher DPAn-6 levels compared to the other three groups, which did not differ significantly from one another. This statistical pattern was similar in total lipid concentrations (Table 3.4-5).

Of note, there was no effect of diet or genotype on brain total lipid or phospholipid fraction levels of saturated acids (palmitic, 16:0; stearic, 18:0) or oleic acid (18:1n-9). ARA levels were altered only by diet in brain total lipid, EthGpl and PtdIns fractions; whereas the PtdSer fraction, there were independent effects of both diet and genotype.
Table 3.4-5: Brain total lipid fatty acid concentrations (nmol/g) of wildtype and fat-1 mice consuming either the 10% safflower oil or 2% fish oil and 8% safflower oil chow.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>10% Safflower Oil</th>
<th>2% Fish Oil, 8% Safflower Oil</th>
<th>Int. Effect (p-value)</th>
<th>Gene Effect (p-value)</th>
<th>Diet Effect (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>222 ± 29</td>
<td>230 ± 11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>22828 ± 2695</td>
<td>22734 ± 545</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>18:0</td>
<td>19126 ± 1857</td>
<td>19079 ± 227</td>
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<td></td>
</tr>
<tr>
<td>20:0</td>
<td>426 ± 106</td>
<td>405 ± 107</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Σ of Saturates*</td>
<td>43066 ± 4716</td>
<td>42890 ± 733</td>
<td></td>
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</tr>
<tr>
<td>16:1n-7</td>
<td>456 ± 43</td>
<td>527 ± 14</td>
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<td></td>
</tr>
<tr>
<td>18:1n-9</td>
<td>16783 ± 2043</td>
<td>17185 ± 1862</td>
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</tr>
<tr>
<td>22:1n-9</td>
<td>198 ± 37</td>
<td>183 ± 35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24:1</td>
<td>278 ± 78</td>
<td>267 ± 80</td>
<td></td>
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</tr>
<tr>
<td>Σ of Monounsaturates*</td>
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<td>18162 ± 1958</td>
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<tr>
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<tr>
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<td>313 ± 43</td>
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<td>581 ± 69</td>
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<td>12795 ± 1065</td>
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<td>nd</td>
<td>nd</td>
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<tr>
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<td>6088 ± 1140</td>
<td>12428 ± 827</td>
<td></td>
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</tr>
<tr>
<td>Σ of n-3 PUFA*</td>
<td>6882 ± 1200</td>
<td>13415 ± 889</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mice consumed chows from weaning until 8 weeks of age. nd = not detected. Data are mean ± SD, n= 6 independent samples per group. Main effects and interactions between genotype (wildtype or fat-1) and diet (10% safflower oil or 2% fish oil and 8% safflower) were determined with
2-way ANOVA. When a significant interaction was detected, means were compared with 1-way ANOVA, with Tukey’s post-hoc test. Means with different superscripts within a row are statistically significant, p<0.05. Int; interaction. *Subtotal values may include fatty acids not shown.
3.5 Discussion

Brain total lipid and phospholipid concentrations for each group are in the range of what is commonly reported in the literature in rodents undergoing similar dietary manipulation (Contreras et al 2001; DeMar et al 2004; Igarashi et al 2007; Green et al 2009). The fat-1 mouse model has been used extensively as an approach to increase tissue n-3 PUFA levels (Hudert et al 2006; Nowak et al 2007; Schmocker et al 2007; Jia et al 2008; Taha et al 2008; Weylandt et al 2008; He et al 2009; Lau et al 2009b, a; Mayer et al 2009). Brain expression of 4.2% of 25,000 measured genes is altered in the fat-1 mouse (Menesi et al 2009). Fat-1 mice have increased hippocampal neurogenesis and spatial learning performance (He et al 2009), and they are protected in the pentylenetetrazol seizure model (Taha et al 2008). In light of these findings, it was important to test if brain DHA levels of fat-1 mice are attainable through diet, the normal route of essential fatty acid acquisition, and thusphysiologically relevant. Our finding that n-3 PUFA feeding in the form of fish oil results in brain DHA levels similar to those in fat-1 mice suggests that the fat-1 mouse has physiologically relevant brain DHA levels.

Within all four phospholipid fractions, as well as total lipids, there was no effect of the fat-1 gene or diet on the major fatty acids 16:0, 18:0 and 18:1n-9. 20:4n-6 levels were not altered by genotype in the total lipid, ChoGpl, EthGpl or PtdIns fractions. However, there was a main effect of genotype on 20:4n-6 in PtdSer, the fraction containing the smallest concentration of 20:4n-6. Overall, a selective effect of the fat-1 gene on absolute brain 20:4n-6 levels suggest this model may be useful to separate the effects of DHA and 20:4n-6, as feeding different levels of n-3 PUFA can alter brain 20:4n-6 levels (Chen et al 2008a).
Previous reports using dietary manipulation have hypothesized that there is a plateau in brain DHA concentrations. This study offers a novel approach by combining a transgenic model with a dietary model in an attempt to increase brain DHA levels. Fat-1 mice have brain DHA levels of around 12,000 nmol/g, and feeding wildtype mice a 2% fish oil chow results in similar brain DHA levels. Yet, fat-1 mice consuming the 2% fish oil chow did not have augmented brain DHA levels, supporting the hypothesis of a brain DHA plateau. However, dose–response studies are required to more definitively test the hypothesis. It is not known how the brain regulates PUFA levels, although one hypothesis proposes that the brain preferentially shunts specific fatty acids to catabolic pathways (oxygenation or b-oxidation) in times of abundance and to esterification pathways in times of need (for review see Chen et al 2008a). Of note, if DHA is shunted to catabolic pathways once the brain is saturated, then there could be an effect on brain function with n-3 PUFA feeding beyond what is measurable in phospholipid fatty acid concentrations. If this is true and holds for other PUFA, such as eicosapentaenoic acid (20:5n-3), then it is one explanation for benefits seen with 20:5n-3 supplementation and neurological disorders, despite the brain having low levels of 20:5n-3 itself (Chen et al 2008b; Ouellet et al 2009).

It is typical of studies using the fat-1 mouse model to feed an n-6 PUFA enriched, n-3 PUFA deprived chow to both fat-1 mice and their wildtype littermates (Hudert et al 2006; Nowak et al 2007; Taha et al 2008; He et al 2009; Lau et al 2009b, a). Consistent with the literature, we found high brain levels of DPAn-6 in wildtype mice fed the SO chow compared to wildtype mice fed the FO chow and fat-1 mice on either the SO or FO chow (Greiner et al 2003; Moriguchi et al 2004). DPAn-6, like DHA, is a 22-carbon chain PUFA, and is preferentially incorporated into the sn-2 position of phospholipids in lieu of DHA during n-3 PUFA deprivation. The brain DPAn-6 concentration in the wildtype mice on the SO chow was in a range indicative of n-3
PUFA deprivation (Contreras et al. 2001; DeMar et al. 2004; Igarashi et al. 2007). N-3 PUFA deprivation leads to neural deficits including impaired neuronal development and learning, and aggressive behaviour.

In conclusion, we found that fat-1 mice have brain DHA levels that are attainable through fish oil feeding, and that were not augmented by feeding a 2% fish oil chow. The fat-1 mouse offers an alternative or complementary approach to dietary modulation of brain n-3 PUFA when testing their role in neurological function and pathologies.
CHAPTER 4:

Unesterified DHA is a precursor to specialized pro-resolving mediators in the brain and protective in neuroinflammation
Adapted from:

**Orr SK, Palumbo S, Bosetti F, Mount HT, Kang JX, Greenwood CE, Ma DWL, Gao F, Serhan CN, Bazinet RP.** Unesterified DHA is a precursor to specialized pro-resolving mediators in the brain and protective in neuroinflammation. *Submitted.*

### 4.1 Abstract

Using chronic transgenic and dietary models we demonstrated that unesterified, but not phospholipid, DHA attenuates neuroinflammation initiated by intracerebroventricular (*icv*) LPS. Hippocampal neuroinflammation was assessed by gene expression and immunohistochemistry. Further, DHA protected against LPS-induced neuronal loss. Acute *icv* infusion of unesterified DHA or its 15-lipoxygenase product and precursor to protectins and resolvins, 17S-HpDHA, mimics anti-neuroinflammatory aspects of chronically increased unesterified DHA. LC-MS/MS metabolomics-lipidomics revealed that PD1/NPD1 and several other novel SPM derived from DHA are present in the hippocampus. Infusing unesterified DHA or 17S-HpDHA *icv* increases PD1/NPD1 concentration concurrent to attenuated neuroinflammation. In conclusion, unesterified DHA is anti-inflammatory in a mouse model of neuroinflammation, at least in part, via its novel SPM; these DHA stores may provide novel targets for the prevention and treatment of neurological disorders with a neuroinflammatory component.

### 4.2 Introduction

DHA; 22:6n-3 is the most abundant n-3 PUFA in the brain and is considered essential for optimal retinal and neural development (Hoffman et al 2009). The importance of DHA in the maintenance of neurological health and disease prevention remains of considerable interest (Cole et al 2010). Results from epidemiological studies are fairly consistent in showing an inverse association between fish or n-3 PUFA intake and risk of neurological disorders and several,
though not all, randomized controlled trials show benefits of n-3 PUFA supplementation (Gao et al 2007; Mehta et al 2009; Appleton et al 2010; Balanza-Martinez et al 2011; Calon 2011).

There are several proposed mechanisms for the role of n-3 PUFA in neurological disorders. For instance, DHA upregulates anti-apoptotic factors Akt, Bcl-2 and Bfl-1 \textit{in vitro} (Akbar et al 2005; Lukiw et al 2005). DHA increases cortical brain derived neurotrophic factor (Rao et al 2007b) and via metabolism to ethanolamide metabolites, promotes synaptic plasticity (Lafourcade et al 2011), and promotes neurite growth and synaptogenesis (Kim et al 2011a). Beyond the aforementioned pathways, it is possible that DHA acts via an anti-neuroinflammatory mechanism, since DHA is known to be anti-inflammatory in non-neural tissues and because most, if not all, neurological disorders have a neuroinflammatory component (Aid et al 2011).

