THE EFFECTS OF DIETARY OMEGA-3 FATTY ACIDS ON BRAIN FUNCTION ARE SEX, AGE AND BRAIN-REGION SPECIFIC

By

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ABSTRACT

Omega (n)-3 polyunsaturated fatty acids (PUFA) have beneficial effects in neuropsychiatric illnesses. The goals of this thesis were to determine the effects of feeding diets varying in n-3 PUFA on brain fatty acid composition, and neurotrophin and myelin-related gene expression of the brain in an age, sex, and region-specific manner. A diet high in n-3 PUFA altered phospholipid docosahexaenoic acid (DHA) and oleic acid composition in an age, sex, and region-specific manner. Diet had no effect on the mRNA expression of brain-derived neurotrophic factor (BDNF) and tropomyosin-receptor kinase-B (TrkB); however, stearoyl-CoA desaturase-1 (SCD1) and myelin basic protein (MBP) gene expression increased in offspring fed a diet high in n-3 PUFA in an age, sex, and region-specific manner. DHA treatment to *ex vivo* cerebral cortical cells showed an increase in BDNF, TrkB, SCD1, and MBP mRNA expression compared to control cells. The mRNA expression of BDNF and SCD1 was higher in DHA treated cells compared to arachidonic acid treated cells. Overall, the data presented in this thesis suggests that the potential benefits of n-3 PUFA on brain function are sex, age and brain-region specific.

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ABBREVIATIONS

AA	Arachidonic acid
ADA	Adrenic acid
ALA	Alpha-linolenic acid
BBB	Blood brain barrier
BDNF	Brain-derived neurotropic factor
CNS	Central nervous system
CREB	cAMP response element binding protein
DHA	Docosahexaenoic acid
ELOVL	Elongases
EPA	Eicosapentaenoic acid
FADS	Fatty acid desaturases
GAD	General anxiety disorder
GC	Gas chromatography
HBSS	Hank's balanced salt solution
LA	Linoleic acid
LPC	Lysophosphatidylcholine
mBDNF	mature brain-derived neurotrophic factor
MBP	Myelin basic protein
MDD	Major depressive disorder
MRI	Magnetic resonance imaging
MUFA	Monounsaturated fatty acid
NGF	Nerve growth factor

PET	Positron emission tomography
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PS	Phosphatidylserine
PLO	Poly-L-ornithine
PL	Phospholipid
p38 MAPK	p38 mitogen-activated protein kinase
PNS	Peripheral nervous system
PUFA	Polyunsaturated fatty acid
n-3 PUFA	Omega-3 polyunsaturated fatty acid
n-6 PUFA	Omega-6 polyunsaturated fatty acid
RT-qPCR	Real-time quantitative polymerase chain reaction
SCD1	Stearoyl-CoA desaturase-1
SFA	Saturated fatty acid
Sph	Sphingomyelin
TrkB	Tropomyosin-receptor kinase B

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Chapter 1: Introduction

1.1 Neuropsychiatric illnesses

The increasing prevalence of neuropsychiatric illnesses is imposing a global economic burden surpassing that of cardiovascular disease and cancer (Collins *et al*., 2011). Commonly known examples of these conditions are depression, general anxiety disorder (GAD), autism, Alzheimer's disease and schizophrenia (Miyoshi & Morimura, 2009). According to a report by the National Institute of Health in 2010 (Vos *et al.*, 2012), neuropsychiatric illnesses are ranked as the leading disorders to affect the global population. When focusing on Canada, 12.6% of Canadians in 2012 met the criteria for having some mood disorder during their lifetime (Pearson *et al.,* 2013). Of these Canadians, depression accounted for 11.3% percent, whereas 8.7% reported symptoms consistent with that of GAD (Pearson *et al.*, 2013).

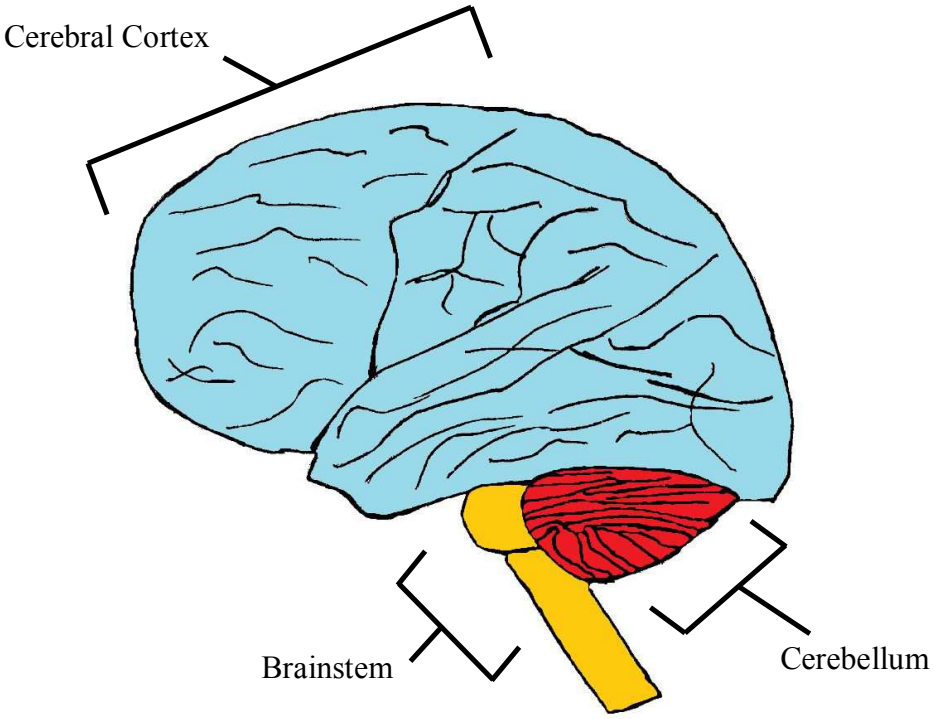
The rates of depression and GAD vary with sex: females suffer from depression and anxiety at a higher rate compared to males (Kessler, 1994). Canadian women show a higher rate of depression than that of Canadian men, especially between the ages of 15 to 24 (Pearson *et al.*, 2013). Moreover, the rates of occurrence of neuropsychiatric illnesses vary with age. For example, the occurrence of GAD and depression is more prevalent in young adults (Pearson *et al.*, 2013); however, the occurrence of Alzheimer's disease is higher in persons of age 65 or older (Xu *et al.*, 2010). Thus, age and sex further add to the complexity of neuropsychiatric disorders.

1.1.1 The complexity of the brain and neuropsychiatric illnesses

The brain is a highly sophisticated and complex organ organized into several regions (Figure 1.1), which govern specific functions. The morphological differences in various regions of the brain are due to the number, density, axonal branching, and synaptic connectivity of neurons (Bansal *et al.*, 2015). The brain also consists of non-neuronal cells, known as glial cells, which maintain homeostasis, myelin formation, and provide support and protection for neuronal cells. Glial cells are also capable to communicate with themselves and neurons through the release of neuroactive substances known as gliotransmitters (Navarrete *et al.*, 2013; Martineau, 2013). The variability in the size and number of glial cells, blood vessels, and the amount of extracellular space can contribute to the differences in brain morphology (Bansal *et al.*, 2015). Furthermore, it is suggested that variances in the morphology of different brain regions can be used to diagnose specific neuropsychiatric disorders (Bansal *et al.*, 2012; Bansal *et al.*, 2015). However, the majority of the studies assessing the function of the brain during periods of illness have focussed on the cerebral cortex; this is due largely to the extent and distribution of abnormal biochemical markers specifically in this region, compared to control subjects (Bowen *et al.*, 1989).

The cerebral cortex covers a vast area of the brain, which is separated into two hemispheres along the sagittal plane. The cerebral cortex is further divided into many sub-regions, which play a role in memory, attention, language and higher-level perception. The cerebral outer layer is highly composed of neuronal cell bodies, forming a large outer gray matter area (Chavko *et al.*, 1993; Söderberg *et al.*, 1990). However, the

Figure 1.1: Anatomical representation of the cerebral cortex, cerebellum and brainstem location in the brain.


Cerebral Cortex
Brainstem
Cerebellum

cerebral inner area contains an inner core of myelinated axons forming the white matter (Chavko *et al.*, 1993; Söderberg *et al.*, 1990). When assessing the volume and size of the human brain, isotopic fractionation has shown the cerebral cortex to make up approximately 81.2% of the total brain mass; however, it only contains 19% of the brain's neurons (Azevedo *et al.*, 2009). Furthermore, the ratio of glial cells to neurons for the cerebral cortex is 3.76, showing a higher population of glial cells than neurons (Azevedo *et al.*, 2009).

With the use of magnetic resonance imagining (MRI), studies have found associations between neuropsychiatric illnesses and the cerebral cortex (Canu *et al.*, 2015; Zielinski *et al.*, 2014). Canu *et al.* (2015) has shown thinning of various sub-regions of the cerebral cortex in patients with major depression disorder (MDD) and GAD, compared to healthy subjects with no history of neuropsychiatric disorder. In addition, autistic children were shown to have cortical thinning throughout mid-childhood compared to the brains of healthy children during the same developmental period (Zielinski *et al.*, 2014), further highlighting the importance of the cerebral cortex in brain function. Interestingly, there is increasing evidence supporting the role of the cerebellum in emotion and cognition (Schutter & Van Honk, 2005). Disruptions in these functions are linked to several neuropsychiatric illnesses (Hoppenbrouwers *et al*., 2008).

The cerebellum, also known as the "little brain", is located ventrally posterior to the cerebral cortices (Figure 1.1). Like the cerebral cortex, the cerebellum is divided into two hemispheres. Most notably, the cerebellum controls motor function and learning, balance, and various cognitive functions. The outside of the cerebellum is composed of gray matter, while the core is composed of white matter (Chavko *et al.*, 1993; Söderberg

et al., 1990). Compared to the cerebral cortex, the cerebellum, however, has a higher content of white matter (Chavko *et al.*, 1993). The human cerebellum makes up 10.3% of the total brain mass; however, it has four times the number of neurons than the cerebral cortex (Andersen *et al.*, 1992; Azevedo *et al.*, 2009). In addition, the human cerebellum has a much lower number of glial cells than the cerebral cortex, with a glial cell to neuron ratio of approximately 0.23 (Azevedo *et al*., 2009). Cerebellar dysfunction has been associated with multiple neuropsychiatric disorders, including autism, schizophrenia, mood disorders, and anxiety disorders *(*Greer *et al.,* 2005; Courchesne *et al.*, 1988; Bottmer *et al.*, 2005). In particular, neuroimaging studies assessing individuals with depression detected significant differences in both cerebellar structure and function (Bench *et al.,* 1992; DelBello *et al*., 1999; Dolan *et al*., 1992). Moreover, individuals suffering from autism have a loss and degeneration of cerebellar purkinje and granule cells (Vargas *et al*., 2005).

Another vitally important region of the brain is the brainstem. The brainstem is a region found ventral to the brain (Figure 1.1), continuous to that of the spinal cord. It includes three subregions: 1) the medulla oblongata; 2) pons; and 3) midbrain. The brainstem tract contains the main cranial nerve nuclei, which innervate various regions of the body, including regions of the respiratory and circulatory system. Most importantly, the brainstem is the route by which motor and sensory information is carried to and from the central nervous system (CNS) to the periperheral nervous system (PNS). Compared to the cerebral cortex and cerebellum, the brainstem consists of mostly white matter; however, it also includes clusters of gray matter (Chavko *et al.*, 1993; Fernandez-Gil *et al*., 2010). In addition, the human brainstem contains less than 1% of the total brain

neurons; however, the glial cell to neuron ratio is approximately 11.35 (Azevedo *et al*., 2009). Brainstem-related symptoms, such as gait and balance impairment (Golbe, 2001), as well as depression (Rub *et al.*, 2000) in Alzheimer's patients suggests the brainstem to play a role in the pathogenesis of Alzheimer's disease (Morcinek *et al.*, 2013). These symptoms have been linked to the build-up of tau proteins, a hallmark of Alzheimer's disease, in the brainstem (Simic *et al*., 2009). Therefore, the brainstem also plays a specific role in the development of neuropsychiatric illnesses.

1.1.2 Neurotrophins and neuropsychiatric illnesses

A group of secreted trophic factors in the brain, known as neurotrophins, are required for neuronal differentiation, survival and growth (Huang & Reichardt, 2001). The four main neurotrophins in the brain are brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3, and neurotrophin-4/5 (Park & Poo, 2013). Neurotrophins begin their actions during gestation; however, the role and activity of neurotrophins varies throughout different stages of growth and development (Birling & Price, 1995). Neurotrophins are necessary for the proper growth and development of the CNS, where they play a vital role in synaptogenesis, synaptic plasticity, neuronal survival and neurogenesis (Chao *et al.*, 2006). The upregulation of neurotrophins was shown to alleviate the symptoms of neuropsychiatric disorders (Razavi *et al.*, 2015; Chao *et al,* 2006).