In the past decade there has been an increasing focus on newly identified enzymatically oxygenated products of DHA as a potential mechanism underlying DHA’s protective actions. While most of ARA-derived lipid mediators, including prostaglandins and leukotrienes, are generally pro-inflammatory, novel SPM derived from n-3 PUFA, such as EPA and DHA, are proving to be anti-inflammatory and pro-resolving (Serhan et al 2008). DHA is metabolized into several classes of SPM including resolvins, protectins, and maresins, which play similar but distinct roles in inflammation and resolution (Serhan et al 2008). To date, these novel mediators have been investigated in a number of tissues and disease models. Neither DHA or DHA-derived SPM have been tested in a model of neuroinflammation, although total brain DHA is known to reduce brain inflammation resulting from insults such as ischemia-reperfusion (Lalancette-Hebert et al 2011), and systemic inflammation (Mingam et al 2008).
D-series resolvins are produced following the action of two lipoxygenases (LO) on unesterified DHA. 15-lipoxygenase-1 (15-LO) first catalyzes the oxygenation of DHA at carbon 17 with the S chirality to produce 17S-HpDHA (17S-hydroperoxy-docosa-4Z,7Z,10Z,13Z,15E,19Z-hexaenoic acid). Resolvin D1 and D2 are formed, via the action of a second 5-Lipoxygenase (5-LO), to form a new 7(8)S-epoxide intermediate (Serhan et al 2008; Yang et al 2011a). In animal models, RvD1 inhibits neutrophil infiltration in peritonitis (Sun et al 2007; Kasuga et al 2008; Recchiuti et al 2011), protects from ischemia-reperfusion induced kidney damage (Duffield et al 2006), reduces oxidative-stress induced inflammation (Spite et al 2009b), and reduces post-operative and inflammation-induced pain (Xu et al 2010; Huang et al 2011); where RvD2 inhibits neutrophil infiltration in murine sepsis and peritonitis (Spite et al 2009a).

17S-HpDHA is also a precursor to PD1/NPD1 (10R,17S-dihydroxydocosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid). 17S-HpDHA undergoes enzymatic epoxygenation followed by hydrolysis to produce PD1/NPD1 (Bannenberg et al 2010). PD1/NPD1 is also coined PD1 when referring to its production outside the nervous system. NPD1/PD1 has protective effects in the retina (Mukherjee et al 2004; Gronert et al 2005; Sheets et al 2010), experimental brain ischemia-reperfusion (Marcheselli et al 2003), and a triple transgenic mouse model of Alzheimer’s disease (Zhao et al 2011). Compared to age-matched neurologically normal human brain, patients with moderate Alzheimer’s disease have two-fold less unesterified DHA in their hippocampi, as well as two-fold lower 15-LO expression and twenty-fold less PD1/NPD1 (Lukiw et al 2005).

In the second single oxygenation route using molecular oxygen, MaR1 (7,14S-dihydroxydocosa-4Z,8,10,12,16Z,19-hexaenoic acid) is formed via human 12-LOX, an enzyme present in macrophages and platelets (Serhan et al 2009). MaR1 displays the potent anti-inflammatory and
pro-resolving properties of SPM, stimulates tissue regeneration and controls pain (Serhan et al 2012), yet MaR1 has not yet been reported in the brain.

DHA and its SPM are anti-inflammatory in non-neural tissues, but only indirect evidence exists for their anti-inflammatory effects in the brain (Orr et al 2008). We therefore tested the effect of increased DHA and 17S-HpDHA levels in a mouse model of local, LPS-induced neuroinflammation. Our analyses are focused in the hippocampus due to its sensitivity in our model of neuroinflammation, its importance in cognitive processes, and since PD1/NPD1 is present in the mouse and human hippocampi (Marcheselli et al 2003; Lukiw et al 2005). In chronic studies we used transgenic and dietary models to increase unesterified DHA and found neuroinflammation was significantly attenuated. Using a direct infusion model, here we find that acute increases in unesterified DHA and 17S-HpDHA are sufficient to attenuate neuroinflammation. Novel SPM derived from DHA were identified in the hippocampus, with higher concentrations of PD1/NPD1 during attenuated neuroinflammation.

4.3 Methods

All procedures were carried out in accordance to the policies set out by the Canadian Council on Animal Care (Canadian Council on Animal Care., 1993) and were approved by the Animal Ethics Committee at the University of Toronto, Toronto, Canada. Mice were housed 3-4 per cage in a facility in which temperature (21°C), humidity and light cycle (12 h light/dark) were controlled, and had ad libitum access to food and water.

4.3.1 Diets

Mice were fed one of three diets: 10% safflower oil diet (SO; D04092701; Research Diets Inc.), or 2% menhaden oil, 8% safflower oil diet (FO; D04092702; Research Diets Inc.), or
standard chow (Teklad 2018; Harlan). Both the SO and FO diets are modified AIN-93-G. The SO diet contains 0.03 and 0.09% of EPA and DHA, respectively, while the FO diet contains 1.97 and 1.61% of EPA and DHA, respectively. The standard chow contained 5.99% \(\alpha\)-linolenate (18:3n-3), 0.06% EPA, and 0.06% DHA. Fatty acid composition was measured by GC-FID.

### 4.3.2 Animals

Fat-1 mice and wildtype littermates were obtained by mating male fat-1 mice (C57BL/6 x C3H background) (Kang et al., 2004) with female C57BL/6 wildtype mice from Charles River Laboratories Canada. Male F1 progeny were used in these studies. Dams were fed the SO diet to reduce transfer of n-3 PUFA to offspring via the placenta or milk. Wildtype and fat-1 F1 progeny were weaned at 21 days of age onto either the SO or FO diet, randomly, depending on the study.

C57BL/6 mice that underwent dietary manipulation were obtained with their dams at 10 days of age from Charles River Laboratories. Dams were immediately placed on the SO diet to reduce n-3 PUFA intake and subsequent transfer to offspring via milk. Mice were weaned at 21 days of age onto either the SO or FO diet. C57BL/6 mice that were acutely infused with fatty acids post-LPS were obtained at eleven weeks of age from Charles River Laboratories and fed standard chow.

### 4.3.3 Total phospholipid and unesterified fatty acid analysis

A separate group of mice from each study was euthanized at 12 weeks of age for hippocampal fatty acid analysis. To maintain integrity of the unesterified fatty acid pool, mice were euthanized by head-focused, high-energy microwave irradiation (6 kW, 0.88–0.99 s, Muromachi brain fixation system, Stoelting Co.). Total lipids were extracted from hippocampi according to the method of Folch (Folch et al 1957).
Total phospholipids were isolated by running a portion of the total lipid extract on thin layer chromatography G-plates (Cat. #10011) in heptane/diethyl ether/glacial acetic acid (60:40:2, by vol) to isolate total phospholipids. Plates were activated by heating at 100°C for 1 h. Total phospholipid bands were visualized under UV light after lightly spraying with 8-anilino-1-naphthalene sulfonic acid (0.1% wt vol-1). Total phospholipid bands were added into a test tube containing a known amount of heptadecanoic acid (17:0) and converted to fatty acid methyl esters with 14% boron trifluoride in methanol at 100°C for 1 h. Fatty acid methyl esters were analyzed using a Varian-430 gas chromatograph (Varian) equipped with a Varian FactorFour capillary column (VF-23ms; 30 m × 0.25 mm i.d. × 0.25 μm film thickness) and a FID. Samples were injected in splitless mode. The injector and detector ports were set at 250°C. FAME were eluted using a temperature program set initially at 50°C for 2 min, increasing at 20°C min-1, and held at 170°C for 1 min, then at 3°C min-1 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-1. Peaks were identified by retention times of authentic FAME standards (Nu-Chek Prep, Inc.). Fatty acid concentrations (nmol g-1 brain) were calculated by proportional comparison of gas chromatography peak areas to that of the 17:0 internal standard (Orr et al 2010).

Unesterified fatty acids were isolated by thin layer chromatography, visualized, converted to fatty acid methyl esters, and quantified by the same methods as total phospholipids. However, a known amount of 17:0 was added to the total lipid extract before plating and unesterified fatty acid methyl esters were manually injected in splitless mode.

4.3.4 Intracerebroventricular administration of LPS
At 12 weeks of age, mice were anesthetized with isoflurane gas (3% induction, 1-2% maintenance) and their head secured into a stereotaxic frame (Stoelting). Before the incision was
made, 50 μl of 0.1% Sensorcaine was injected subcutaneously at the incision site. The skull was exposed and a small hole was drilled (-1.0 mm medial/lateral and -0.5 mm anterior/posterior from bregma). A 1 μl injection of vehicle (saline) or LPS (5 μg in 1 μl; E. coli serotype 055:B5; Sigma-Aldrich, St. Louis, MO) was infused at a constant rate over a 5 minute period into the right lateral ventricle (-2.3 dorsal/ventral) using a 33-gauge beveled injection needle (World Precision Instruments). The needle was slowly drawn out of the brain to avoid infusate backflow. Twenty-four hours after the injection, mice were euthanized by CO2, brains were excised, and hippocampi were isolated then stored at -80°C until analysis.

4.3.5 Implantation of osmotic pump

One approach required the insertion of an icv catheter attached to an osmotic pump (model 1003D; Alzet, DURECT Corporation) that delivered treatment over 24h post-LPS. Treatments included artificial cerebrospinal fluid (aCSF; 150 mM Na, 3 mM K, 1.4 mM Ca, 0.8 mM Mg, 1 mM P, 155 mM Cl), 40 μg of DHA (#90310, Cayman Chemical), 1 μg of 17S-HpDHA (#13185; Cayman Chemical), 40 μg ARA (U-71-A, Nu-Chek Prep Inc.), or 40 μg DPAn-6 (U-102-A, Nu-Chek Prep Inc.). Mice were first treated with acute saline or LPS as described above.

Immediately following this and at the same coordinate, an icv catheter attached to an osmotic pump containing one of the listed treatments. Osmotic pumps were implanted subcutaneously on the dorsal side, posterior to the head.

4.3.6 Radioactive tracer

Two mice were implanted with an osmotic pump containing 10 μCi of 14C-DHA ([1-14C]-DHA, specific activity: 52 mCi mmol-1) in order to determine where unesterified fatty acids diffuse to within the brain. Twenty four hours following catheter implantation, mice were euthanized and the brain dissected into hippocampus, cortex, and the remainder of the brain.
Total lipids were extracted and run on thin layer chromatography to isolate total phospholipid and unesterified fatty acid fractions. Radioactivity was quantified by a Packard TRI-CARB2900TR liquid scintillation analyzer (Packard) with a detector efficiency of 48.8%. Radioactivity was expressed in units of decays per minute (dpm); then converted to nCi tissue-1.

4.3.7 Gene expression analysis

Total RNA was isolated from hippocampi using Trizol (Invitrogen) according to the manufacturer’s protocol. RNA purity and quantity were determined using the 260 nm to 280 nm UV absorbance ratio and 260 nm absorbance, respectively, measured with a Nanodrop 1000 (NanoDrop Technologies Inc.). One microgram of RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Quantitative real-time PCR was performed using the TaqMan Low Density Array (TLDA; Applied Biosystems) on the 7900HT Real-Time PCR Systems (Applied Biosystems). Briefly, 50 ng of cDNA was diluted with RNAse-free water to a volume of 50 μL and combined with an equal volume of TaqMan Universal PCR Master Mix (Applied Biosystems), then loaded onto a pre-configured 384-well TLDA plate according to the manufacturer’s protocol. TaqMan Gene Expression Assays were used to assess ARA cascade enzymes including Group IVA cPLA2 (assay ID Mm00447040_m1), COX-2 (Mm00478374_m1), and (mPGES Mm00452105_m1); cytokines IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), and TNF-α (Mm00443258_m1); chemokines CCL2 and CCL3 (Mm00441242_m1 and Mm00441258_m1); activated microglial cell markers CD11b and CD45 (Mm00434455_m1 and Mm00448463_m1); astrocyte marker GFAP (Mm00546086_m1); and oxidative stress marker iNOS (Mm00440485_m1). Endogenous controls measured included 18S RNA and phosphoglycerate
kinase 1 (PGK1; Mm00435617_m1). All genes were normalized to PGK1. Similar results were found when normalizing to 18S.

4.3.8 Immunohistochemistry

For immunohistochemistry, mice were euthanized by transcardial perfusion with phosphate buffered saline followed by a 4% paraformaldehyde solution. Brains were post-fixed overnight in the same paraformaldehyde solution and then placed in a 30% sucrose solution, frozen in 2-propyl butane (-60 °C) and stored in -80 °C. Fixed frozen brains were sectioned (30 µm) using a cryostat Leica CM1950 (Leica Microsystems).

Fluoro-jade B (FJB) (Millipore) was used to identify degenerating neurons following the protocol provided by the company. FJB positive cells were counted in the hippocampus within the dentate gyrus and quantified as previously described (Choi and Bosetti, 2009).

Anti-GFAP glial fibrillary acidic protein (GA5) mouse primary antibody (1:400, Cell Signaling, Beverly, MA) and anti-Iba1 (2 µg ml-1; Wako Chemicals) were used as markers of astrocytes and macrophages/microglia respectively using the Vectastain ABC kit following the protocol provided by the same company (Vector Laboratories). The number of positive GFAP and Iba1 cells was counted within 0.3 mm² of DG hippocampus area in 3 consecutive sections from each brain; two blinded investigators repeated the counting.

4.3.9 Genotyping

Genotyping was performed as previously reported (Orr et al 2010).

4.3.10 Lipid Mediator Metabolo-lipidomics

The mouse hippocampus was homogenized by adding 0.5 mL cold methanol. Deuterated internal standards ((d4-PGE₂, d8-5S-HETE, and d4-LTB₄) were added to each homogenate.
After protein precipitation for 12 hr, samples were extracted by SPE column and methyl formate fractions were taken for LC-MS/MS-based lipidomics.

LC-MS/MS was performed with an Agilent 1100 HPLC (Agilent Technologies) equipped with an Agilent Eclipse Plus C-18 column (4.6 mm×50 mm×1.8 µm) paired with an ABI Sciex Instruments 5500 QRAP linear ion trap triple quadrupole mass spectrometer (Applied Biosystems). Instrument control and data acquisition were performed using AnalystTM 1.5 software (Applied Biosystems). The mobile phase consisted of methanol/water/acetic acid (55/45/0.01; v/v/v) and was ramped to 88/12/0.01 (v/v/v) after 10 min, 100/0/0.01 (v/v/v) after 18 min, and 55:45:0.01 (v/v/v) after 1 minute to wash and equilibrate the column. Mass spectrometry analyses were carried out in negative ion mode using multiple reaction monitoring (MRM) of established specific transitions for 17-HDHA (m/z 343>245), PD1/NPD1 (m/z 359>153), MaR1 (m/z 359>250), RvD1 (m/z 375>215), RvD2 (m/z 375>233), RvD5 (m/z 359>199), 14-HDHA (m/z 343>205), PGE2 (m/z 351>189), PGD2 (m/z 351>351), LXB4 (m/z 351>221), and 15-HETE (m/z 319>179). The criteria used for positive identification of mediators of interest were matching retention time and matching of 6 diagnostic ions to synthetic standards (Yang et al 2011a). Quantification was performed using standard calibration curves for each compound, and recovery was calculated using deuterated internal standards.

4.3.11 Statistics
Results are expressed as means ± SEM unless otherwise stated. Fatty acid concentration means were compared by Student’s t-test by genotype, genotype and diet, or diet depending on the study. Gene expression and immunohistochemistry means in the first three studies were compared by two-way ANOVA with genotype and/or diet as one main factor and treatment (saline or LPS) as the other. Significant interactions were further analyzed by one-way ANOVA.
with Bonferroni’s multiple comparisons post-hoc test. In the osmotic pump study, gene expression means were compared by one-way ANOVA with a Bonferroni’s multiple comparison post-hoc. Lipid mediators were analyzed by Student’s t-test comparing treatment (DHA or 17S-HpDHA) to control (aCSF). Statistical significance was set as p < 0.05 for all analyses.

4.4 Results

4.4.1 Fat-1 mice have higher hippocampal DHA levels

We first characterized the brain fatty acid profile of fat-1 mice and their wildtype littermates. All mice were fed a 10% safflower oil (SO) diet from weaning, as were their dams throughout breeding and lactation (for composition see Table 4.4-1). Under ischemic conditions, calcium-dependent phospholipase A2 cleaves fatty acids from membrane phospholipids, dramatically increasing unesterified fatty acid concentrations. Therefore to preserve the integrity of the unesterified fatty acid pool, mice were euthanized by head-focused, high-energy microwave irradiation. Fat-1 mice have twice the amount of DHA in their total phospholipids with a much lower amount of docosapentaenoic acid n-6 (DPAn-6) compared to wildtype littermates (p<0.001; Figure 4.4-1b). Wildtype littermates are n-3 PUFA inadequate, indicated by their high level of phospholipid DPAn-6, which is preferentially esterified into membranes in the absence of DHA. There were no other phospholipid fatty acid differences, except for the slightly higher oleic acid (18:1n-9) concentrations in fat-1 mice compared to wildtype littermates (p=0.05).

The unesterified fatty acid pool is gaining recognition as the “active” pool from which fatty acids are metabolized to novel bioactive mediators, and it is 5,000-10,000 times smaller than the total phospholipid pool. We found that fat-1 mice have significantly higher levels of unesterified DHA compared to wildtype littermates, but there was no difference in unesterified ARA.
### Table 4.4-1: Fatty acid (FA) percent composition of total fat in 10% safflower oil (SO), 2% fish oil, 8% safflower oil (FO), and standard chow diets.

<table>
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</tr>
<tr>
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<td>0.16</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>22:6n-3</strong></td>
<td><strong>0.09</strong></td>
<td><strong>1.61</strong></td>
<td><strong>0.06</strong></td>
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</table>

Fatty acid percent compositions are means of triplicate analysis. Data are % of total fatty acids; other fatty acids are not shown.
Figure 4.4-1: Fatty acid and neuroinflammatory profile of the fat-1 mouse

(a) Study design. F0 mice and F1 progeny consumed 10% safflower oil (SO) diet. At 12 weeks of age mice received an intracerebroventricular (icv) injection of lipopolysaccharide (LPS) or vehicle (saline). Twenty-four hours following surgery, brains were collected and hippocampi isolated for analysis. (b) Hippocampal fatty acid concentrations (nmol g⁻¹) of wildtype and fat-1 mice consuming the SO diet. 18:3n-3 was not detected in either group, 20:5n-3 was not detected in wildtype mice. n= 5 independent samples per group. Significantly different means represented by *p<0.05, **p<0.01 and ***p<0.001 (Student’s t-test). (c) Hippocampal gene expression of neuroinflammatory markers interleukin-1β (IL-1β), glial fibrillary acidic protein (GFAP), chemokine (c-c motif) ligand 3 (CCL3), and inducible nitric oxide synthase (iNOS) are significantly attenuated in LPS-treated fat-1 mice compared to LPS-treated wildtype. (d)
Arachidonic acid cascade markers calcium-dependent cytosolic phospholipase A$_2$ (cPLA$_2$), cyclooxygenase-2 (COX-2), and microsomal prostaglandin E synthase (mPGES) are significantly down-regulated in fat-1 mice compared to wildtype littermates. Gene expression in C and D compared by 2-way ANOVA followed by Bonferroni’s post-hoc if a significant interaction was found. n=5-7 independent samples per group. Differences between wildtype and fat-1 within treatment (icv saline or LPS): ***p<0.001. All data are mean ± SEM.
4.4.2 Fat-1 mice display attenuated neuroinflammatory responses

To test if increased DHA is protective against neuroinflammation, fat-1 mice and their wildtype littermates were challenged with an acute 5 μg intracerebroventricular (icv) injection of LPS. LPS was injected directly into the left lateral cerebral ventricle of mice because systemic injections cause robust systemic inflammation, fever, and increased mortality (Rudaya et al 2005). We did not observe any significant difference in body temperatures of LPS-treated (36.7 ± 0.4 °C) versus vehicle-treated mice (36.8 ± 0.7 °C), confirming that our LPS dose directly activated brain innate immunity minimizing other side effects like fever or peripheral immune signals to the brain that might be attenuated by increases in peripheral DHA (Calder 2009; Yong et al 2009). The study design is described in figure 4.4-1a, with age at surgery, inflammatory agent, dose, and time points based on pilot data and published literature (Choi et al 2008).