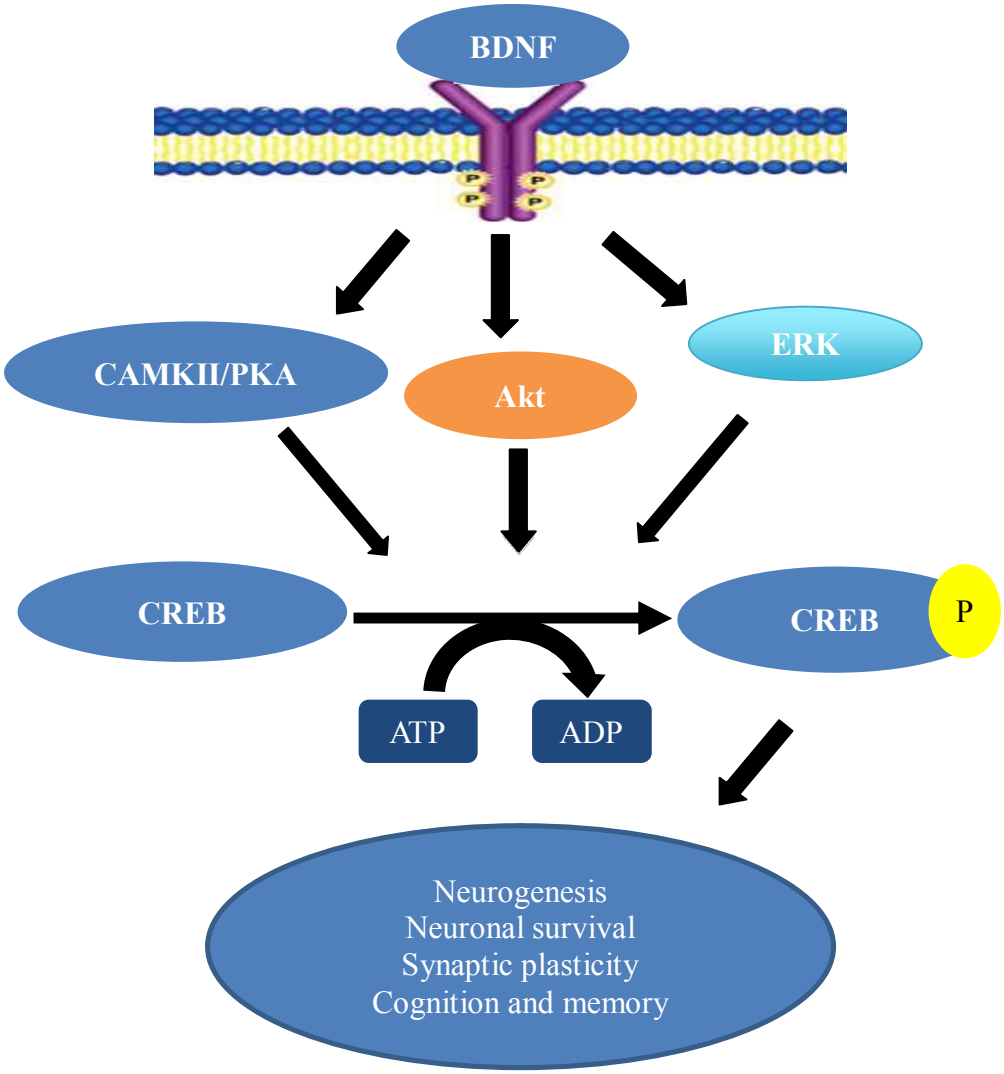
1.1.3 BDNF and brain function

Among all neurotrophins in the brain, BDNF is one of the main secretory proteins in the CNS (Chao *et al*., 2006). A dysfunction in the expression of BDNF was previously shown to render significant deficits in neuronal signalling activities (Mariga *et al.*, 2015).

Furthermore, reduced BDNF expression predisposed humans and rodents to have an altered cognitive-behavioural functions, as well as increasing their risk for neuropsychiatric illnesses (Castrén & Rantamäki, 2008; Chen *et al.*, 2006). BDNF is first translocated into the endoplasmic reticulum as a precursor protein pre-proBDNF (Mowla *et al*., 1999). The pre-signalling sequence is then cleaved to render proBDNF, which is transported to the Golgi; the proBDNF is then cleaved by furin and endoprotease into an approximately 14 kilodalton (kDa) mature BDNF (mBDNF) (Mowla *et al.*, 1999). Cleavage of proBDNF into mBDNF can also occur in secretory granules by convertases before being exocytosed into the synaptic cleft (Mowla *et al.*, 1999). BDNF expression was shown to be stimulated with or without an increase in neuronal activity (Bramham, 2008; Bramham & Messaoudi, 2005). Once secreted, mBDNF can bind to its high-affinity receptor tropomyosin-receptor kinase B (TrkB), promoting an intracellular signalling cascade (Figure 1.2) (Kaplan & Miller, 2000). Upon binding, TrkB dimerizes and autophosphorylates, which initiates downstream phosphorylation of several proteins involved in the PI3K/Akt pathway, Ras/Raf pathway, and the cAMP response element binding protein (CREB) (Kaplan & Miller, 2000). Phosphorylation of CREB then leads to the transcription of genes involved in neuronal survival, neurogenesis and synaptic plasticity (Kaplan & Miller, 2000).

The importance of BDNF during development was established using a BDNF knockout mouse that developed brain abnormalities, and died soon after birth (Ernfors *et al.*, 1994). In addition, mice lacking fully functional hippocampal TrkB signalling during neuronal development showed an increase in anxiety-like behaviours in later life (Bergami *et al.*, 2008). Thus, abnormalities in mBDNF synthesis, release, and signalling

Figure 1.2: BDNF signal transduction pathway. *BDNF binds to its high-affinity receptor tropomyosin-receptor kinase B (TrkB) to initiate the activation of* Ca^{2+} */calmodulin-dependent protein kinase (CAMKII), protein kinase A (PKA), protein kinase B (PKB) AKT, and extracellular-signal-regulated kinase (ERK) pathways. This leads to phosphorylation of cAMP response element-binding protein (CREB), which enters the nucleus and activates transcription of genes involved in neurogenesis, neuronal survival, synaptic plasticity, and cognition and memory. (Adapted from Kaplan & Miller, 2000, Current Opinions in Neurobiology, 10; 381)*


BDNF
CAMKII/PKA
Akt
ERK
CREB
CREB
P
ATP
ADP
Neurogenesis
Neuronal survival
Synaptic plasticity
Cognition and memory

have been shown to be a culprit in the development of neuropsychiatric illnesses (Rehn & Rees, 2005; Chen *et al*., 2006). Patients with MDD showed reduced serum protein levels of mBDNF (Matrisciano *et al.*, 2009), which was mitigated with antidepressants (Castrén & Rantamäki, 2010). Our laboratory has previously established that dietary factors, such as omega (n)-3 polyunsaturated fatty acids (PUFA), increase the gene expression of mBDNF (Balogun & Cheema, 2014); however, the exact mechanism is still not clear.

1.1.4 Risk factors associated with neuropsychiatric illnesses

Multiple factors, such as genetics, sex, and environment can play critical roles in neuropsychiatric illnesses. Furthermore, sex-specific differences related to structural and hormonal differences between males and females add to the complexity of neuropsychiatric illnesses (Bao & Swaab, 2010). A recent meta-analysis found that males have 8-13% larger brain volumes compared to females (Ruigrok *et al.*, 2014). Furthermore, MRI studies have also shown that various regions of the brain differ in size and volumes between adult males and females (Goldstein *et al.*, 2001; Ruigrok *et al.*, 2014).

Environmental factors may play an important role in the development of neuropsychiatric illnesses. Among the many environmental factors, nutritional status during critical periods of brain development was shown to affect the outcomes of the offspring's mental health (Jacobson *et al.*, 2008; Crawford, 1992; Weiser *et al*., 2015). The brain is largely composed of lipids; 60% of the brain's structural material is lipid (Crawford, 1992). Studies have shown that the amount and type of dietary fatty acids can affect the lipid composition of various regions of the brain, thereby influencing the onset of neuropsychiatric illnesses in later life (Bhatia *et al.*, 2011; Balogun & Cheema, 2014).

1.2 Fatty acid composition of the brain

Fatty acids in the brain come from the peripheral circulation, and are either derived from the diet or from hepatic *de novo* synthesis (Legarde *et al.*, 2001; Theis *et al.*, 1994). In addition, the brain is also capable of synthesizing fatty acids (Aeberhard & Menkes, 1967). The majority of the saturated fatty acids (SFA) in the neuronal membrane phospholipids (PLs) of the brain are palmitic acid (C16:0) and stearic acid (C18:0), making up approximately 19% and 17% of total SFA, respectively (Edmond *et al.*, 1998; McNamara & Carlson, 2006). The most abundant monounsaturated fatty acids (MUFA) are oleic acid (C18:1) and eicosenoic acid (C20:1n-9), comprising of approximately 19% and 1% of total MUFA, respectively (Edmond *et al.*, 1998; McNamara & Carlson, 2006). Finally, the most abundant PUFAs in the brain are the n-3 PUFA docosahexaenoic acid (DHA; C22:6n-3) and the n-6 PUFA arachidonic acid (AA; C20:4n-6) (Martínez & Mougan, 1998). DHA and AA account for 50% and 40% of the total PUFAs in the brain, respectively (Lauritzen *et al.*, 2001). In general, the brain is composed of these SFAs, MUFAs and PUFAs, but various regions of the brain differ in their specific fatty acid compositions.

1.2.1 Region-specific differences in the fatty acid composition

The neuronal membrane is highly enriched in lipids, particularly PLs (Svennerholm, 1968). The PLs that primarily make-up the neuronal membrane are phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), and sphingomyelin (Sph) (Svennerholm, 1968). In addition, PC and PE are the most abundant PLs in the neuronal membranes of an adult whole brain (Sun & Horrocks, 1967). There is only one study to date to show that the

fatty acid composition of the specific PLs of the brain can change during development and in later life (Svennerholm, 1968). In this study, it was reported that the gray matter PE contains C18:0 as the most abundant fatty acid at all ages; although, in white matter PE, C18:1 made up over 40% of four year-old children's and adult's brains, while C18:0 comprised only about 10%. Interestingly, white matter PE was shown to have a sparse amount of DHA after childhood and it did not increase with age. Furthermore, gray matter PC esterified with DHA increased with age; however, in white matter PC, DHA and AA decreased with age. This study only attributes the age-specific effects of the specific PL fatty acid composition to white and gray matter from the cerebral cortex. However, we postulate the specific PL fatty acid compostion of the cerebellum, cerebral cortex, and brainstem will differ due to the differences in the regional composition of gray and white matter. As previously mentioned, the brainstem has the highest amount of white matter, followed by the cerebellum and then cerebral cortex (Chavko *et al.*, 1993). White matter is highly enriched with SFA and MUFAs, and depleted of PUFAs, which is due to the region-specific differences in the specific PL species (Kishimoto *et al.*, 1969). Thus, the regional and age-related fatty acid composition of the brain appears to pertain to that of the content of white versus gray matter. It would be important to know the age-related regulation of the specific PL fatty acids of different regions of the brain, as the development of various regions of the brain is age dependent. In addition, whether the specific fatty acid composition of different regions of the brain can be altered by environmental factors, such as diet, and whether age plays an important role is not known.

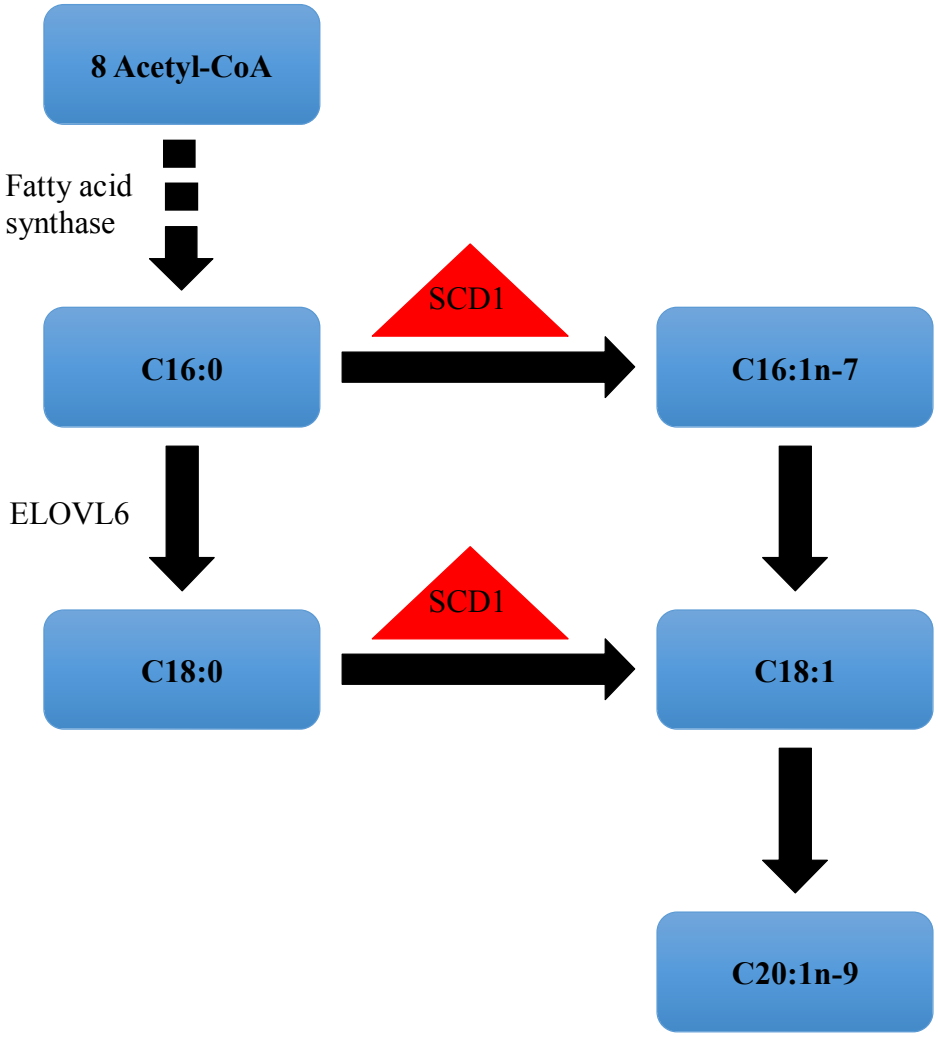
1.2.1.1 Effect of age on brain spatial fatty acid composition

A handful of studies have shown age specific effects on the fatty acid composition of the brain. A study by Xiao *et al.* (2005) found that the cerebral cortex from 3-day-old rat pups had similar percentages of approximately 11.0% DHA in the cerebral cortex, cerebellum, and brainstem PLs; however, at postnatal day 21, the PL DHA was higher in the cerebral cortex (15.3%) than the cerebellum (13.8%) and brainstem (13.6%). This trend of regional DHA distribution carried into the adult-life, and it stayed significantly higher in the cerebral cortex compared to the cerebellum and brainstem, while the amount of AA remained similar in all regions of the brain and decreased with age. However, Carrié *et al.* (2000) has reported that the amount of AA was highest in the cerebral cortex compared to the brainstem and cerebellum in the adult brain. The amount of SFA and MUFA has also been shown to vary with age. The adult mouse cerebral cortex has a higher amount of SFA, particularly C18:0, followed by the cerebellum and brainstem (Carrié *et al.*, 2000). Furthermore, C16:0 levels were similar in the cerebral cortex, cerebellum, and brainstem; however, C16:0 decreased with age (Xiao *et al.*, 2005). The amount of C18:1 was higher in the brainstem and cerebellum compared to the cerebral cortex, and it increased with age in all regions of the brain (Carrié *et al.*, 2000; Xiao *et al.*, 2005). During development and throughout life, SFAs and MUFAs have been shown to serve important structural and physiological functions (O'Brien & Sampson, 1965; Rodriguez-Rodriguez *et al.*, 2004). These include the formation of the myelin sheath (Edmond et al. 1998; Muse et al. 2001), dendritic differentiation (Rodriguez-Rodriguez *et al.*, 2004), and inflammation (Kwon *et al.*, 2014). Thus, understanding the synthesis of SFAs and MUFAs is vitally important.