We found either i) a significant interactive effect of genotype (fat-1 or wildtype littermates) and treatment (saline or LPS), or ii) significant main effects of genotype and treatment on the gene expression of cytokines, chemokines, and other neuroinflammatory markers all indicating an attenuated neuroinflammatory response in fat-1 mice (Figure 4.4-1c). The attenuated increase in pro-inflammatory cytokine IL-1β, astrocyte marker GFAP, chemokine CCL3, and oxidative stress marker iNOS in LPS-treated fat-1 mice compared to LPS-treated wildtype littermates is shown in figure 4.4-1c. Similar results were seen with pro-inflammatory IL-6 and TNF–α, chemokine CCL2, activated microglia markers CD11b and CD45, and microbicidal oxidase system component cytochrome b beta (CYBB) (data not shown).

The ARA cascade is activated during neuroinflammation and is upregulated in response to DHA deprivation (Rao et al 2007a). Group IVA cPLA2 preferentially cleaves ARA out of the phospholipid membrane into its unesterified form where it is available for metabolism. Fat-1
mice have significantly lower cPLA₂ gene expression in the hippocampus compared to wildtype littermates (Figure 4.4-1d). COX-2 and mPGES, in sequence, metabolize ARA to PGE₂. LPS-treated fat-1 mice have significantly lower gene expression of both COX-2 and mPGES compared to LPS-treated wildtype littermates (Figure 4.4-1d).

In addition to gene expression measures, we measured glial activation and neuronal degeneration by immunohistochemistry (Figure 4.4-2). GFAP and ionized calcium binding adaptor molecule 1 (Iba1) were measured in the dentate gyrus. GFAP protein reflected gene expression results in that LPS-treated fat-1 mice had a significantly attenuated increase compared to LPS-treated wildtype littermates. Iba1 is expressed on the cell surface of microglia and its expression increases with microglial activation. Iba1 protein expression showed lowered microglial activation in fat-1 mice following LPS compared to wildtype littermates, confirming CD11b and CD45 gene expression measures. Similar results were found with Iba1 and GFAP in the CA1 region of the hippocampus (data not shown). FJB staining indicates significantly less neuronal degeneration in the dentate gyrus of fat-1 mice following LPS compared to wildtype littermates. As expected, there was no marked neuronal degeneration in saline treated wildtype and fat-1 mice. Interestingly, saline-treated fat-1 mice had significantly lower levels of CYBB, GFAP, CD11b, CD45, mPGES, and cPLA₂ compared to saline-treated wildtype littermates (Student’s t-test, p<0.05; data not shown), indicating that DHA affects inflammation pathways separate from its established roles in neurotrophic and neural apoptosis pathways.
**Figure 4.4-2**: Effect of fat-1 genotype on hippocampal neuroinflammatory and neurodegenerative markers by immunohistochemistry (IHC)

Representative photomicrographs of (a-d) glial fibrillary acidic protein (GFAP), (f-i) ionized calcium binding adaptor molecule 1 (Iba1), (k-m) and fluoro-jade B (FJB) in wildtype and fat-1 mice 24 h after *icv* injection of LPS or saline. Quantitative analysis of (e) GFAP IHC, (j) Iba1 IHC, and (o) FJB IHC. Analyzed by 2-way ANOVA followed by 1-way ANOVA with Bonferroni’s post-hoc if a significant interaction was found. n=8 independent samples per group. Scale bars = 100 μm. Differences between wildtype and fat-1 within treatment (*icv* saline or LPS): ***p<0.001.
4.4.3 FO diet restores hippocampal DHA of wildtype littermates

Earlier findings from our lab show that whole brain total DHA concentrations of fat-1 mice can be mimicked by feeding a 2% fish oil, 8% safflower oil (FO; for composition see Table 4.4-1) diet to their n-3 PUFA inadequate wildtype littermates (Orr et al 2010). For this experiment, wildtype littermates were weaned onto the FO diet at 3 weeks of age, while fat-1 mice continued on the SO diet (see study design Figure 4.4-3a). At 12 weeks of age mice were euthanized and fatty acids were analyzed. There were no statistical differences in phospholipid fatty acid profile, or unesterified ARA or DHA between fat-1 mice on the SO and wildtype littermates on the FO diet, except a statistically higher level of phospholipid linoleic acid (18:2n-6) in fat-1 mice on the SO diet compared to wildtype littermates on the FO diet (Figure 4.4-3b).

4.4.4 FO diet restores protection in wildtype littermates

To determine if mimicking the fatty acid profile of fat-1 mice is sufficient to attenuate the neuroinflammatory response in wildtype littermates fed the FO diet, we challenged mice with icv LPS in the 2x2 factorial design already described (Figure 4.4-3a). We found no significant differences in the expression of the IL-1β, GFAP, CCL2, or iNOS genes between LPS-treated wildtype littermates on the FO diet and LPS-treated fat-1 mice on the SO diet (Figure 4.4-3c). The only significant finding was an effect of LPS. There were similar findings in IL-6, TNF-α, CD11b, CD45, CCL3, and CYBB where the only significant effect is of LPS treatment (data not shown). Similar effects were seen in enzymes of the ARA cascade cPLA2 and COX-2, although there was increased mPGES gene expression in LPS-treated fat-1 mice on the SO diet compared to wildtype littermates on the FO diet (Figure 4.4-3d).
Figure 4.4-3: Fatty acid and neuroinflammatory profile of wildtype littermates fed FO compared to fat-1 mice

(a) Study design is the same as in Fig 1A except at weaning wildtype mice are placed on a 2% fish oil, 8% safflower oil (FO) diet. Fat-1 mice remained on a 10% safflower oil diet (SO). Hippocampal fatty acid concentrations (nmol g⁻¹) of FO-fed wildtype mice and SO-fed fat-1 mice. 18:3n-3 was not detected in either group. n = 9 independent samples per group. Significantly different means represented by *p<0.05 (Student’s t-test). (b) Hippocampal neuroinflammatory responses of SO-fed fat-1 mice and FO-fed wildtype 24 h following icv administration of lipopolysaccharide (LPS) or vehicle (saline). Gene expression of neuroinflammatory markers interleukin-1β (IL-1β), glial fibrillary acidic protein (GFAP), chemokine (c-c motif) ligand 2 (CCL2), and inducible nitric oxide synthase (iNOS) is not
significantly different between LPS-treated SO-fed fat-1 mice and LPS-treated FO-fed wildtype. (d) Arachidonic acid cascade markers calcium-dependent cytosolic phospholipase A\textsubscript{2} (cPLA\textsubscript{2}), cyclooxygenase-2 (COX-2), and microsomal prostaglandin E synthase (mPGES) are similar in SO-fed fat-1 mice and FO-fed wildtype littermates. Gene expression in C and D compared by 2-way ANOVA followed by Bonferroni’s post-hoc if a significant interaction was found. n = 4-6 independent samples per group. Differences between wildtype and fat-1 within treatment (icv saline or LPS): ***p<0.01. All data are mean ± SEM.
4.4.5 FO diet increases phospholipid DHA in n-3 PUFA adequate mice
Having shown that the fat-1 mouse has an attenuated neuroinflammatory response, which is mimicked by feeding fish oil to its n-3 PUFA inadequate wildtype littermates, we set out to determine if a dietary intervention in n-3 PUFA adequate C57BL/6 mice could attenuate neuroinflammation. Brain DHA has a long half-life that is increased in times of n-3 PUFA deprivation (DeMar et al 2004). To address this C57BL/6 mice and their dams were placed on the SO diet at 10 days of age when they arrived at our facility (see study design Figure 4.4-4a). At weaning, mice were randomized to either the SO or FO diet. A subset of mice was euthanized at 12 weeks of age to determine their hippocampal fatty acid profile. FO-fed C57BL/6 mice had significantly higher phospholipid DHA levels compared to SO-fed C57BL/6 mice (p<0.01), and an expected equally lowered level of phospholipid DPAn-6 (p<0.001). There was no significant difference in either unesterified ARA or DHA levels (Figure 4.4-4b). The ability to modulate unesterified DHA when feeding the FO diet to wildtype littermates of fat-1 mice (Figure 4.4-3), when these same diets did not affect C57BL/6 mice, is likely due to the state of n-3 PUFA inadequacy in wildtype littermates of fat-1 mice prior to n-3 PUFA feeding.