1.2.2 SFA and MUFA synthesis and their functions in the brain

Oligodendrocytes have the capacity to synthesize the requirement of SFAs and MUFAs for the neuronal membrane PLs (Garbay *et al.*, 1997). Therefore, the synthesis of SFAs and MUFAs in the brain is independent of exogenous SFAs and MUFAs in the circulation (Edmond *et al.*, 1998). In the brain, C16:0 is the final product of fatty acid synthase (Figure 1.3), which can be elongated to make C18:0; these two SFAs can then be desaturated via delta-9 desaturase or stearoyl-CoA desaturase-1 (SCD1) to synthesize MUFAs. The main MUFAs produced after desaturation are C16:1n-7 from C16:0, C18:1 synthesized from C18:0, and C20:1n-9 from the elongation of C18:1 by elongase-6. During development, there is a rapid accumulation of SFAs and MUFAs in the brain, along with an increased activity of the rate-limiting enzyme SCD1 to form more MUFAs (DeWille & Farmer, 1992). Interestingly, studies assessing brain PL fatty acid composition after feeding diets varying in C18:1 found no change in C18:1 PL fatty acid composition (Rioux & Innis, 1992; Bourre & Dumont, 2003; Edmond *et al.*, 1998). Therefore, brain C18:1 production may come from SCD1-mediated production, as opposed to uptake from circulation. MUFAs are needed by the brain as a source of energy, ligands for various physiological functions (German, 2011) and the synthesis of PLs for the developing neuronal membrane (Lengi & Corl, 2015). Furthermore, there is a rapid production and accumulation of MUFAs, primarily C18:1 and C20:1n-9 during brain development for the formation of myelin sheath (Svennerholm, 1968). During the first 12-30 days after birth in rodents, the CNS oligodendrocytes promote massive synthesis of long-chain SFAs and MUFAs to promote the formation of the myelin sheath (Edmond *et al.*, 1998; Muse *et al.*, 2001).

Figure 1.3: Pathways for biosynthesis of saturated and monounsaturated fatty acids in the brain. *Palmitic acid (C16:0) is the main end product of brain fatty acid synthase which can be elongated and desaturated to form C16:1n-7, C18:1, and C20:1n-9. Stearoyl-CoA desaturase-1 (SCD1) is the rate-limiting enzyme for the desaturation of C16:0 and C18:0. ELOVL6 = elongase-6; ELOVL3 = elongase-3*


8 Acetyl-CoA
Fatty acid synthase
C16:0
SCD1
C16:1n-7
ELOVL6
C18:0
SCD1
C18:1
C20:1n-9

Myelin sheath formation in the rodent brain occurs between days 10-12 after birth, with a maximal rate of formation at day 20 (Cuzner & Davison, 1968). In particular, C18:1 is an important fatty acid for proper myelin sheath formation during development and later life (Rioux & Innis, 1992; Bourre & Dumont, 2003). In fact, 20-40% of the total fatty acids that comprise the myelin sheath are C18:1 (Norton & Cammer, 1984), and neuronal membrane C18:1 increases with the development of myelin (Garbay *et al.*, 1998). Thus, any alterations in the rate of synthesis of MUFAs and SFAs in the brain will affect the brain fatty acid composition.

A dysregulation in fatty acid homeostasis was associated with neuropsychiatric disorders (Lin *et al.*, 2010; Hamazaki *et al.*, 2012; Hamazaki *et al.*, 2013). In particular, significantly higher amounts of C16:0 and lower amounts of C18:1 were found in the amygdala from post-mortem MDD patients compared to control subjects (Hamazaki *et al.*, 2012).

A possible explanation for this alteration is thought to be due to changes in SFA and MUFA biosynthesis in the brain of MDD patients (McNamara & Liu, 2011). Specifically, there is only one human study to date to show a reduction in the mRNA expression of SCD1 in the prefrontal cortex of patients with MDD (McNamara & Liu, 2011). McNamara & Liu (2011) promote the importance of SFA and MUFA biosynthesis in the brain via SCD1, but more research needs to be undertaken. In addition, it is not clear whether the synthesis of SFA and MUFA in different regions of the brain is differentially regulated.

1.2.3 N-3 and n-6 PUFA in the brain

Although the brain can synthesize its own SFAs and MUFAs, PUFA synthesis

and transport to the brain is still debatable. Several mechanisms have been postulated for the entry of PUFAs into the brain from peripheral circulation (Figure 1.4). It has been suggested that PUFAs arrive at the blood brain barrier (BBB), a highly selective endothelial barrier, in either a non-esterified or esterified form (Lagarde *et al.*, 2001). Esterified PUFAs can arrive to the BBB as lysophosphatidylcholine (LPC) attached to albumin (Thies *et al.*, 1994), or by lipoproteins packaged in the liver (Spector, 2001). However, whether or not lipoproteins are involved in the transport of PUFA for the brain remains controversial (Chen *et al*., 2008; Williams *et al.*, 1997). The controversy arises from the biological relevance of PUFA lipoprotein transport to the brain, as LDL (Chen *et al*., 2008) and VLDL (Rahman *et al*., 2010) receptor knock-out mice shown no changes in brain PUFA regulation. DHA, which is esterified to LPC, is more efficiently transferred than the non-esterified form (Bernoud *et al.*, 1999). However, non-esterified fatty acids, derived from adipose tissue stores attached to albumin and lipoproteins, have also been shown to contribute to brain n-3 and n-6 PUFA pool (Thies *et al.*, 1994; Brecher & Kuan, 1979; Ouellet *et al.*, 2009). The recent discovery of the major-facilitator superfamily domain-containing protein-2 (Mfsd2a) has established that Mfsd2a transports the majority of DHA in the form of LPC to the brain (Nguyen *et al.*, 2014).

The brain is also capable of synthesizing DHA endogenously via endothelial cells comprising the BBB and neuroglial cells known as astrocytes (Moore *et al*., 1990). These cells are capable to begin the initial elongation and desaturation of the essential n-3 PUFA alpha-linolenic acid (C18:3n-3;ALA) (Moore *et al.*, 1990). The essential fatty acids ALA and linoleic acid (C18:2n-6; LA) go through desaturation and elongation to make longer-chain fatty acids by sharing the same desaturase and elongase enzymes (Figure 1.5).

Figure 1.4: The omega-3 and - 6 PUFA transport across the blood brain barrier. *PUFAs can be transported to the brain esterified to lipoproteins or they can be transported to the blood brain barrier esterified to lysophosphatidylcholine (Lyso-PC) or as non-esterified fatty acid attached to albumin. Lyso-PC can be transported across the BBB via the major-facilitator superfamily domain-containing protein-2 (Mfsd2a). NE-PUFA = non-esterified polyunsaturated fatty acid; E-PUFA = esterified polyunsaturated fatty acid; LDL-R = Low-density lipoprotein receptor. (Adapted from Liu et al., 2015, Brain Research, 1597:220)*

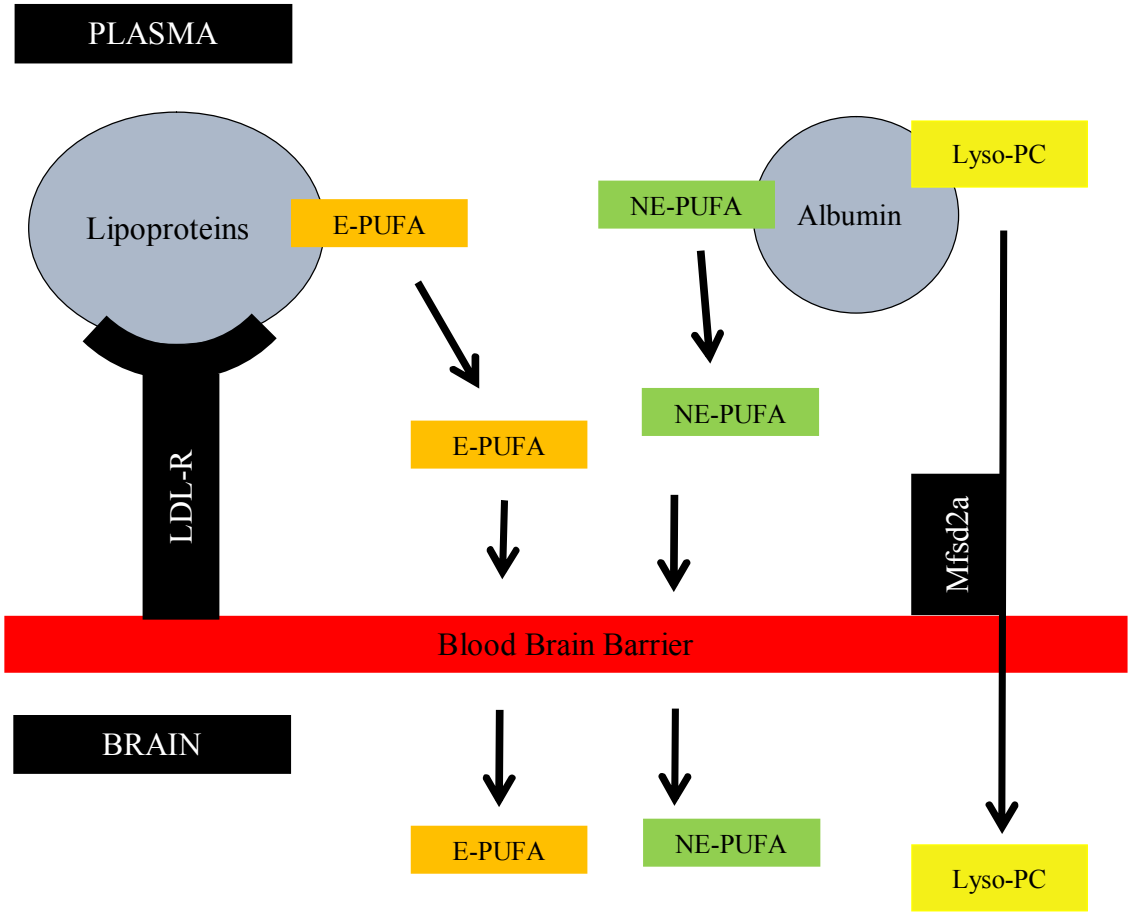
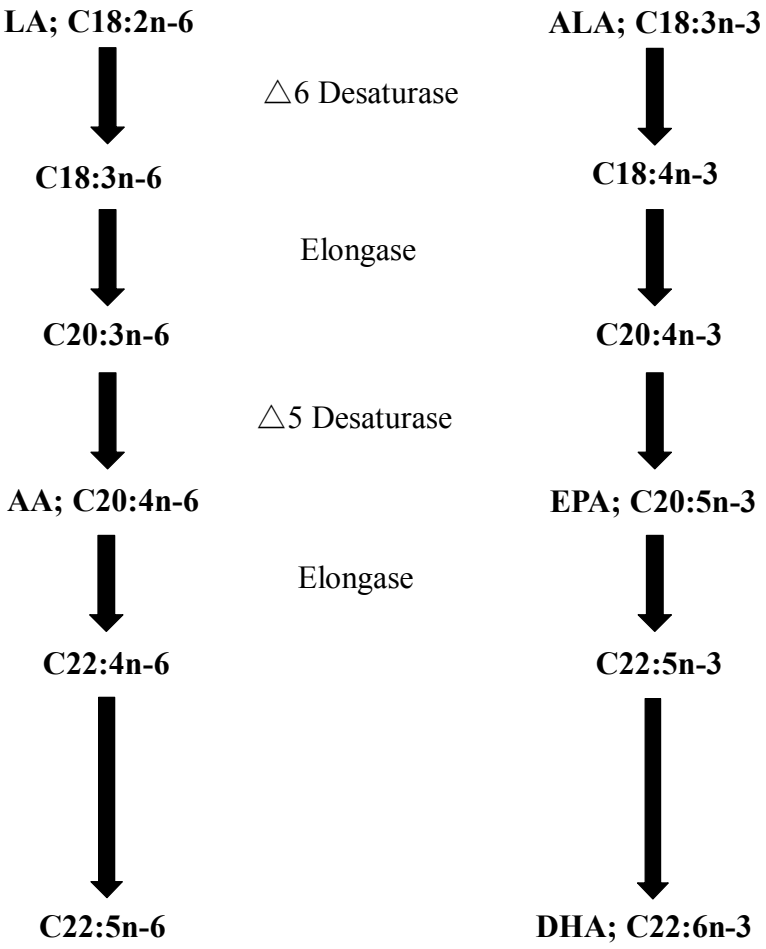