4.4.6 Neuroinflammation not altered by increased phospholipid DHA
To determine whether a moderate increase in total phospholipid DHA but not unesterified DHA results in an attenuated neuroinflammatory response we challenged 12 week old FO- and SO-fed C57BL/6 mice with an icv injection of LPS in a 2x2 factorial design as described in Figure 4.4-4a. We found no difference in the neuroinflammatory response of FO-fed mice compared to SO-fed mice as measured by a compliment of gene markers including IL-1β, GFAP, CCL2, and iNOS (Figure 4.4-4c). There were also no significant differences in CCL3, CYBB, IL-6, CD11b, CD45, or TNF-α (data not shown). Also, there were no differences ARA cascade enzymes, with
**Figure 4.4-4**: Fatty acid and neuroinflammatory profile of C57BL/6 mice on SO and FO diets

(a) **Study design.** C57BL/6 mice consumed 10% safflower oil diet (SO) from 10 days of age. At weaning mice were randomized to either the SO diet or a 2% fish oil, 8% safflower oil (FO) diet. Remainder of design is similar to Fig 1A. (b) **Hippocampal fatty acid concentrations (nmol g⁻¹) of SO- or FO-fed C57BL/6 mice.** 18:3n-3 was not detected in either group. n= 8 independent samples per group. Significantly different means represented by *p<0.05, **p<0.01, ***p<0.001 (Student’s t-test). (c) **Hippocampal neuroinflammatory responses of SO- or FO-fed C57BL/6 mice 24 h following icv administration of lipopolysaccharide (LPS) or vehicle (saline).** Gene expression of neuroinflammatory markers interleukin-1β (IL-1β), glial fibrillary acidic protein (GFAP), chemokine (c-c motif) ligand 2 (CCL2), and inducible nitric oxide synthase (iNOS) is not significantly different between LPS-treated SO-fed mice and LPS-treated FO-fed mice. (d) **COX-2 and cPLA2 expression in LPS- or saline-treated SO- or FO-fed C57BL/6 mice.**
mice. (d) Arachidonic acid cascade markers calcium-dependent cytosolic phospholipase A$_2$ (cPLA$_2$), cyclooxygenase-2 (COX-2), and microsomal prostaglandin E synthase (mPGES) are similar in SO-fed and FO-fed C57BL/6. Gene expression in C and D compared by 2-way ANOVA followed by Bonferroni’s post-hoc if a significant interaction was found. n = 6-8 independent samples per group. All data are mean ± SEM.
the exception of COX-2 (Figure 4.4-4d). These results indicate that modulating the unesterified DHA pool is necessary to affect neuroinflammation.

4.4.7 icv DHA or 17S-HpDHA attenuates neuroinflammation

In the earlier studies, chronically increased unesterified DHA resulted in attenuated neuroinflammatory responses. We sought to determine if an acute increase in hippocampal unesterified DHA during neuroinflammation would attenuate the response. N-3 PUFA adequate C57BL/6 mice on standard chow were challenged with LPS and implanted with an icv catheter delivering 40 μg of DHA, 1 μg of its metabolite 17S-HpDHA, or vehicle over 24 hours (see study design Figure 4.4-5a). N-3 PUFA adequacy was confirmed by measuring hippocampal fatty acid composition (Table 4.4-2). 17S-HpDHA was chosen since it is the precursor to both protectins and D-series resolvins. To ensure that icv delivered DHA reaches the hippocampus, we implanted two mice with catheters delivering 10 μCi of 14C-DHA. Radioactivity was detectible in hippocampal unesterified fatty acids, with low but detectible levels also found in total phospholipids (Figure 4.5-6), indicating we could test the hypothesis that increased hippocampal unesterified DHA levels would attenuate the neuroinflammatory response.

Neuroinflammatory markers including IL-1β and CCL3 had significantly attenuated expression levels in the hippocampi of DHA- and 17S-HpDHA-infused mice following LPS compared to control (Figure 4.5-5b). Similar results were seen for CYBB, CD45, and TNF–α (data not shown). 17S-HpDHA alone attenuated GFAP (Figure 4.5-5b) and CD11b (data not shown). CCL2, iNOS, and IL-6 were not significantly altered by DHA or 17S-HpDHA treatment post-LPS, indicating chronic increases in unesterified DHA may have a more broad effect on inflammatory pathways compared to acute increases in unesterified DHA and 17S-HpDHA.
Figure 4.4-5: Neuroinflammatory profile of mice infused with DHA or 17-HpDHA

(a) At 12 weeks of age mice received an intracerebroventricular (icv) injection of lipopolysaccharide (LPS) then an icv catheter was implanted at the same coordinate. Catheters were attached to pumps containing 40 μg DHA, 1 μg 17S-HpDHA, 40 μg ARA, 40 μg DPAn-6, or vehicle (aCSF). (b) Hippocampal neuroinflammatory responses of mice treated with aCSF [blue bars], 40 μg of DHA [orange bars], or 1 μg 17S-HpDHA [yellow bars] for 24 hours post-LPS. Gene expression of neuroinflammatory markers shown are interleukin-1β (IL-1β), glial fibrillary acidic protein (GFAP), chemokine (c-c motif) ligand 3 (CCL3), and inducible nitric oxide synthase (iNOS). There is a significantly attenuated neuroinflammatory response in mice.
treated with DHA or 17S-HpDHA post-LPS compared to vehicle with no effect on the arachidonic acid (ARA) cascade markers (c) cytosolic phospholipase A2 (cPLA2), cyclooxygenase-2 (COX-2), and microsomal prostaglandin E synthase (mPGES). (d) In a separate study, mice were implanted with a pump containing aCSF [blue bars], ARA [green bars], or DPAn-6 [red bars]. Gene expression of neuroinflammatory markers shown are IL-1β, GFAP, CCL3, iNOS. ARA and DPAn-6 have no effect on neuroinflammatory markers, although DPAn-6 trends towards significantly increasing pro-neuroinflammatory gene expression. One-way ANOVA p values are italicized in each graph. Fold change is relative to mice that underwent a sham surgery (icv injection of saline then implantation of an aCSF-containing pump [dotted line]. n = 4-5 independent samples per group. All data are mean ± SEM.
Table 4.4-2: Hippocampal fatty acid composition of n-3 PUFA adequate mice.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
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<tr>
<td></td>
<td></td>
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<tr>
<td><strong>Total Phospholipids</strong></td>
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<tr>
<td>16:0</td>
<td>22,827 ± 875</td>
</tr>
<tr>
<td>18:0</td>
<td>19,044 ± 827</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>14,816 ± 753</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>417 ± 28</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>nd</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>5,758 ± 275</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>nd</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>155 ± 24</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>6,758 ± 858</td>
</tr>
<tr>
<td><strong>Unesterified Fatty Acids</strong></td>
<td></td>
</tr>
<tr>
<td>20:4n-6</td>
<td>3.61 ± 0.41</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>1.62 ± 0.39</td>
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Mice consumed diets from weaning until 8 weeks of age. nd = not detected. Data are mean ± SD, n= 7 independent samples per group.
Ten week old male C57BL6 mice were obtained from Charles River Canada and fed a standard chow. At 12 weeks of age mice underwent a stereotaxic surgery to implant an intracerebroventricular catheter attached to an osmotic pump containing 10 μCi $^{14}$C-DHA. Catheters were left in for 24 h at which time mice were euthanized, brains isolated and dissected into hippocampus, cortex, and the remaining brain. Fatty acids were separated into unesterified fatty acids and total phospholipids using thin layer chromatography. Radioactivity was counted on a liquid scintillation counter. (a) Radioactivity was detectible in all regions in the unesterified pool. (b) Some radioactivity was detectible in total phospholipids, indicating some of the radiolabelled unesterified fatty acid was incorporated into the membrane. The tracer-to-tracer ratios are 0.28 and $6.51 \times 10^{-6}$ for the hippocampus unesterified and phospholipids pools, respectively, supporting selective targeting of the unesterified pool with little change in the phospholipid pool.
Interestingly, acute infusion of DHA or 17S-HpDHA did not modulate ARA cascade enzyme gene expression as seen in our chronic transgenic and dietary models (Figure 4.4-5c); suggesting that down-regulating the ARA cascade is not necessary for protection.

4.4.8 DHA and 17S-HpDHA selective in attenuating neuroinflammation

To determine if the attenuation of neuroinflammation observed in mice infused with DHA and 17S-HpDHA is selective, we infused mice with 40 μg of ARA, 40 μg of DPA-6, or vehicle in the same study design (Figure 4.4-5a). There was no significant difference in the gene expression of neuroinflammatory markers, but there was a trend towards exacerbation in DPA-6 treated mice (Figure 4.4-5d). GFAP was one exception with significant differences between treatments (p=0.03).

4.4.9 Novel SPM increase in hippocampus during neuroinflammation

A novel family of bioactive lipid mediators has recently been identified and characterized (Hong et al 2003; Serhan et al 2008; Serhan et al 2012). These novel DHA-derived autacoids, termed resolvins, protectins, and maresins, are thought to underlie most of the protective and beneficial actions of DHA in setting where inflammation is involved (Serhan et al 2008; Serhan et al 2012). In this study, we analyzed these novel lipid mediators using LC-MS/MS in mouse hippocampus. Mice were euthanized with head-focused, high-energy microwave irradiation. In pilot studies we established that several novel lipid mediators measured increase dramatically in the hippocampus during ischemia and are not degraded by microwave irradiation.

PD1/NPD1 was identified in mice hippocampi (Figure 4.4-7c). 17-hydroxy-DHA (17-HDHA), a biosynthesis marker of 17S-HpDHA production, was also identified. Interestingly, 14-hydroxy-DHA (14-HDHA), the marker for 14S-HpHDA production the precursor of maresin 1 (MaR1),
and MaR1 were also found in the mouse hippocampus ([Figure 4.4-7c](#)). RvD5 was also identified. RvD1 and RvD2 were not found within the hippocampus ([Figure 4.4-7c](#)), adding to our pilot data showing RvD1 and RvD2 do not increase in the hippocampus in response to ischemia at death. The absence of RvD1 and RvD2 suggests that they are not responsible for the attenuated neuroinflammation in our experiments and possibly that the leukocytes that produce them did not infiltrate the tissue.

As expected, prostaglandin D$_2$ (PGD$_2$) and PGE$_2$ were identified in the hippocampus at low levels ([Figure 4.5-8c](#)). Lipoxin B$_4$ (LXB$_4$) was also identified in the hippocampus along with 15-hydroxyeicosatetraenoic acid (15-HETE), a marker of the LX pathway ([Figure 4.5-8c](#)).