PLASMA
Lipoproteins
E-PUFA
NE-PUFA
Albumin
Lyso-PC
LDL-R
E-PUFA
NE-PUFA
Mfsd2a
Blood Brain Barrier
BRAIN
E-PUFA
NE-PUFA
Lyso-PC

Figure 1.5: The omega-3 and -6 PUFA synthesis from the essential fatty acids. *Alpha-linolenic acid (ALA) and linoleic acid (LA) from the diet are desaturated and elongated to longer-chain fatty acids via the same desaturases and elongases. DHA = docosahexaenoic acid; AA = arachidonic acid; EPA = eicosapentaenoic acid* (*Adapted from Liu et al*., 2015, *Brain Research,* 1597:220)


LA; C18:2n-6
ALA; C18:3n-3
△6 Desaturase
C18:3n-6
C18:4n-3
Elongase
C20:3n-6
C20:4n-3
△5 Desaturase
AA; C20:4n-6
EPA; C20:5n-3
Elongase
C22:4n-6
C22:5n-3
C22:5n-6
DHA; C22:6n-3

Once desaturated and elongated, DHA and AA are released into the surrounding cerebrospinal fluid, where both can be used by the brain for various structural and physiological roles (Moore *et al*., 1990; Crawford, 1992; Bhatia *et al*., 2011). However, when tracking the entry of radiolabelled ALA into the brain, it was completely metabolized via β-oxidation into aqueous β-oxidation products (DeMar *et al.*, 2005). This renders a conversion of less than 0.2% of ALA into DHA (DeMar *et al.*, 2005). As opposed to a low conversion of ALA to DHA, LA was shown to be converted to AA with an efficiency of approximately 15% in the brain (Green & Yaven, 1993). Thus, it appears that the brain depends on the exogenous supply of PUFA, such as AA and DHA, rather than the endogenous synthesis.

1.2.3.1 Role of n-3 and n-6 PUFAs in the brain

During development, there is a large accumulation of DHA and AA that is critical for the proper functioning and development of the mammalian CNS (Xiao *et al.*, 2005; Stillwell *et al.*, 2005). Recent studies using radiolabelled fatty acids for the use of positron emission tomography (PET) have made human brain PUFA studies achievable (Thambisetty *et al*., 2012; Umhau *et al.*, 2013). After intravenously infusing radiolabelled non-esterified DHA into healthy adult patients and using the PET method, Umhau *et al.* (2009) found that a greater amount of DHA was taken up by the gray matter compared to the white matter neuronal membrane PLs from the whole brain. These levels of DHA incorporation may represent active DHA metabolism during synaptogenesis and active neurotransmission (DeGeorge *et al.*, 1989; Jones *et al*., 1996). DHA and AA compete for neuronal membrane placement, thus, the higher intake of n-6

PUFA can render a greater accretion of AA thereby altering membrane functionality. After infusing radiolabelled AA into the plasma of healthy adults, the PET scan showed greater accretion of AA in gray matter compared to white matter (Giovacchini *et al.*, 2002). PET studies on healthy human adult brains have further established that the uptake of radiolabelled AA is higher than that of radiolabelled DHA (Giovacchini *et al.*, 2004); patients with Alzheimer's disease showed a further increase in the uptake of AA compared to DHA (Esposito *et al*., 2008). DHA and AA are mobilized from the *sn*-2 position of the neuronal membrane PLs via the action of phospholipases (Farooqui *et al*., 2004). They have the capability to modulate signalling pathways through their association with the neuronal membranes and synapses (Murphy, 1985). For example, studies have shown that PUFAs interact with pathways responsible for the regulation of neurotransmitters (Owens & Innis, 1999), neurotrophins (Balogun & Cheema, 2014), myelin formation (Peters *et al*., 2012), and neuronal survival (Akbar, 2005).

It is important to note that n-6 and n-3 PUFAs elicit different physiological functions in the brain. More specifically, when assessing the effects of both DHA and AA on rat neural stem cells, only DHA treatments increased the rate of rat neural stem cell differentiation (Katakura *et al.*, 2013). Another study assessing the effects of AA and DHA on *ex vivo* rat cerebral cortical cells showed DHA treatment reduced the uptake of the neurotransmitter glutamate by cortical astrocytes (Grintal *et al.*, 2009); however, AA had no effect. Thus, an imbalance in the intake of dietary n-3 and n-6 PUFA may alter the proportion of n-3 and n-6 PUFA in the brain, which may then lead to an alteration in the functioning of the brain.

1.3 Importance of dietary n-3 and n-6 PUFA in the brain

Recent evidence shows that the fatty acid composition of the brain can be changed by dietary lipids (Bhatia *et al.*, 2011). Since brain SFA and MUFA are automonous to dietary intake, most studies have focused on the importance of dietary PUFAs in brain development and their involvement in the underlying pathogenesis of neuropsychiatric disorders (Carrié *et al.*, 2000; Bhatia *et al.*, 2011). The main PUFAs in the brain are DHA and AA, which account for 50% and 40 % of the brain total PUFA, respectively (Lauritzen *et al.*, 2001). The maintenance of this fatty acid profile, mostly through dietary PUFAs, is very important for the proper functioning of the brain (Chung *et al.*, 2008; Rao *et al.*, 2007). Many studies have aimed to determine the beneficial effects of n-3 PUFA on the brain (Carrié *et al.*, 2000; Bhatia *et al.*, 2011), especially because the dietary intake of n-3 PUFA has been declining in the Western society. Since the industrialization of the modern world, dietary imbalance has shifted the intake of n-6 to n-3 PUFAs ratio of 1:1 from the Hunter-Gatherer time to approximately 20:1 in a typical North American diet (Simopoulos, 2011). Evidence shows that countries with lower fish intake have a higher rate of bipolar disorder and post-partum depression, compared to countries that have higher intakes of fish (Noaghiul & Hibbeln, 2003; Hibbeln, 2002), suggesting the importance of dietary n-3 PUFA in maintaining proper brain function. Studies suggest this may be due to altering the brain fatty acid DHA/AA ratio, which then will alter the biological function of the neuronal membrane (Bhatia *et al.*, 2011; Balogun & Cheema, 2014). However, most studies determining the biological outcome from changing the DHA/AA ratio of the neuronal membrane focus mainly on the cerebral cortex and hippocampus (Balogun & Cheema, 2014; Bhatia *et al.*, 2011; Rao *et al.*,

2007). Unlike the cerebellum and brainstem, both these regions of the brain are highly enriched in PS and PE esterified with DHA (Svennerholm, 1968; Chavko *et al.*, 1993). Therefore, the potential benefits from altering the PL DHA/AA ratio may be specific to the PL class of the neuronal membrane. However, it is not known whether altering the DHA/AA ratio in the cerebellum and brainstem will alter any biological outcome.

During the last trimester of pregnancy and throughout lactation, the brain is rapidly developing, and there is a high demand for DHA for development of the neuronal membrane (Martinez, 1992). Once established, the neuronal membrane PL fatty acid composition is quite rigid throughout life and typically remains unchanged; however, n-3 PUFA dietary supplementation can have a huge impact on the membrane fatty acid profile. Most of the PUFA used for neuronal membrane synthesis originates from dietary DHA and AA (Clandinin *et al*., 1980). Our laboratory has previously shown that feeding a diet high in n-3 PUFA to mice caused an increase in the cerebral cortex DHA (Balogun & Cheema, 2014). A study assessing baboon neonates showed feeding a diet enriched with DHA for 4-months after birth increased the accretion of DHA in the cerebral cortex (Hseih *et al.*, 2007). The brain, however, is composed of several regions that serve different functions, and there are only a handful of studies to show the effect of a diet high in n-3 PUFA on the fatty acid composition of different regions of the brain (Xiao *et al.*, 2005; Carrie *et al.*, 2000). Furthermore, these studies did not investigate age specific effects of a diet high in n-3 PUFA on the fatty acid composition of various regions of the brain. This is vitally important because as the brain ages the region-specific PL fatty acid composition of brain changes (Martinez, 1992; Xiao *et al.*, 2005); thus it would be important to investigate whether a diet high in n-3 PUFA would induce alterations in fatty

acid composition in an age and brain region-specific manner.

Studies have indicated that altered brain development during the perinatal period is consequentially a high risk factor for the development of neuropsychiatric illnesses (Barlow *et al.*, 2007; Miller & O'Callaghan, 2008; Palubinsky *et al.*, 2012). The upregulation of neurotrophins mitigates the symptoms of neurological illnesses (Huang & Reichardt, 2001). Moreover, alterations in the synthesis and release of neurotrophins has been shown to be involved in the development of neuropsychiatric illnesses (Rehn & Rees, 2005). In recent years, one of the effects induced by DHA in the brain has been shown to be the regulation of the BDNF signalling pathway (Rao *et al.*, 2006).

1.3.1 Dietary n-3 PUFA and BDNF regulation

Studies assessing the effects of n-3 PUFAs on depression and anxiety in humans have found a positive correlation between n-3 PUFA intake and serum BDNF levels (Ferreira *et al.*, 2014). Furthermore, rodent models of depression and anxiety have found a reduction in anxiety and depression like symptoms, and an increase in BDNF expression after fish oil supplementation (Arbabi *et al.*, 2014; Mizunoya *et al.*, 2013). An increase in the maternal consumption of n-3 PUFA showed an increase in the levels of BDNF in their offspring (Weiser *et al.*, 2015). Our laboratory has previously established that feeding varying amounts of dietary n-3 PUFA to females during pregnancy altered the cortical fatty acid composition of the offspring (Balogun & Cheema, 2014). This alteration in neuronal membrane fatty acid composition is due to the competition between n-6 and n-3 PUFA for incorporation in the membranes. Others have shown that a diet deficient in n-3 PUFA during the perinatal and postnatal period decreased BDNF protein expression in the male rat offspring hypothalamus and hippocampus (Bhatia *et al.*, 2011).

In addition, this was associated with higher anxiety-like behavior compared to their counter parts fed a high n-3 PUFA diet (Bhatia *et al.*, 2011). Multiple studies have tried to elucidate the underlying mechanisms by which DHA increases the expression of BDNF. Rats fed a diet deficient in n-3 PUFA for 15 weeks exhibited a decrease in the phosphorylation of the p38 mitogen-activated protein kinase (MAPK), CREB DNA binding activity, and ultimately BDNF expression in the frontal cortex (Rao *et al.*, 2006). Our laboratory has previously shown that a maternal diet high in n-3 PUFA increased the phosphorylation of CREB in the offspring cortex compared to the low n-3 PUFA group (Balogun & Cheema, 2014). Rao *et al.* (2006) established DHA treatment to primary rat cortical astrocytes increased the BDNF protein expression, which was shown to be via the p38 MAPK pathway. However, it is not clear whether the regulation of BDNF is specific towards DHA, or can be regulated by other fatty acids. In addition, no study to date has characterized the relationship between DHA and BDNF expression in different regions of the brain.

Most studies assessing the effects of DHA on the brain have focused on the cerebral cortex and hippocampus as the region of choice (Avramovic *et al*., 2013; Chang *et al*., 2015; Firlag *et al*., 2013; Bhatia *et al.*, 2011; Rao *et al.*, 2006). This may be due to the functionality of these regions, as both play a role in higher level thinking, short, working and long-term memory, and because most behavioural and cognitive symptoms associated with neuropsychiatric illnesses coincide with the functions of these regions. However, the brainstem and cerebellum have also been linked to the development of various neuropsychiatric diseases (Morcinek *et al.*, 2013; Marinova *et al.*, 2015). Thus, it will be important to determine if a diet high in n-3 PUFA will alter BDNF expression in a

region-specific manner.

1.3.2 The effects of dietary n-3 PUFA on the myelin sheath

Studies have correlated a higher intake of fish or fish oil supplements with a significant increase in the quality of life for those with multiple sclerosis (Jelinek *et al.*, 2013), which is a neurodegenerative disease rendering the deterioration of the myelin sheath. Furthermore, the beneficial effects of fish oil on the progression of multiple sclerosis have been shown to improve the future outcomes for newly diagnosed patients throughout their clinical course (Nordvik *et al.*, 2000). The positive effects of n-3 PUFA on protection of myelin are thought to be due to the stimulation of the myelinogenesis process (Salvati *et al.*, 2008). Interestingly, an *in vitro* study supplementing oligodendroglia cells with n-3 PUFAs have found an increase in their differentiation (van Meeteren *et al.*, 2006). This would be very important for myelin formation, as oligodendroglia cells are responsible for this process in the CNS (Baumann & Pham-Dinh, 2001). Another factor that is vitally important in the formation of the myelin sheath is the myelin basic protein (MBP) (Boggs, 2006).

MBP is the second most abundant protein in the CNS myelin and it comprises 30% of the total protein (Boggs, 2006). MBP is the only structural protein found thus far to be essential for the formation of myelin (Boggs, 2006), and is accepted as the 'executive molecule of myelin' (Moscarello, 1994). MBP is a positively charged protein, with a molecular mass of approximately 18.5 kDa (Boggs, 2006). MBPs positive charged allows MBP to interact with the oligodendroglial negatively charged PLs (Müller *et al.*, 2013). The interaction between the oligodendroglial PLs and MBP has been proposed to compact myelin membranes (Müller *et al.*, 2013). Shiverer (*shi*) mice lack

the functional MBP, due to a autosomal recessive mutation, which renders a hypomyelination within the CNS, shivering symptoms, and eventually a premature death (Readhead & Hood, 1990). Injecting 10 mM eicosapentaenoic acid (EPA; C20:5n-3) and DHA intracerebroventricularly into rat pup brains showed an increase in the mRNA expression of MBP in the cerebellum and medulla, compared to the control animals (Salvati *et al.*, 2008), thus supporting the importance of n-3 PUFA in MBP regulation and myelin development. After myelin maturation during development, the adult CNS has the propensity to remyelinate itself after a demyelinating injury occurs; however, this mechanism is less understood (Gallo & Deneen, 2014). Adult mice suffering from a traumatic brain injury who were fed a diet high in n-3 PUFA two months prior to the injury had reduction in the loss of MBP compared to animals fed a diet low in n-3 PUFA diet (Pu *et al.*, 2013), suggesting protective effects of a diet high in n-3 PUFA on the myelin sheath of the brain. However, whether a diet high in n-3 PUFA will show different effects on myelin formation is not known. In addition, it is important to establish whether a diet high in n-3 PUFA has region-specific regulation of MBP gene expression.

1.4 N-3 PUFA on brain structure and function: effects of sex and age

Studies have shown that females have a higher tissue accumulation of n-3 PUFAs compared to males (Decsi & Kennedy, 2012; Extier *et al.*, 2010). This is due to a higher conversion rate of ALA to DHA (Decsi & Kennedy, 2012) in females because the key rate-limiting enzymes, delta-5 and delta-6 desaturases, have a higher expression in females (Extier *et al.*, 2010). Isotope studies have clearly revealed young women are more efficient at converting ALA into the longer chain metabolites DHA and EPA

(Burdge & Wootton, 2002; Burdge *et al.*, 2002). Furthermore, a study assessing both male and female adults from the United States found that a high n-3 PUFA status was linked to a much slower progression of depressive symptoms in women compared to men (Beydoun *et al.*, 2015). This sex-specific n-3 PUFA tissue distribution and metabolism is thought to be due to estrogen (Childs *et al.*, 2008); 17β-estradiol treatment has been found to increase the formation of ALA to EPA and DHA (Alessandri *et al.*, 2008). The female estrogens are well documented for their association with depression (Studd, 2011). Furthermore, estradiol has been shown to upregulate the gene expression of lipid metabolism genes (Alessandri *et al.*, 2008). FADS, ELOVL, and SCD1 have been shown to be regulated by estradiol, consequently leading to an increase in the rate of desaturation and elongation of their respective fatty acids (Alessandri *et al.*, 2008; Marks *et al.,* 2013). Women suffer from depression more frequently than men, especially during periods of hormonal fluctuation, such as the premenstrual, postpartum, and menopausal periods (Noble, 2005). Moreover, estrogens have also been shown to play a vital role in neuronal protection and survival, along with enhancing synaptic plasticity and synaptogenesis through its interactions with the neurotransmitters dopamine and serotonin (Maki & Dumas, 2009; Mateos *et al.*, 2012). In addition, estrogens have also been shown to increase the expression of BDNF (Mateos *et al.*, 2012), and increase the phosphorylation of its down-stream transcription factor CREB in the hippocampus of ovariectomized rats (Panickar *et al.*, 1997). Estrogens mediate their biological function through activation of the extranuclear estrogen receptor, which leads to the induction of the ERK/AKT/CREB/BDNF pathway in the hippocampus (Yang *et al.*, 2010). Furthermore, ovariectomized rats injected with estrogen and fed a diet high in n-3 PUFA showed a

synergistic effect on antidepressant-like behaviour and CREB activation in the hippocampus (Jin & Park, 2015). Thus, estrogen plays a role in BDNF signalling in the brain; however, it is not known whether this interaction is dependent on the specific region of the brain. Neuropsychiatric illnesses have been shown to have region and sex-specfic effects (Beydoun *et al.*, 2015; Morcinek *et al.*, 2013; Greer *et al.*, 2005). Furthermore, abnormalities in BDNF synthesis and release has been shown to be the culprit in the development of mulitple neuropsychiatric symptoms (Rehn & Rees, 2005). Therefore, it would be important to know whether BDNF expression differs in a sex and region specific manner.

Although the effects of sex on the regulation of n-3 PUFA in the brain are quite noteable, age has also been shown to have an effect on n-3 PUFA metabolism in the brain. As the human brain develops during the last trimester of pregnancy, the fetal brain begins to rapidly accumulate DHA (Martinez, 1992). This continues throughout the first 2 years of life, although the postnatal DHA accumulation occurs more slowly, compared to the DHA accumulation during perinatal period (Moriguchi & Harauma, 2013). In rodents, this period of rapid brain growth and DHA accumulation is between prenatal day 7 to postnatal day 16 (Green *et al.*, 1999). When assessing the effects of dietary n-3 PUFA on DHA accumulation during brain development, human studies found prenatal and postnatal n-3 PUFA supplementation had inconsistent results, showing either beneficial effects or no neurodevelopmental effects (Henriksen *et al.*, 2008; Isaacs *et al.*, 2011). However, animal studies have provided information that supports the beneficial effects of a diet high in n-3 PUFA on brain development (Luchtman & Song, 2013; McNamara & Carlson, 2006). Moreover, studies found the brain fatty acid composition

of rodents to change after feeding a diet high in n-3 PUFA (Ozias *et al.*, 2007; Balogun & Cheema, 2014). For example, when pregnant female rats were fed a diet either deficient or adequate in the n-3 PUFA ALA throughout gestation and lactation, the whole brain from male offspring obtained from the deficient mother had a decrease in the percentage of DHA, compared to male offsprings on a maternal diet adequate in ALA (Ozias *et al.*, 2007). Similarly, when a maternal diet is deficient in n-3 PUFA during gestation and lactation, the adult male rats have a reduction in cerebral cortex DHA compared to adult male rats who were fed a maternal diet adequate in n-3 PUFA during this period (Garcia-Calatayud *et al.*, 2005). Although the accumulation of DHA into the neuronal membrane is only rapid during brain growth and development, the accretion of DHA can also occur slowly in the adult brain (Moriguchi & Harauma, 2013). The neuronal membrane PLs are continuously recycled in the adult brain, thus the PL fatty acid composition of the neuronal membrane has the propensity to change (Rapoport, 2001). Plasma infusion studies using unesterfied radiolabelled DHA found adult male rats to replace 2-8% of esterified DHA daily (Rapoport, 2001). Interestingly, many animal, epidemiological and clinical studies have shown that the risk of Alzheimer's disease was reduced with a higher intake of DHA (Green *et al.* 2006; Luchtman & Song, 2013; Kiso, 2011). Thus, DHA has the propensity to protect the brain during adult years.

To date, no study has determined whether the effects of a diet high in n-3 PUFA will alter the fatty acid composition of various regions of the brain in an age and sex related manner. Therefore, whether male or female brains have an alteration in the regulation n-3 PUFA metabolism as they age is unknown.

1.5 Mouse as an animal model

The genetic background of a mouse is the most characterized out of all mammals (Paigen *et al.*, 1990). The mouse model has been the favourable model for animal experimentation because of its short reproduction period (Fazio & Linton, 2001). We chose the C57BL/6N mouse for our study because it has been well established to investigate the effects of diet on neuronal and behaviour studies (Crawley *et al.*, 1997). Furthermore, the C57BL/6N mouse has been used as the strain of choice to generate numerous transgenic models to study the pathological conditions of various neuropsychiatric illnesses (Flint, 2006). Therefore, using this mouse model leaves our options open to extend our study with different neuropsychiatric transgenic mouse models in the future.

1.6 Rationale

Majority of the studies supporting beneficial effects of n-3 PUFA on the function of brain have focussed on the cortex. Other regions of the brain also play important functions, however there is limited evidence on the effect of n-3 PUFA on other regions of the brain. In addition, as most neuropsychiatric diseases have underlying age and sex-specific risk factors, it is important to establish whether diets enriched in n-3 PUFA have similar effects on the fatty acid composition of different regions of the brain, and whether the effects are age and sex-specific. It is also important to investigate whether altering the fatty acid composition of different regions of the brain alters the gene expression of neurotrophins and other genes regulating the myelin sheath formation.

1.6.1 Overall hypothesis

The overall hypothesis for this thesis is that a diet high in n-3 PUFA will increase the accretion of DHA in the brainstem and cerebellum, leading to an increase in the mRNA expression of BDNF and genes related to myelin sheath formation.

1.6.2 Specific aims

Aim 1: To investigate the dose, age, and sex-specific effects of dietary n-3 PUFA on the accretion of phospholipid fatty acids in cerebellum and brainstem, and the regulation of mRNA expression of BDNF, TrkB and the genes involved in myelin sheath formation.

Hypothesis: The prevalence of neuropsychiatric illnesses in females and males differ with age. However, no study to date has investigated the importance of a diet high in n-3 PUFA on the fatty acid composition, along with the mRNA expression of BDNF, TrkB and the genes involved in myelin sheath formation in a sex, age and region-specific manner. It was hypothesized that the effects of a diet high in n-3 PUFA on the accretion of phospholipid fatty acids, and the mRNA expression of BDNF, TrkB and genes involved in myelin sheath formation will be age, sex and region-specific.

Aim 2: To investigate whether prolonged exposure to diets high in n-3 PUFA will increase the accretion of DHA in adult cerebral cortex.

Hypothesis: The fatty acid composition of the adult-brain is tightly regulated and is thought to be stable; however, it is not clear whether prolonged exposure to diets rich in n-3 PUFA can affect the accretion of DHA in an adult cerebral cortex. It was hypothesized that a diet high in n-3 PUFA will increase the accretion of DHA in the cerebral cortex with longer exposure to the diet.

Aim 3: To investigate the mechanisms by which n-3 PUFA regulate the cerebral cortex

mRNA expression of BDNF, TrkB and genes involved in myelin sheath formation using primary cortical cells.

Hypothesis: The cerebral cortex is highly enriched in both DHA and AA that play an important role in brain function mediated by neurotrophic factors. However, it is not known whether DHA and AA have similar effects on the regulation of neurotrophic factors. It was hypothesized that BDNF and TrkB will be regulated by DHA in cerebral cortical neurons, while AA will have no effect. It was further hypothesized that SCD1 and MBP will be regulated by DHA, while AA will have no effect.

Chapter 2: Materials and Methods

2.1 Diets and animals

A former Master's student Raniru Randunu and the Ph.D. student Kayode Balogun were responsible for the described animal feeding trials and tissue collections (Balogun 2015; Randunu 2012).

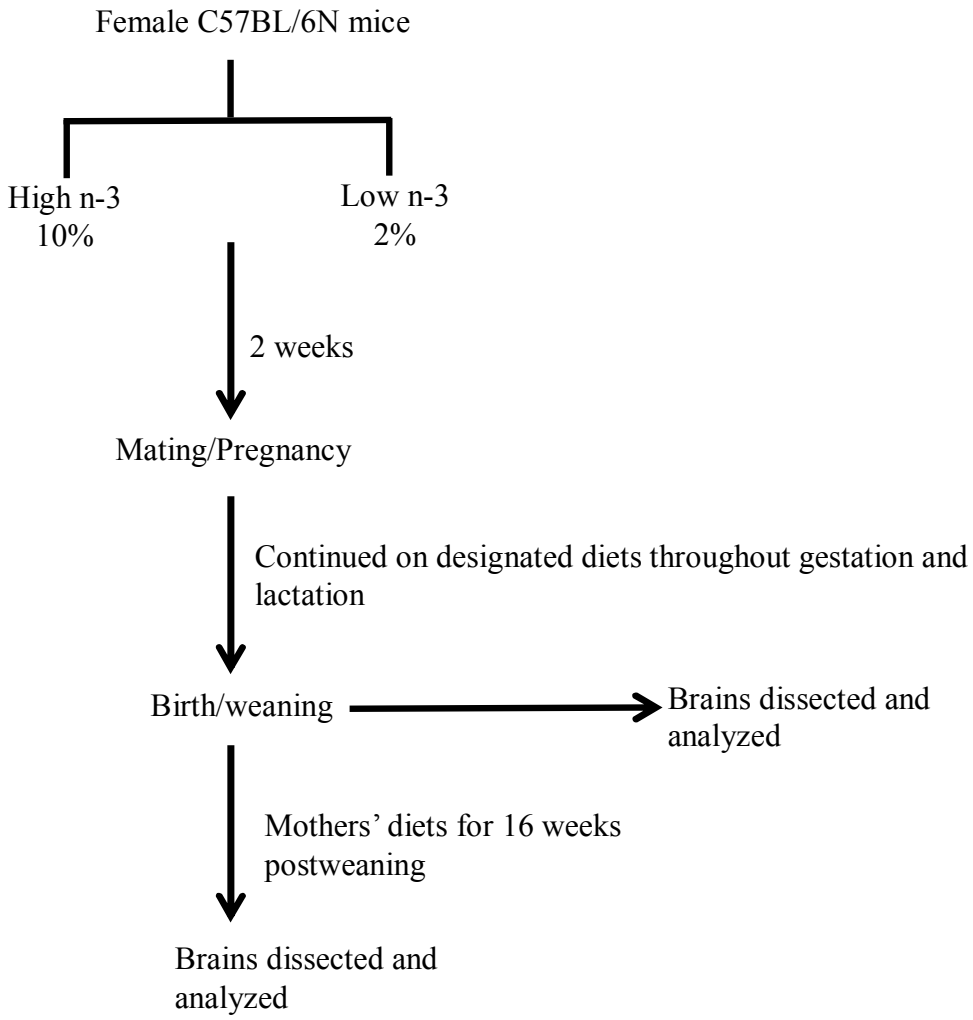
2.2 Diets

A high fat diet was prepared using a base fat-free semi-synthetic diet (MP Biomedicals, Santa Ana, CA, USA) designed to control for fat levels at 20% w/w. The composition of the semi-synthetic base diet is given in Appendix 2. Menhaden fish oil (Sigma-Aldrich, St. Louis, MO, USA), safflower oil, extra-virgin olive oil, and lard (purchased from local grocery stores) were used as sources of n-3 PUFA, n-6 PUFA, MUFA, and SFA, respectively. These oils were used in specific combinations to make two different diet mixtures containing either 10% w/w (high n-3 PUFA) or 2% w/w (low n-3 PUFA) n-3 PUFA of the total dietary fat (Balogun & Cheema, 2014). The 10% and 2% w/w n-3 PUFA diets represent the suggested and the current North American intake of n-3 PUFA, respectively (Simopoulos, 2011; Kris-Etherton *et al*., 2000). The amounts of total SFA, MUFA, and PUFA were kept constant; however, the amount of n-6 and n-3 PUFA varied depending on the diet. The fatty acid composition of the experimental diets was analyzed using gas chromatography (GC), and is given in Appendix 1. The high-fat diets with different amounts of n-3 PUFA were stored at -20°C under nitrogen to prevent fatty acid oxidation.