EPA-derived products resolvin E1 as well as 5- and 18-hydroxy-EPA were detected in the hippocampus. This was unexpected since it is well established that EPA itself occurs at low to nearly non-detectible levels in neural tissue (Chen et al 2011), and may be the product of EPA derived from endothelial cells or infiltrating blood immune cells. E-series resolvins and mono-hydroxy-EPAs, which possess anti-inflammatory properties in non-neural tissues, may therefore play an important role in brain inflammation and should be explored in future research.
Figure 4.4-7: Specialized pro-resolving mediators (SPM) derived from DHA in mouse hippocampus: LC-MS/MS based lipid mediator metabolo-lipidomics.

(a) 3D chromatograms showing the separation of SPMs derived from DHA in mouse hippocampus, with retention time (min) and m/z values for 17-H(p)DHA and 14-H(p)DHA.

(b) Mass spectra of 17-HDHA and 14-HDHA, showing the molecular ions and fragment ions at different m/z values.

(c) Relative intensity of PD1 and MaR1, with ng/g hippocampus as the y-axis and nd (not determined) indicated for some samples.

(d) PD1 levels following acute LPS plus 24 hour icv infusion, with ng/hippocampus on the y-axis and aCSF, DHA, and 17(S)-HpDHA on the x-axis, showing a significant increase with 17(S)-HpDHA infusion.
(a) MRM chromatograms (359>250, 359>153 m/z 343>205 and 343>245). (b) Representative tandem mass spectra of 17-hydroxy-DHA (17-HDHA), 14-hydroxy-DHA (14-HDHA), neuroprotectin D1 (PD1/NPD1), and maresin1 (MaR1) in samples of mouse hippocampus. (c) DHA-derived SPM in the mouse hippocampus; n=3. (d) Mice were acutely injected icv with 5 μg LPS then implanted with an icv pump containing artificial cerebrospinal fluid (aCSF), 40 μg DHA, or 1 μg 17S-hydroperoxy-DHA (17S-HpDHA) (see treatment design fig. 5A). PD1/NPD1 measured by HPLC-MS/MS after 24 h. NPD1 levels were significantly increased with 17S-HpDHA infusion compared to aCSF (p<0.01). DHA treatment trended towards an increase in NPD1 levels (p=0.07). n = 2 independent samples per group. All results are expressed as the mean ± SEM.
**Figure 4.4-8**: LC-MS/MS lipidomics of lipid mediators derived from arachidonic acid ARA in mouse hippocampus

(a) Representative MRM chromatograms of monohydroxy markers from ARA, prostaglandins (PGs), and lipoxins (LXs). (b) Structures and pathways in ARA metabolism. (c) Lipid mediators in the mouse hippocampus. n=3. All data are mean ± SEM.
4.4.10 PD1/NPD1 increases with 17S-HpDHA administration

We established that neuroinflammation could be attenuated by infusion of unesterified DHA and 17S-HpDHA icv for 24 hours post-LPS (Figure 4.4-5). We treated mice according to the described study design (Figure 4.4-5a) to determine whether PD1/NPD1 is increased in the hippocampus of mice infused with unesterified DHA or 17S-HpDHA following LPS. There was a significant increase in hippocampal PD1/NPD1 levels in mice treated with 17S-HpDHA compared to control (p=0.01) (Figure 4.4-7d). PD1/NPD1 in unesterified DHA-treated mice trended towards an increase but did not reach statistical significance (p=0.07) (Figure 4.4-7d).

4.5 Discussion

DHA protects against neuronal damage in several models of brain injury and disease, however, this study was the first to show that brain DHA directly attenuates neuroinflammation. Through a series of chronic and acute studies we have individually increased phospholipid and unesterified DHA levels and identified unesterified DHA as necessary to attenuate neuroinflammation. Increases in phospholipid DHA is not sufficient to attenuate neuroinflammation, although this pool is an important source of unesterified DHA through deacylation. Unesterified DHA is a precursor to SPM, which are novel anti-inflammatory, pro-resolving molecules. We identified SPM 14-HDHA, MaR1, and RvD5 in the brain for the first time, but neither RvD1 nor RvD2, demonstrating that DHA metabolism in the brain is unique from other tissues and requires independent study. The production of DHA-derived SPM, exemplified by PD1/NPD1, was augmented by directly infusing the brain with either of their precursors, DHA or 17S-HpDHA. PD1/NPD1 levels were inversely associated with neuroinflammation, suggesting that DHA is anti-neuroinflammatory via its local conversion to SPM.
This study is unique because we injected LPS directly into the brain lateral ventricle, thereby initiating a primarily local brain inflammatory response. Earlier studies have found that DHA and DHA-derived lipid mediators decrease inflammatory markers in the central nervous system following systemic LPS administration (Mingam et al 2008), brain ischemia-reperfusion (Marcheselli et al 2003; Lalancette-Hebert et al 2011) and spinal cord injury (Huang et al 2007b), however, we cannot conclude from these studies that DHA was acting directly in neuroinflammatory pathways, since they also show a consistent attenuation of primary injury. The present results provide clear evidence that unesterified DHA directly attenuates neuroinflammation, at least in part by acting as a precursor to bioactive SPMs. Further, unesterified DHA and 17S-HpDHA have protective effects in n-3 PUFA adequate mice, arguing against the hypothesis that the use of n-3 PUFA is limited to conditions of inadequacy.

Only recently has there been an interest in enzymatically oxygenated n-3 PUFA-derived SPM (Serhan et al 2000). To date, complete lipid mediator profiles have been established in only a few tissues and disease models. PD1/NPD1 and 17-HDHA have been monitored in the hippocampus following ischemia-reperfusion (Marcheselli et al 2003). PD1/NPD1 has also been identified in the hippocampus of a triple transgenic mouse model of Alzheimer’s disease (Zhao et al 2011) and in the hippocampus of patients with Alzheimer’s disease and neurologically normal controls (Lukiw et al 2005). We report herein that PD1/NPD1 levels in the hippocampus are inversely related to neuroinflammation. Notably, there is a lack of RvD1 and RvD2 in the hippocampus, whether at baseline or in response to ischemia, therefore it is unlikely that they mediated the protection from neuroinflammation in our LPS model. Because 17S-HpDHA treatment mimicked the gene expression effects of increasing unesterified DHA and enhanced the
production of PD1/NPD1, it suggests that the 17S-HpDHA conversion to PD1/NPD1 pathway is responsible for the majority of the protective impact of unesterified DHA in our LPS models.

One confounder when chronically increasing brain DHA levels is the closely related decrease in DPAn-6 levels, an association that was confirmed in our studies. Little is known about the biological effect of DPAn-6 (Akbar et al. 2005). In addition, chronically reduced DHA levels increase ARA metabolism and may exacerbate neuroinflammation indirectly via this mechanism (Rao et al. 2007a). The results of our acute icv infusion of DHA and 17S-HpDHA allow us to attribute direct anti-neuroinflammatory effects to DHA-derived biosynthesis of local autacoids. However, not all inflammatory markers were altered by DHA or 17S-HpDHA infusion, including genes of the ARA cascade, suggesting that some of the anti-neuroinflammatory effects of DHA are due to chronic modulation of inflammatory pathways. Finally, ARA and DPAn-6 were injected acutely. There was no effect of ARA or DPAn-6, although DPAn-6 showed a trend towards exacerbating neuroinflammation. Thus, unesterified DHA and 17S-HpDHA are attenuating neuroinflammation, although chronic modulation of ARA pathways and a potentially detrimental effect of DPAn-6 cannot be discounted.

The present results show that chronically low DHA predisposes the brain to more robust and damaging icv LPS-induced inflammation. This may be one mechanism by which n-3 PUFA supplementation is beneficial in the prevention and treatment of chronic neurological disorders with a neuroinflammatory component. Direct infusion of unesterified DHA or 17S-HpDHA also attenuates neuroinflammation, and elucidates novel pathways in the brain through which DHA is exerting its anti-inflammatory effects.
4.6 Acknowledgements

Thad Vickery and Jonathan FitzGerald (CNS lab BWH), as well as Liz Cumyn (HTM lab) provided excellent technical assistance.
CHAPTER 5:
General Discussion
5.1 Overall Findings

The research presented herein supports the main hypothesis of this thesis, that DHA has anti-inflammatory effects in the brain, mediated, at least in part, by its SPM derivatives. Because the vast majority of research conducted during my PhD was compiled as a single manuscript, the integration of the individual studies with each other and within the literature appears in the discussion of that paper (thesis section 4.5). This section will discuss the overall findings of my research in relation to the objectives presented in Chapter 2.

Objective 1: To determine if the fat-1 mouse has a physiologically relevant brain fatty acid profile.

The fat-1 mouse was the first model of chronic brain DHA modulation used to test the effects of brain DHA in neuroinflammation. No reports existed on the relevance of tissue n-3 PUFA levels of fat-1 mice in relation to n-3 PUFA levels that can be attained through the diet, which is the normal route by which mammals attain essential fatty acids. To address this gap, a 2% fish oil, 8% safflower oil diet was fed to wildtype littermates of fat-1 mice from weaning until 12 weeks of age. This “dosage” is equivalent to incorporating two commonly available fish oil capsules per day, or three servings of dark meat fish per week, into the diet of the average Canadian (see section 2.3). Wildtype littermates fed the 2% fish oil, 8% safflower oil diet had similar total brain DHA levels as fat-1 mice fed a 10% safflower oil diet. From this study it can be concluded that the fat-1 mouse has physiologically relevant brain fatty acid levels, and provides a reasonable model to test the effects of brain DHA levels on neuroinflammation.
**Objective 2:** To determine if chronic increases in hippocampal DHA, both in the phospholipid and unesterified pools, attenuate neuroinflammation.