2.3 To investigate the effects of perinatal and post-weaning diets high or low in n-3 PUFA on different regions of the brain

Male and female C57BL/6 N mice (seven weeks old) were purchased from Charles Rivers Laboratories (Wilmington, MA, USA). The mice were housed in separate cages under controlled temperature (21 ± 1 °C) and humidity (35 ± 5%) conditions with a 12 hour light/ 12 hour dark period cycle. Mice were kept on a standard chow diet (Prolab RMH 3000; PMI nutrition, USA) for a one week acclimatization period. Female mice were then randomly divided into two dietary groups, and fed diets high (10% w/w) or low (2% w/w) in n-3 PUFA for two weeks before mating. Male mice consumed a standard chow diet, except during one-week of mating when they consumed the same diet as females. Mice were continued on the experimental diets during mating, throughout gestation, lactation, and up until weaning (Figure 2.1). At weaning, offspring from both experimental diet groups (male, n=8 and female, n=8) were fasted overnight for 12 hours then sacrificed using isoflurane as an anesthetizing agent and cardiac puncture. In another study, the offspring from both experimental diet groups (male, n=8 and female, n=8) were continued on their mother's diet for an additional 16 weeks post-weaning. Throughout the experimental period, the animals were provided water and fresh food *ab libitum*, which was replenished every other day. Body weights were measured once a week, while food intake was recorded every other day. Recorded food intake and body weight showed no significant differences between groups, as shown in a previous study (Balogun, 2015). At the end of the experimental period, the animals were fasted overnight and anesthetized using isoflourane. These animals were also used by the former

Figure 2.1: Experimental design for feeding a diet high or low in n-3 PUFA to female C57BL/6N mice during gestation and lactation.


Female C57BL/6N mice
High n-3
10%
Low n-3
2%
2 weeks
Mating/Pregnancy
Continued on designated diets throughout gestation and lactation
Birth/weaning
Brains dissected and analyzed
Mothers' diets for 16 weeks postweaning
Brains dissected and analyzed

Ph.D. student Kayode, who undertook metabolic studies for which it was important to fast the animals. The head was decapitated using large scissors and transferred to an ice-chilled petri dish to dissect out the whole brain. The whole brain was placed onto a separate ice-chilled petri dish to segregate the brainstem, cerebellum and cerebral cortex (Figure 2.2). The brain regions were snap frozen in liquid nitrogen, and stored at -80°C until further use. Experimental procedures were done in accordance with the principles and guidelines of the Canadian Council on Animal Care and were approved by Memorial University's Animal Care Committee.

2.4 To investigate the effects of diets high or low in n-3 PUFAs on adult C57BL/6N cerebral cortex

Female C57BL/6 N mice (seven weeks old) were purchased from Charles River Laboratories and housed as explained in Section 2.3. After one-week acclimatization period, female mice were randomly divided and placed on either a high (10% w/w) or a low (2% w/w) n-3 PUFA diet (Figure 2.3). The specified diets were fed for either 4 weeks or 8 weeks. Animals were provided with deionized water and fresh food *ad-libitum,* which was replenished every other day. Body weights were recorded once a week, while food intake was recorded every other day; diet had no effect on body weight or food intake (Randunu, 2012). At the end of the treatment period, mice were decapitated and brain sections were removed as explained in **Section 2.3**.

Figure 2.2: Dissection of whole mouse brain to isolate cerebral hemispheres, cerebellum, and brainstem.

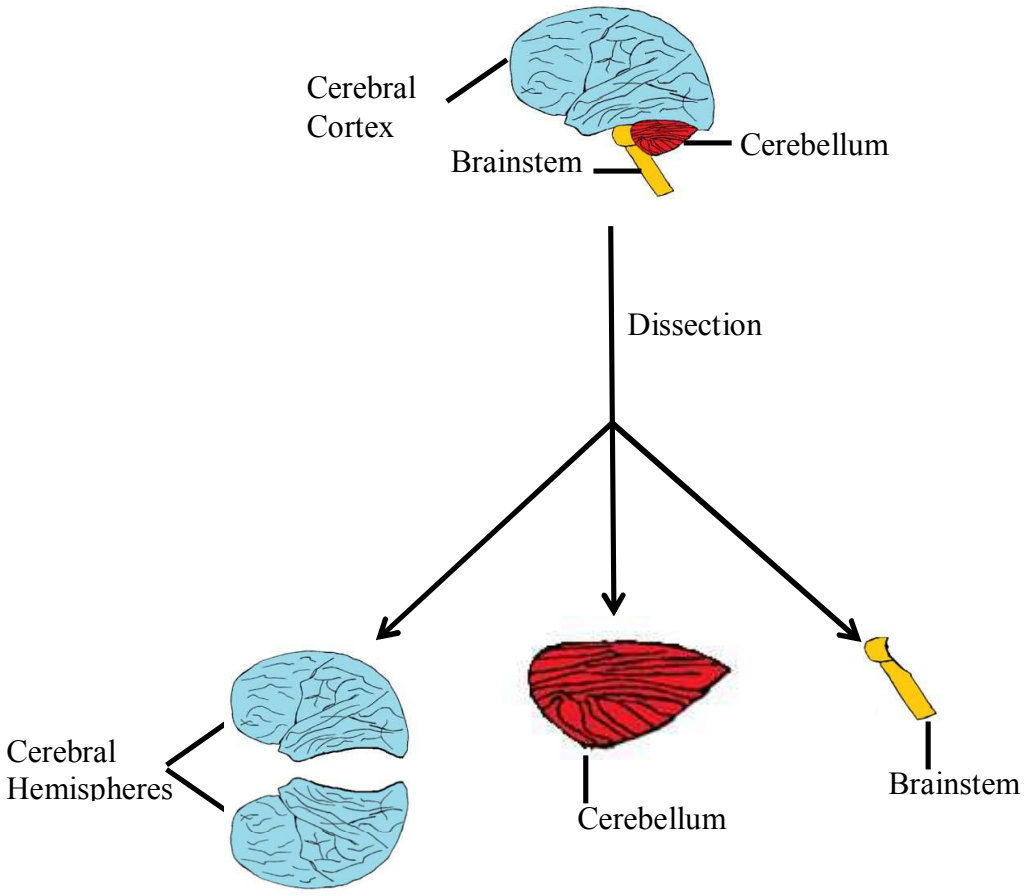
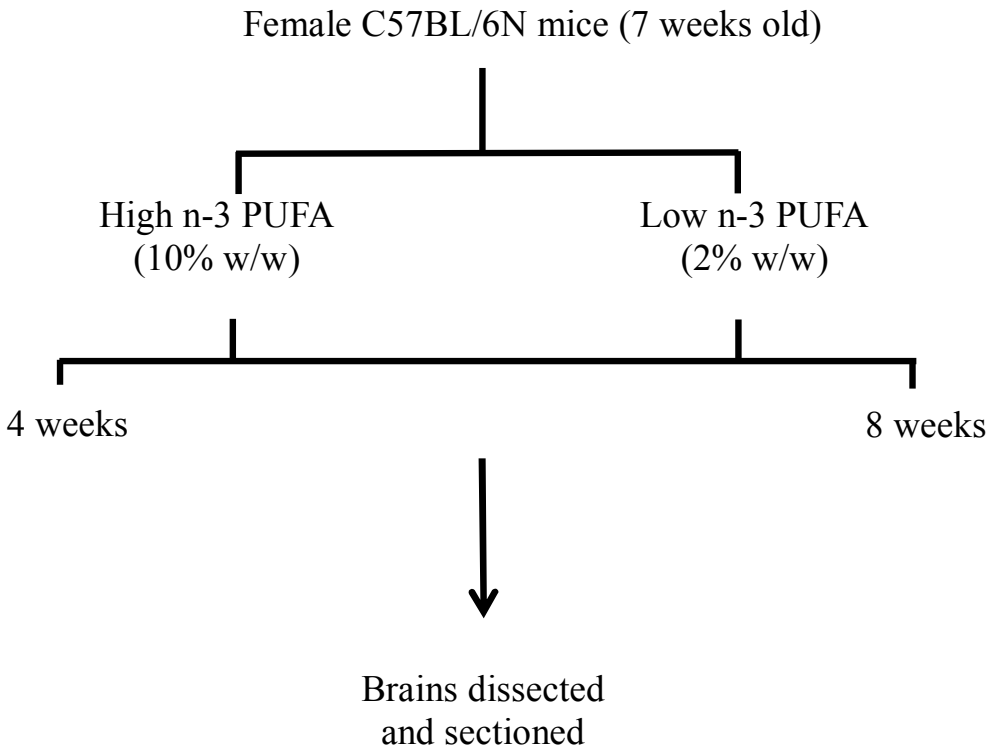

Cerebral
Cortex
Brainstem
Cerebellum
Dissection
Cerebral
Hemispheres
Cerebellum
Brainstem

Figure 2.3: Experimental design for feeding a diet high or low in n-3 PUFA to adult female C57BL/6N mice for 4 or 8 weeks.


Female C57BL/6N mice (7 weeks old)
High n-3 PUFA
(10% w/w)
Low n-3 PUFA
(2% w/w)
4 weeks
8 weeks
Brains dissected
and sectioned

2.5 Lipid extraction

Total lipids were extracted from specific regions of the brain using the method of Folch *et al.* (1957). PLs were then separated from total lipids using thin-layer chromatography (Keenan, 1982). Total extracted lipids were dissolved in 100 μL of chloroform and spotted on 20 x 20 cm flexible plates coated with silica gel (Cat #:4420222, Whatman Ltd. UK). PLs were separated using the solvent system: hexane:ethyl ether:acetic acid (70:30:20 v/v; Keenan, 1982). PL spots were extracted using chloroform: methanol (2:1 v/v) and fatty acid methyl esters were prepared using the method of Arvidson and Olivecrona (1962). Trans-methylation reagent (2 mL of 94% methanol, 6% of 98% sulfuric acid) was added to the extracted PLs along with a few crystals of the anti-oxidant hydroquinone for 2 hours at 65°C. Methyl esters were extracted using hexane and water, then placed at -20°C overnight. The supernatant was dried under nitrogen and re-suspended in carbon disulphide prior to GC analysis. Samples were run for 60 minutes on an Omega-wax X 320 (30 m x 0.32 mm) column from Supelco (Sigma-Aldrich) using a flame ionization detector. The GC parameters were set as follows: oven temperature, 200°C; injector temperature, 240°C; and detector temperature 260°C. The GC was ignited and allowed to run until the baseline was stable. PUFA standards -2 and -3 (Sigma-Aldrich) were used as standards for identification of fatty acids peaks by retention time.

2.6 RNA extraction and real-time quantitative polymerase chain reaction

2.6.1 Total RNA isolation

Total RNA was extracted from the cerebellum and brainstem tissues using the Trizol method (Invitrogen, Carlsbad, CA, USA) (Chomczynski & Sacchi, 1987).

Contaminating genomic DNA was removed by treating with RNase-free DNAse 1 enzyme (Promega, Madison, WI, USA). RNA concentration and purity was assessed using the Nano-drop spectrophotometer 2000 (Thermo Scientific, Waltham, MA, USA) and RNA integrity was checked using 1.2% agarose gel. The synthesis of cDNA was carried out using the isolated RNA, random hexamer primers (0.5µg/µL; Promega) and a reverse-transcription master-mix [(5x Reverse Transcriptase buffer (Promega), recombinant RNAsin (Promega), M-MLV Transcriptase (Promega), and 10mM dNTPs (10 mM dATP, 10 mM dGTP, 10 mM dCTP and 10 mM dTTP; Invitrogen)]. The conditions and settings for cDNA synthesis were carried-out as described by Promega. Samples were then stored at -20°C.

2.6.2 Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) as described by the manufacturer. All primers used for qPCR were designed using NCBI primer blast (www.ncbi.nlm.nih.gov/ tools/primer-blast/) and obtained from IDT Technologies (Coralville, IA, USA); primer sequences, amplicon sizes, and reaction efficiencies are given in Table 2.1. The reactions were run at a total volume of 10 µL with 25 ng cDNA per reaction. Samples were run for 40 cycles (95°C for 15 seconds for denaturation, 58°C for 15 seconds for annealing and 72°C for 15 seconds for extension) using the CFX96^{TM} Real-Time System. No template controls were used for each gene per experiment to ensure amplification was specific to the gene of interest. Data output was managed using the CFX Manager^{TM} Software Version 3.0.