Using both a transgenic and dietary approach, we found that mice with higher hippocampal phospholipid and unesterified DHA concentrations experienced attenuated neuroinflammation and were protected against the resulting neuronal degeneration. However, in these models the comparison group was severely DHA-deprived wildtype littermates, indicated by their low brain DHA levels (on 10% safflower oil diet) and high brain DPAn-6 levels. Very low brain DHA level are thought to be extreme and can only be achieved through artificial n-3 PUFA-deprived rearing or multi-generational dietary n-3 PUFA deprivation (Ward et al 1996). The latter was done in the fat-1 mouse colony by feeding dams the 10% safflower oil diet. Due to potential limitations in the application of severe DHA inadequacy on neuroinflammation, we further explored the impact of severe vs. moderate n-3 PUFA deprivation through single-generation dietary n-3 PUFA deprivation. In these mice, 9 weeks of dietary n-3 PUFA modulation resulted in significantly different hippocampal phospholipid DHA but not unesterified DHA. There was no attenuated neuroinflammation seen in mice with higher phospholipid but similar unesterified DHA levels. This experiment gave us two important insights, i) that severe but not moderate n-3 PUFA deprivation predisposes mice to exacerbated neuroinflammation, and ii) that increased phospholipid DHA alone is not sufficient to attenuate neuroinflammation. The increase in phospholipid DHA but not unesterified DHA in this study was unexpected but beneficial because it highlighted the potential importance of the unesterified DHA pool.

**Objective 3:** To determine if acute increases in unesterified DHA or 17S-HpDHA attenuate neuroinflammation.
To assess the importance of the unesterified DHA pool and the necessity of phospholipid DHA levels in attenuating neuroinflammation, we infused unesterified DHA directly into the cerebral ventricles of mice during neuroinflammation. We found that acutely administering unesterified DHA icv post-LPS significantly attenuates hippocampal neuroinflammation. This experiment provides the first direct evidence to support the hypothesis that unesterified DHA is the key pool, an idea that is supported indirectly by i) unesterified DHA being available to metabolism by LO, unlike (esterified) phospholipid DHA; and ii) the increased production of DHA-derived SPM during an immune response to pathogens or injury. If DHA is indeed attenuating neuroinflammation through metabolism to SPM, then infusing its 17S-HpDHA derivative, which is committed to pathways that produce resolvins and PD1/NPD1, will result in a similar anti-neuroinflammatory effect; and this is exactly what was found. Importantly, the mice used in the acute infusion studies were n-3 PUFA adequate mice. This establishes something that our previous experiments did not; that there is a benefit to augmenting unesterified DHA and/or 17S-HpDHA beyond conditions of inadequacy. To confirm that the anti-inflammatory effect we were observing would not result from the infusion of any unesterified fatty acid or a fatty acid hydroperoxy derivative, we infused unesterified ARA or unesterified DPAn-6 in the same experimental model. ARA-infusions did not alter neuroinflammation, and DPAn-6 trended towards exacerbating neuroinflammation but due to a large variation in gene expression measures it was not significantly different. Overall, our acute infusion studies showed that i) increased phospholipid DHA is not necessary to attenuate neuroinflammation; ii) increased unesterified DHA is sufficient to attenuate neuroinflammation; iii) DHA is likely anti-inflammatory through 17S-HpDHA derived SPM; and iv) anti-inflammatory effects are selective to DHA.
**Objective 4:** To determine the metabolomics-lipidomic profile of the hippocampus, and if infusing DHA or 17S-HpDHA augments the production of SPM.

Ours is the first report of comprehensive metabolo-lipidomics profiling of the hippocampus. We established the presence of 17S- and more newly identified 14S-hydroperoxy pathways. In line with previous reports (Marcheselli et al 2003; Zhao et al 2011), we found that 17-HDHA (a stable metabolite and thus marker of 17S-HpDHA) and its metabolite PD1/NPD1 are produced in the hippocampus. For the first time we report the identification of MaR1 and a marker of its 14S-HpDHA precursor, 14-HDHA, in the brain. In the hippocampus, MaR1 may play an anti-neuroinflammatory role through the reduction in PMN infiltration, as has been established in murine peritonitis (Serhan et al 2012). However, our results showing that 17S-HpDHA produces a similar, if not more robust, anti-neuroinflammatory effect as unesterified DHA suggest that MaR1 and other 14S-HpDHA-derived SPM either play a minimal role in neuroinflammation, have overlapping effects with the 17S-HpDHA pathway, or have separate effects that were not measured in our model. Another novel finding of this work is the lack of RvD1 and RvD2 in the hippocampus at baseline or in response to global brain ischemia following euthanasia by carbon dioxide. RvD1 has been shown to reduce IL-1β production in glial cells *in vitro* (Serhan et al 2002; Hong et al 2003), however, its endogenous production in brain tissue had not been reported and our data suggests that it may not be produced in the hippocampus. RvD1 and RvD2 appear to play prominent roles in anti-inflammatory pathways of non-neural tissues (Spite et al 2009a; Spite et al 2009b), demonstrating that we should not generalize inflammatory mechanisms between tissues.

Another important mechanistic finding was the increased production of PD1/NPD1 in mice that received icv 17S-HpDHA post-LPS, since these mice also experience a significantly attenuated
neuroinflammatory response. Mice receiving icv DHA had PD1/NPD1 levels that trended towards an increase (p=0.07), although there was a limited sample size (n=2/group). Assuming that significance would have been reached with a larger n, with a similar magnitude of the effect, then PD1/NPD1 production would correlate inversely with neuroinflammatory measures in our model. Without this assumption, we may still conclude that the production of PD1/NPD1 appears to be protective against excessive neuroinflammation.

5.2 Limitations

As with all experimental models, it is not known if the findings are applicable to other species or types of neuroinflammation. Although my experiments were conducted in mice, there are several lines of research supporting the applicability of these findings to humans. In humans, increased dietary n-3 PUFA intake and plasma n-3 PUFA levels have been linked to attenuated peripheral inflammatory responses. The production of DHA-derived SPM has been shown ex vivo in human blood, and PD1/NPD1 has been detected in post-mortem human brain (Lukiw et al 2005). These studies combined with the studies presented herein suggest that DHA may be anti-neuroinflammatory in humans via its metabolites, but no conclusion on the importance of DHA in human neuroinflammation can be made. Also, we used an LPS model of neuroinflammation and whether DHA or its derivatives are anti-neuroinflammatory when neuroinflammation is initiated by other stimuli, particularly ischemia, trauma, Aβ, or other endogenous compounds present in neurodegenerative disorders, is of great interest. Whether the modulation of hippocampal neuroinflammation is applicable to other brain regions remains unknown.

Another limitation of this research is our inability to conclude that DHA-derived SPM attenuate LPS-stimulated neuroinflammation. The presence of PD1/NPD1 and MaR1, in consideration with the anti-neuroinflammatory effects of infusing unesterified DHA or 17S-HpDHA, suggests
but does not demonstrate that SPM themselves are anti-neuroinflammatory. Further, support comes from the inverse association between PD1/NPD1 and neuroinflammatory markers in icv 17S-HpDHA-treated mice.

While this study used the most effective method of isolating the *in vivo* innate inflammatory response of the brain, it is still impossible to conclude that the DHA-linked reductions in neuroinflammatory gene markers are due to the direct involvement of unesterified DHA and 17S-HpDHA in inflammatory pathways. *In vitro*, unesterified DHA acts not only in neuroinflammatory, but also neurotrophic, apoptotic, and synaptic plasticity pathways (Section 2.5). It is possible that once neuroinflammation was initiated by LPS, DHA protected against neuronal cell apoptosis and that this resulted in less immune-stimulating cell debris. However, neuronal degeneration was not significantly different between saline-treated fat-1 mice and their wildtype littermates, while gene expression of several neuroinflammatory markers was significantly elevated in the latter; in the absence of neuronal degeneration there was a protective effect of high brain DHA levels against neuroinflammation. Therefore, the actions of DHA on neuroinflammation are likely direct, rather than via attenuated neuronal degeneration pathways.

Unesterified DHA and its enzymatically oxygenated derivatives were the only thoroughly explored mechanism of action in this research, and it is possible that DHA is anti-neuroinflammatory through other derivatives or pathways. For example, ethanolamide derivatives of DHA have been measured in murine brain. Docosahexaenoyl ethanolamide binds to cannabinoid receptor type 2, and reduce *in vitro* PMN chemotaxis and platelet-leukocyte aggregation, both of which play important roles in inflammation (Yang et al 2011b). Docosahexaenoyl ethanolamide also promotes synaptogenesis *in vitro* (Kim et al 2011b). Aside
from DHA derivatives that might be anti-neuroinflammatory via cannabinoid signaling pathways, there may be other pathways that have not yet been identified.

With brain SPM measurement in its infancy, there has not been extensive research on how to best capture their true tissue concentrations. It is an important consideration due to the unique nature of the brain, which sees rapid increases in unesterified ARA and DHA levels in ischemic euthanasia. This potential rapid increase is the rationale for euthanizing via high-energy, head-focused irradiation (fixation) since it denatures phospholipases responsible for creating this artifact in less than one second. We chose to measure SPM following fixation based on two findings from pilot data, i) SPM concentrations increased significantly following ischemic euthanasia, and ii) SPM were detectible following microwave fixation. While our pilot data suggest that fixation is the best method, it is possible that there was significant SPM degradation that lowered our values from their true in vivo concentrations. Thus, it is more accurate to use our concentration measures for comparison between and within groups than as absolute values. Similar to interpreting values for unesterified DHA, extra caution should be used when comparing SPM values from un-fixed and fixed brains.

Markers of neuroinflammation were examined at a single time-point, therefore we do not know the state of inflammation before or following our measures 24 h post-LPS. It is possible that neuroinflammation was either not different or exacerbated in DHA-treated mice at other time-points. However, exacerbated neuroinflammation at different time-points is unlikely given the many studies reviewed (section 2.7) that find increased n-3 PUFA have either no effect or an attenuating effect on neuroinflammation during brain disease and injuries.
5.3 Future Research

Studies are needed that address several of the limitations discussed above. Further elucidating the mechanism of DHA of action in neuroinflammation is one line of research that would help to more fully define our LPS-stimulated neuroinflammation model. What is the temporal course of events? How are the levels of SPM affected by inflammation in the brain? Inflammatory reactions are defined by an early phase of PMN infiltration, a middle phase of monocyte and macrophage infiltration, and finally a clearance of debris and immune cells. Once defined, it is possible to measure if and how they are affected by unesterified DHA or DHA-derived SPM. One of the main mechanisms of action of DHA-derived SPM is the reduction of PMN infiltration (Serhan et al 2008). PMN influx to the brain in an animal model of stroke is significantly reduced by PD1/NPD1 treatment (Marcheselli et al 2003). PMN influx was not measured in our studies, but our findings combined with these studies suggest that DHA-derived SPM would reduce brain PMN infiltration resulting from icv LPS.

Complementary to determining how DHA affects the course of cellular events, positron emission tomography (PET) imaging and post-mortem tissue measures of SPM could be used to define the course of DHA metabolism during neuroinflammation. PET provides a technique to image in vivo DHA metabolism. After an i.v. infusion of radiolabelled unesterified DHA, quantitative autoradiography can measure the rate of DHA incorporation into brain from plasma, an established biomarker of brain PUFA metabolism (Basselin et al 2012). In animal models, drugs that might attenuate SPM production, for example a 15-LOX blocker, could be tested for their effects on in vivo DHA metabolism using PET. In humans, this technique offers the ability to measure brain DHA metabolism in response to short-term and long-term dietary n-3 PUFA supplementation. This measure would be insightful for studies using n-3 PUFA supplements in
randomized control trials of neurological disorders, even if only in a subset of participants. PET measures of altered DHA metabolism would be even more insightful in studies seeing no effects of n-3 PUFA supplementation, since it is possible that the supplementation has not altered brain DHA metabolism.

Determining if PD1/NPD1 and MaR1 have anti-neuroinflammatory effects in the brain is necessary to conclude that SPM are indeed a mechanism of DHA’s anti-inflammatory actions. Further, investigating if they have differential effects in the neuroinflammatory response will help determine which targets might be useful in a clinical treatment for neuroinflammation. Specifically, if an increase in both the 17S- and 14S-HpDHA pathways is necessary for robust anti-neuroinflammatory effects, then targets would be needed that increase unesterified DHA. However, if only PD1/NPD1 is necessary to attenuate neuroinflammation, then a PD1/NPD1 mimetic would be a specific and potentially more beneficial treatment for excessive neuroinflammation.

The unique SPM profile detected in the brain warrants closer examination. RvD1 plays a particularly important role in peripheral immune responses (Spite et al 2009b), yet it was not detected in mice hippocampi in response to LPS or ischemia. PD1/NPD1 seems to be the predominant DHA-derived SPM produced in brain tissue, but there have been far fewer reports of a systems approach with LC-MS-MS lipidomics and informatics in the brain than in the periphery, thus it is possible that there are yet-undefined brain-specific DHA-derivatives. This is plausible since neuroinflammation is dominated by microglia, immune cells unique to the brain that may have unique DHA metabolic pathways. Identification of brain-specific SPM could provide novel targets for the modulation of neuroinflammation.
It is also possible that EPA plays a role in neuroinflammation, which we did not focus on due to the very low to non-detectible levels of brain phospholipid and unesterified EPA concentrations (Igarashi et al 2007; Chen et al 2011). Against expectations, EPA derivatives were detected in the LC-MS-MS lipidomics analysis of the hippocampus during neuroinflammation; particularly interesting were the increases in RvE1 and its precursor 18-HETE. In keeping with this limitation, other fatty acids, aside from unesterified ARA and DPAn-6, might also affect neuroinflammation.

Receptors for SPM are just beginning to be identified. RvD1 binds with high affinity to two G-protein coupled receptors, FPR2/ALX, which also mediates ARA-derived lipoxin A₄ signaling, and the orphan receptor GPR32 (Krishnamoorthy et al 2010). There have been no receptors identified for PD1/NPD1 or MaR1, the two DHA-derived SPM identified in the hippocampus during LPS-induced neuroinflammation.

5.4 Implications

The results of this work have direct implications for research, and potential implications for clinical and public health practice. Research investigating the effects of DHA in the brain should pay particular attention to whether they achieved a difference in unesterified DHA between comparison groups, given its necessity for biological effects in our studies. The brain is notoriously resistant to changes in total DHA concentrations, requiring multigenerational dietary n-3 PUFA deprivation to achieve low levels (Ward et al 1996), and accreting dietary DHA following depletion slower than other tissues including retina, liver, and serum (Moriguchi et al 2001). This study adds another layer of complexity by identifying the need to modulate the brain unesterified DHA pool, which in our chronic dietary study was more resistant to change compared to the brain phospholipid DHA pool.
In animal research it is possible to capture brain unesterified DHA levels and incorporate the measure into the overall findings. There is also an interest in considering this concept in human research, as it may explain the differential findings of clinical and epidemiological research investigating the effect of dietary n-3 PUFA on neurological disorders. Clinical studies do not consistently support a beneficial effect of n-3 PUFA supplementation in Alzheimer’s disease, Parkinson’s disease, or Multiple Sclerosis, among other neurological disorders. Epidemiological studies more consistently find a benefit of long-term n-3 PUFA consumption in lowering the risk of developing these disorders. Our dietary studies suggest that it may not be feasible to increase brain unesterified DHA levels through the diet in clinical research studies. This is consistent with the finding that human total brain DHA has a long turnover rate, based on a half-life of 2.1 years (Rapoport et al 2007). The most common dietary n-3 PUFA supplementation period is well under 1 year and thus is unlikely to modulate participants’ brain DHA levels or provide any treatment effect. In the case of chronic neurological disorders, increasing the bioavailability of unesterified DHA might be a more effective strategy, if attenuating neuroinflammation can benefit the course of a fully developed neurological disorder at all. Prevention of neuroinflammation might be more feasible through dietary guidelines recommending adequate n-3 PUFA consumption throughout the lifespan, and is discussed below.

Our findings may have implications for the treatment of acute brain injury. Neuroinflammation was reduced when DHA or 17S-HpDHA was administered post-LPS, indicating that a chronic upregulation of unesterified DHA pathways prior to injury is not necessary to benefit from post-injury increases in DHA and 17S-HpDHA levels. This idea is supported by two other lines of research. First, post-ischemia icv unesterified DHA or PD1/NPD1 attenuates stroke volume in experimental ischemia-reperfusion (Marcheselli et al 2003). In experimental spinal cord injury,
post-injury i.v. DHA significantly reduces lesion size (King et al 2006; Huang et al 2007b). In both of these acute injury models, neuroinflammatory markers were reduced in DHA-treated animals along with the primary outcome. Our work showing an attenuating effect of unesterified DHA and 17S-HpDHA in an isolated neuroinflammatory response indicates that the reduction in neuroinflammation seen in these models can, in part, be directly attributable to the actions of DHA; and further, that targeting neuroinflammation post-injury can promote beneficial clinical outcomes, whether by DHA or by other anti-neuroinflammatory compounds and pathways. The second line of research supporting a potential clinical benefit of unesterified DHA or 17S-HpDHA in the treatment of acute brain injuries is the ability of human cells to produce DHA-derived SPM. D-series resolvins, PD1/NPD1, and MaR1 are produced ex vivo in human PMN and macrophages (Hong et al 2003; Serhan et al 2012), PD1/NPD1 is produced in exhaled breath condensates of exacerbated human asthma (Levy et al 2007), and is found in human post-mortem hippocampus (Lukiw et al 2005). With benefits to acute unesterified DHA and 17S-HpDHA in experimental neuroinflammation, an association between attenuated neuroinflammation and neuronal damage in experimental acute brain and spinal cord injury, and the presence of DHA-derived SPM pathways in humans, it is possible that unesterified DHA or drug analogues might be used clinically to target anti-inflammatory pathways in acute human brain injury. Future clinical research in this area is warranted.

While this research has even less direct implication for public health than it does clinically, it supports a growing body of literature promoting the necessity of DHA for optimal health (Deckelbaum et al 2012), particularly of the brain (Simopoulos 2011). While there is no known optimal level of DHA intake, our results showed that mice exposed to low levels of dietary DHA had low brain DHA concentrations and were predisposed to more robust neuroinflammatory
responses. Given the presence of neuroinflammation in neurodegenerative diseases (Glass et al 2010), it is possible that chronically low brain DHA levels can predispose or exacerbate neurodegenerative disorders. Therefore, dietary guidelines that encourage the adequate consumption of DHA, or n-3 PUFA, may be one population strategy for the prevention of neurodegenerative disorders. Currently, there are no dietary reference intakes for DHA or EPA, but ALA has an adequate intake level of 1.6 and 1.1 g/d for males and females, respectively, of which DHA and EPA account for up to 10%. What level of ALA intake is necessary to maintain a currently undefined optimal brain level of DHA in humans is not known and requires further study.

5.5 Conclusion

The overall hypothesis of this thesis is that DHA has anti-inflammatory effects in the brain, mediated, at least in part, by its SPM derivatives. The research presented supports the overall hypothesis.

In relation to the objectives of this thesis, the following conclusions can be made:

1. The fat-1 mouse has a physiologically relevant brain fatty acid profile.

2. Chronic increases in hippocampal unesterified DHA, but not phospholipid DHA, attenuate neuroinflammation.

3. Acute increases in unesterified DHA or 17S-HpDHA in the brain attenuate neuroinflammation.

4. PD1 and MaR1, but not RvD1 or RvD2, are present in the hippocampus. Augmenting 17S-HpDHA leads to increased PD1 levels concurrent to attenuating neuroinflammation.
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