Table 2.1: Sequences and specifications of primers used for RT-qPCR

Gene	***Forward Primer (5'-3')***	***Reverse Primer (5'-3')***	***Reference Sequence***	***Amplicon Size***	***Efficiency***
BDNF	tacttcggttgcatgaaggcg	gtcagacctctcgaacctgcc	NM_001316310.1	137 bp	100%
TrkB	cggcacataaatttcacacg	ttacccgtcaggatcaggtc	XM_006517152	92 bp	100%
SCD1	agagtagctgagctttgggc	acaccccgacagcaatatccag	NM_009127.4	105 bp	99%
MBP	agagtccgacgagcttcaga	caggtacttggatcgctgtg	XM_006526452.2	106 bp	101%
β – actin	cacgcagctcattgtagaagg	atggtgggaatgggtcagaag	NM_007393.5	157 bp	100%

All primers were designed using NCBI primer blast and obtained from IDT technologies. Abbreviations: BDNF = brain-derived neurotrophic factor; TrkB = tropomyosin-receptor kinase B; SCD1 = stearoyl-CoA desaturase-1; MBP = myelin basic protein; β – actin = beta-actin

Relative gene expression was normalized to β-actin, then calculated and recorded using the comparative ΔΔCt method (Livak & Schmittgen, 2001).

2.7 Primary cortical cell culture

2.7.1 Preparation of Poly-L-Ornithine coated plates

The cell culture plates were coated with poly-L-ornithine (PLO) to allow primary cortical cells adhesion to the surface. A solution of PLO (Sigma-Aldrich) was prepared with autoclaved deionized water to a concentration of 10 mg/mL, filter sterilized using a 0.22 μm sterile syringe filter and stored at -20°C until further use. The stock solution of PLO was further diluted with autoclaved deionized water to a final concentration of 500 μg/mL. PLO (1 mL and 50 μL) was added to a 6 well and 96 well plate, respectively. Plates were kept in the laminar flow hood for 2 hours after plating; PLO was removed and the plates were allowed to dry completely. This step was repeated once again, plates were then rinsed with autoclaved deionized water, allowed to dry and stored at 4°C until further use.

2.7.2 Cortical cell culture

C57BL/6N mice pups (1-2 days old) were decapitated using sterilized large scissors and dissected using a procedure adapted from Nafar and Mearow (2014) and Vyas *et al.* (2013). A dorsal incision from posterior to anterior was made to remove the skin from the head. From the foramen magnum, the skull was cut laterally on both sides, then dorsally from posterior to anterior. The skull was then pulled off and the brain was carefully removed, and placed in a petri dish containing 1x Hank's Balanced Salt Solution (1xHBSS, Ca^{2+} and Mg^{2+} free; Life Technologies, Carlsbad, CA, USA)

supplemented with penicillin-streptomycin (pen-strep; 100 μg/mL; Life Technologies). Using a scalpel and 24 ½ gauge syringe needle, the cerebellum was removed followed by the separation of the two remaining cerebral hemispheres. Using fine-tip tweezers, the corpus callosum and basal ganglia were then removed to isolate the cerebral cortex. Meninges and blood vessels were removed from both cerebral hemispheres, followed by teasing of the tissue using fine-tip tweezers. Cerebral hemispheres were placed into a 15 mL conical tube containing 1x HBSS supplemented with pen-strep on ice.

Cortical tissue was allowed to settle at the bottom of the 15 mL tube and the supernatant was aspirated. Fresh 1x HBSS supplemented with pen-strep was added to the cortical tissue; the cells were allowed to settle to the bottom of the tube and the the supernatant was aspirated. Fresh 1xHBSS supplemented with pen-strep, Mg^{2+} (0.49 mM), Ca^{2+} (1.26 mM), Trypsin-EDTA (0.01%; Life Technologies), and DNAse (5 mg/mL; Sigma) was added to the cortical cells and incubated at 37°C for 20 minutes. The cells were allowed to settle and the trypsin containing supernatant was aspirated and replaced with Basal Medium Eagle growth medium (Life Technologies) containing 10% horse serum (Life Technologies), an antibiotic− antimycotic solution (100 units/mL penicillin G, 100 μg/mL streptomycin sulphate and 250 ng/mL amphotericin B; Life Technologies), 0.5% glucose (Sigma), 1 mM sodium pyruvate (Life Technologies) and 1% N2 supplements (Life Technologies)] for 10 minutes to inhibit trypsin (Life Technologies). The medium was aspirated and replaced with fresh growth medium. The tissue was then triturated using a flame molded Pasteur pipette until a homogenous cellular solution was obtained. Cells were counted using a haemocytometer and plated to a concentration of $2.0x10^6$ cells/mL. After two hours, the medium was completely

removed and replaced with fresh growth medium containing serum. After 48 hours, half of the medium was removed and replaced with fresh serum-free Basal Medium Eagle culture medium [(Life Technologies) containing an antibiotic− antimycotic solution (100 units/mL penicillin G, 100 µg/mL streptomycin sulphate, and 250 ng/mL amphotericin B; Sigma), 0.5% glucose (Sigma), 1 mM sodium pyruvate (Invitrogen), 2 mM Glutamine (Sigma) and 2% B27 supplements (Invitrogen)]. Only half of the culture medium was changed every 2 days with fresh culture medium due to the fragile nature of neuronal cells. Cells were checked daily and used on day 10 *in vitro* (DIV).

2.7.3 Fatty acid treatments

Fatty acid treatments were prepared by conjugating specific fatty acids with fatty acid free bovine serum albumin (BSA) (Vaidya & Cheema, 2015). Briefly, a 2% BSA solution was prepared using serum-free culture medium as described in section 2.7.2. DHA and AA were dissolved in 100% ethanol (100 mM) and mixed with 2% BSA solution to a final concentration of 2.5 mM for each fatty acid. Prepared fatty acids were filter sterilized using a 0.22 µm sterile syringe filter and stored at 4°C.

2.7.4 Cell viability assay

Cortical cells were cultured in a PLO pre-coated 96-well plate. At DIV 10, the cells were treated with DHA (10, 25, 50, 75, 100, 250 and 500 µM) for 24 hours in an incubator at 37°C/5% CO_2. Ethanol was used as a vehicle control at a final concentration of 0.02%. After the treatment period, the cells were incubated with 10% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) for 4 hours at 37 °C/5% CO_2. The MTT solution was aspirated and the remaining formazan crystals were re-suspended in 0.4 N HCl/isopropanol and shaken in the Fisher Brand Tempmaster for 3

minutes at room temperature. The assay was read in the Biotek Power Wave XS using a wavelength of 570 nm. Data were expressed as a percent of MTT activity in control cells.

2.7.5 Fatty acid treatment of cortical cells to measure fatty acid analysis and gene regulation

Cortical cells were cultured in PLO pre-coated 6-well plates. At DIV 10, the cells were treated with DHA (10, 25, 50, 75, and 100 μM) for 24 hours in an incubator at 37°C/5% CO_2. Ethanol was used as a vehicle control at a final concentration of 0.02% corresponding to the highest amount of ethanol used in fatty acid treatments. After the treatment period, the medium was removed and the cells were washed twice with ice-cold 1xPBS (pH 7.4) and scraped in 1xPBS (pH 7.4). The cellular suspension was aliquoted into a 1.5 mL Eppendorf tube and spun at 3000 rpm for 5 minutes at 4°C to sediment the cells, the supernatant was removed and the cells were re-suspended in 300 μL of 50 mM NaCl. Lipids were extracted and trans-methylated as described in methods section 2.5.

Cortical cells were cultured in PLO pre-coated 6-well plates. At DIV 10, the cells were treated with 50 μM of either DHA or AA for 24 hours in an incubator at 37°C/5% CO_2. Ethanol was used as a vehicle control at a final concentration of 0.01%, which represented the percentage of ethanol in the 50 μM DHA and AA treatments. After the treatment period, the medium was removed and the cells were washed twice with ice-cold 1xPBS (pH 7.4). One mL of Trizol Reagent was added to the wells and cells were scraped. RNA extraction, cDNA synthesis and RT- qPCR was performed as described in methods section 2.6. The RT-qPCR reactions were run at a total volume of 10 μL with

100 ng cDNA per reaction. Relative gene expression was calculated using the published comparative ΔΔCt method (Livak & Schmittgen, 2001). The Ct value of the target gene is normalized to that of the reference gene (β-actin), and the change in Ct is normalized to a change in Ct of a chosen calibrator sample. Data were expressed as relative to the gene expression of β-actin.

2.8 Statistical analysis

Data were analyzed using IBM SPSS Statistics version 21.0. For all *in vivo* studies, means were compared using three-way analysis of variance (ANOVA) to determine the main effects of diet, age and sex. Pairwise comparisons were conducted using Bonferroni correction to determine differences among the groups only when there was an observed interaction between sex and diet, sex and age, diet and age, and sex, diet, and age. Results were expressed as mean ± standard deviation (SD).

For all *in vitro* experiments (cell culture studies) one way ANOVA was used to compare the differences between control and treatment groups. Significant effects were further analyzed using Bonferroni multiple comparisons post-hoc statistical tests. Fatty acid compositions were expressed as relative weight percentage of the total extracted fatty acids; fatty acid composition data were then arcsine transformed before subjecting to statistical analysis to normalize data. For all statistical analysis, differences were considered to be statistically significant if the associated P value was <0.05.

Chapter 3: Results

3.1 Effects of a diet high or low in n-3 PUFA on the phospholipid fatty acid composition of the cerebellum

The cerebellum PL SFA composition of the offspring, obtained from mothers fed a diet high or low in n-3 PUFA, at weaning and 16 weeks postweaning is given in **Table 3.1.** There was no independent effect of the diet for total or individual SFA amongst the two dietary groups. However, a significant interaction was observed between sex and age in total PL SFA and individual SFA ($P<0.05$). Interestingly, there was an age dependent decrease in the percentage of total SFA in the female offspring cerebellum ($P<0.05$); however, this effect was not observed in the male offspring. There was a decrease in C14:0 and C16:0 from weaning to 16 weeks in both males and females ($P<0.05$). Females at weaning had a higher percentage of C16:0 and C18:0 compared to the males ($P<0.05$).

The cerebellum PL MUFA composition of the offspring, obtained from mothers fed a diet high or low in n-3 PUFA at weaning and 16 weeks postweaning, is given in **Table 3.2.** There was a significant interaction between sex and age for C16:1n-7 ($P<0.0001$), while there was no independent effect of the diet. At weaning, male offspring had a higher percentage of C16:1n-7 compared to the female offspring ($P<0.05$); however, this difference disappeared at 16 weeks. It was interesting to note that the male offspring had an age dependent decrease in C16:1n-7 from weaning to 16 weeks ($P<0.05$), while this effect was not observed in the females. Furthermore, there

Table 3.1: Effects of a diet high or low in n-3 PUFA on the phospholipid saturated fatty acid composition of the cerebellum from male and female offspring at weaning and 16 weeks postweaning

			SFA (%w/w)			
			C14:0	**C16:0**	**C18:0**	**ΣSFA**
Male	Weaning	High n-3	0.65 ± 0.09[a]	24.30 ± 1.33[b]	22.23 ± 1.96[b]	47.19 ± 3.03[b]
		Low n-3	0.63 ± 0.06[a]	22.87 ± 1.01[b]	23.29 ± 1.82[b]	46.80 ± 2.08[b]
	16 Weeks	High n-3	0.26 ± 0.06[b]	21.42 ± 1.32[c]	23.54 ± 1.48[b]	45.22 ± 2.73[b]
		Low n-3	0.40 ± 0.16[b]	20.94 ± 1.72[c]	23.91 ± 1.41[b]	45.25 ± 2.93[b]
Female	Weaning	High n-3	0.78 ± 0.14[a]	26.35 ± 1.73[a]	26.33 ± 2.02[a]	53.47 ± 3.47[a]
		Low n-3	0.74 ± 0.11[a]	26.26 ± 1.81[a]	25.04 ± 0.93[a]	52.05 ± 2.43[a]
	16 Weeks	High n-3	0.29 ± 0.05[b]	21.11 ± 0.89[c]	23.82 ± 0.61[ab]	45.23 ± 1.43[b]
		Low n-3	0.28 ± 0.14[b]	21.55 ± 1.52[c]	25.94 ± 1.94[ab]	47.78 ± 3.27[b]
Sex			NS	P < 0.001	P < 0.0001	P < 0.0001
Diet			NS	NS	NS	NS
Age			P < 0.0001	P < 0.0001	NS	P < 0.0001
Sex*Diet			NS	NS	NS	NS
Sex*Age			P < 0.05	P < 0.01	P < 0.05	P < 0.05
Diet*Age			NS	NS	NS	NS
Sex*Diet*Age			NS	NS	P < 0.05	NS

Data are expressed as weight percentage of the total extracted phospholipid fatty acids. Mean ± SD, n=8. Main effects and interactions were determined by three-way ANOVA; pairwise comparisons using Bonferroni correction was used to determine differences when there was an observed statistical significant interaction. Within a column, statistically significant values are marked with unlike superscripts when a significant interaction was observed (P<0.05) between sex and age (a,b,c). NS= non-significant; ΣSFA = sum of saturated fatty acids.

Table 3.2: Effects of a diet high or low in n-3 PUFA on the phospholipid monounsaturated fatty acid composition of the cerebellum from male and female offspring at weaning and 16 weeks postweaning

MUFA (%w/w)						
			C16:1n-7	**C18:1**	**C20:1n-9**	**ΣMUFA**
Male	Weaning	High n-3	1.52 ± 1.01^{a}	20.31 ± 0.94^{B}	1.44 ± 0.57	23.27 ± 1.97
		Low n-3	0.96 ± 0.07^{a}	21.49 ± 1.36^{B}	2.26 ± 0.64	24.71 ± 1.96
	16 Weeks	High n-3	0.50 ± 0.04^{c}	26.75 ± 1.32^{A}	3.91 ± 0.81	31.15 ± 1.96
		Low n-3	0.53 ± 0.15^{c}	24.99 ± 1.51^{A}	4.16 ± 0.94	29.68 ± 2.38
Female	Weaning	High n-3	0.77 ± 0.11^{b}	20.85 ± 3.35^{B}	1.26 ± 0.90	22.88 ± 4.32
		Low n-3	0.76 ± 0.19^{b}	21.58 ± 1.56^{B}	1.62 ± 0.57	23.97 ± 2.12
	16 Weeks	High n-3	0.64 ± 0.13^{bc}	26.08 ± 1.16^{A}	3.51 ± 0.60	30.22 ± 1.64
		Low n-3	0.60 ± 0.20^{bc}	24.85 ± 2.45^{A}	3.87 ± 1.27	29.32 ± 3.67
Sex			NS	NS	NS	NS
Diet			NS	NS	P<0.05	NS
Age			P < 0.0001	P < 0.0001	P < 0.0001	P<0.0001
Sex*Diet			NS	NS	NS	NS
Sex*Age			P < 0.0001	NS	NS	NS
Diet*Age			NS	P < 0.05	NS	NS
Sex*Diet*Age			NS	NS	NS	NS

Data are expressed as weight percentage of the total extracted phospholipid fatty acids. Mean ± SD, n=8. Main effects and interactions were determined by three-way ANOVA; pairwise comparisons using Bonferroni correction was used to determine differences when there was an observed statistical significant interaction. Within a column, statistically significant values are marked with unlike superscripts when a significant interaction was observed (P<0.05) between sex and age (a,b,c) and diet and age (A,B). NS= non-significant; ΣMUFA = sum of monounsaturated fatty acid.

was a significant interaction between age and diet for C18:1 ($P<0.05$), with no significant independent effect of diet. Interestingly, C18:1 and C20:1n-9 increased from weaning to 16 weeks ($P<0.0001$) in both males and females. There was also an independent effect of the diet, where both male and the female offspring fed a diet high in n-3 PUFA showed higher percentage of C20:1n-9 compared to the low n-3 PUFA group ($P<0.05$). Finally, the cerebellum from both male and female offspring revealed approximately 7% increase in total MUFA from weaning to 16 weeks ($P<0.0001$).

The cerebellum PL n-6 PUFA composition of the offspring, obtained from mothers fed a diet high or low in n-3 PUFA, at weaning and 16 weeks postweaning is given in **Table 3.3.** Diet had no significant effect on total n-6 PUFA at weaning in both male and the female offspring. However, there was a decrease in LA and total n-6 PUFA from weaning to 16 weeks, and the effect was more pronounced in mice fed a diet high in n-3 PUFA compared to the low n-3 PUFA group at 16 weeks ($P<0.0001$). A reduction in LA was seen in the male and female offspring fed a diet high in n-3 PUFA, compared to the low n-3 PUFA diet group. There was an independent effect of age and diet on adrenic acid (ADA; C22:4n-6), with ADA decreasing from weaning to 16 weeks ($P<0.0001$); there was a lower percentage in the male and female offspring fed a diet high in n-3 PUFA compared to the low n-3 PUFA group ($P<0.0001$). Perinatal and postweaning exposure to a diet high in n-3 PUFA led to a decrease in AA in the male and female cerebellum both at weaning and 16 weeks, compared to mice fed the low n-3 PUFA diet ($P<0.0001$). There was also a significant interaction between age and sex on AA in the cerebellum ($P<0.005$). At weaning, male offspring had a higher percentage of AA compared to the females ($P<0.05$) but the effect of sex was not observed at 16 weeks.

Table 3.3: Effects of a diet high or low in n-3 PUFA on the phospholipid n-6 polyunsaturated fatty acid composition of the cerebellum from male and female offspring at weaning and 16 weeks postweaning

			N-6 PUFA (%w/w)			
			C18:2n-6 (LA)	**C20:4n-6 (AA)**	**C22:4n-6 (ADA)**	**Σn-6PUFA**
Male	Weaning	High n-3	2.31 ± 0.48^{Aa}	9.49 ± 0.64^{a}	1.97 ± 0.52	13.77 ± 0.90^{Aa}
		Low n-3	1.96 ± 0.21^{Aa}	10.63 ± 1.06^{a}	2.52 ± 0.29	15.12 ± 1.04^{Aa}
	16 Weeks	High n-3	0.99 ± 0.24^{Cc}	5.44 ± 0.71^{c}	1.04 ± 0.28	7.47 ± 1.06^{Cc}
		Low n-3	1.26 ± 0.21^{Bc}	6.87 ± 0.52^{c}	2.17 ± 0.88	10.29 ± 1.30^{Bc}
Female	Weaning	High n-3	1.81 ± 0.18^{Ab}	8.12 ± 0.94^{b}	1.47 ± 0.26	11.39 ± 1.02^{Ab}
		Low n-3	1.57 ± 0.22^{Ab}	9.23 ± 0.72^{b}	2.13 ± 0.57	12.93 ± 0.89^{Ab}
	16 Weeks	High n-3	1.00 ± 0.42^{Cc}	5.56 ± 0.49^{c}	1.29 ± 0.64	7.86 ± 1.10^{Cc}
		Low n-3	1.35 ± 0.25^{Bc}	6.64 ± 1.05^{c}	1.72 ± 0.31	9.71 ± 1.18^{Bc}
Sex			$P < 0.05$	$P < 0.005$	NS	$P < 0.0001$
Diet			NS	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Age			$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Sex*Diet			NS	NS	NS	NS
Sex*Age			$P < 0.01$	$P < 0.005$	NS	$P < 0.001$
Diet*Age			$P < 0.0001$	NS	NS	$P < 0.05$
Sex*Diet*Age			NS	NS	NS	NS

Data are expressed as weight percentage of the total extracted phospholipid fatty acids. Mean ± SD, n=8. Main effects and interactions were determined by three-way ANOVA; pairwise comparisons using Bonferroni correction was used to determine differences when there was an observed statistical significant interaction. Within a column, statistically significant values are marked with unlike superscripts when a significant interaction was observed (P<0.05) between sex and age (a,b,c) and diet and age (A,B,C). LA = linoleic acid; AA = arachidonic acid; ADA = adrenic acid; NS= non-significant; Σn-6 PUFA = sum of omega-6 polyunsaturated fatty acids.

There was also a significant effect of age on cerebellar AA, which revealed a decrease in AA in both male and female offspring, respectively, from weaning to 16 weeks ($P<0.05$).

The cerebellum PL n-3 PUFA composition of the offspring, obtained from mothers fed a diet high or low in n-3 PUFA, at weaning and 16 weeks postweaning is given in **Table 3.4.** We were unable to detect and quantify ALA, EPA and C22:5n-3, likely due to very low concentrations of these fatty acids. The only n-3 PUFA detected in the cerebellum was DHA, which exhibited a significant interaction between sex, diet, and age ($P<0.05$). The driving factor for the three-way interaction is sex, as sex is driving a difference between DHA accretion between male and female offspring. A diet high in n-3 PUFA caused an increase in DHA at weaning and 16 weeks in both males and females ($P<0.0001$). Interestingly, at weaning, females had lower levels of DHA compared to the males. However, the amount of DHA increased from weaning to 16 weeks in the females ($P<0.05$). There was no significant effect of sex on DHA at 16 weeks.

3.2 Effect of a diet high or low in n-3 PUFA on the mRNA expression of BDNF and TrkB of the cerebellum

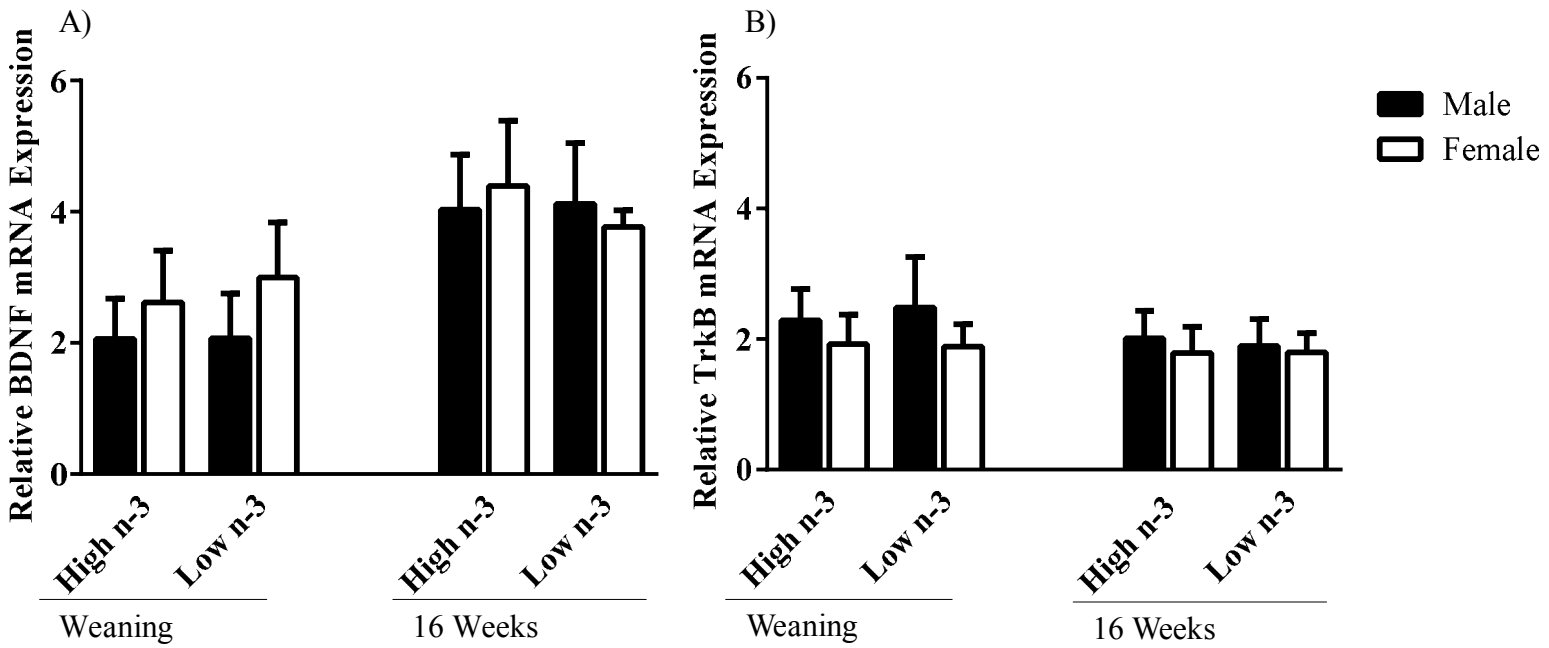
The effects of a perinatal and postweaning diet high or low in n-3 PUFA on cerebellar mRNA expressions of BDNF and TrkB were assessed using real-time qPCR analyses. There was an independent significant effect of age ($P<0.0001$) (Figure 3.1A) on the mRNA expression of BDNF. The mRNA expression of BDNF increased from weaning to 16 weeks postweaning in both the female and male offspring ($P<0.0001$)

Table 3.4: Effects of a diet high or low in n-3 PUFA on the phospholipid n-3 polyunsaturated fatty acid composition of the cerebellum from male and female offspring at weaning and 16 weeks postweaning

N-3 PUFA (%w/w)						
			C18:3n-3 (ALA)	**C20:5n-3 (EPA)**	**C22:5n-3**	**C22:6n-3 (DHA)**
Male	Weaning	High n-3	ND	ND	ND	15.78 ± 1.27[a]
		Low n-3	ND	ND	ND	13.38 ± 1.86[a]
	16 Weeks	High n-3	ND	ND	ND	16.15 ± 2.56[a]
		Low n-3	ND	ND	ND	14.78 ± 1.48[a]
Female	Weaning	High n-3	ND	ND	ND	12.26 ± 1.47[b]
		Low n-3	ND	ND	ND	11.05 ± 0.56[b]
	16 Weeks	High n-3	ND	ND	ND	16.57 ± 1.01[a]
		Low n-3	ND	ND	ND	13.19 ± 2.56[a]
Sex						P < 0.0001
Diet						P < 0.0001
Age						P < 0.0001
Sex*Diet						NS
Sex*Age						P < 0.001
Diet*Age						NS
Sex*Diet*Age						P < 0.05

Data are expressed as weight percentage of the total extracted phospholipid fatty acids. Mean ± SD, n=8. Main effects and interactions were determined by three-way ANOVA; pairwise comparisons using Bonferroni correction was used to determine differences when there was an observed statistical significant interaction. Within a column, statistically significant values are marked with unlike superscripts when a significant interaction was observed (P<0.05) between sex and age (a,b). ALA = alpha-linolenic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; NS = non-significant; ND = not detected.

Figure 3.1: Effects of a diet high or low in n-3 PUFA on the mRNA expression of brain-derived neurotrophic factor and tropomyosin-receptor kinase B of the cerebellum: *Data represents female and male cerebellar mRNA expression of brain-derived neurotrophic factor (BDNF) (A) and tropomyosin-receptor kinase B (TrkB) (B) at weaning and 16 weeks, normalized to β-actin as the house-keeping gene. Main effects and interactions were determined by three-way ANOVA; pairwise comparisons using Bonferroni correction was used to determine differences when there was an observed statistical significant interaction. Mean (n=8) ± SD. NS = non-significant.*



Diet: NS
Age: P<0.05
Sex: NS
Diet*Age: NS
Diet*Sex: NS
Age*Sex: NS
Age*Sex*Diet: NS

Diet: NS
Age: P<0.05
Sex: P<0.05
Diet*Age: NS
Diet*Sex: NS
Age*Sex: NS
Age*Sex*Diet: NS

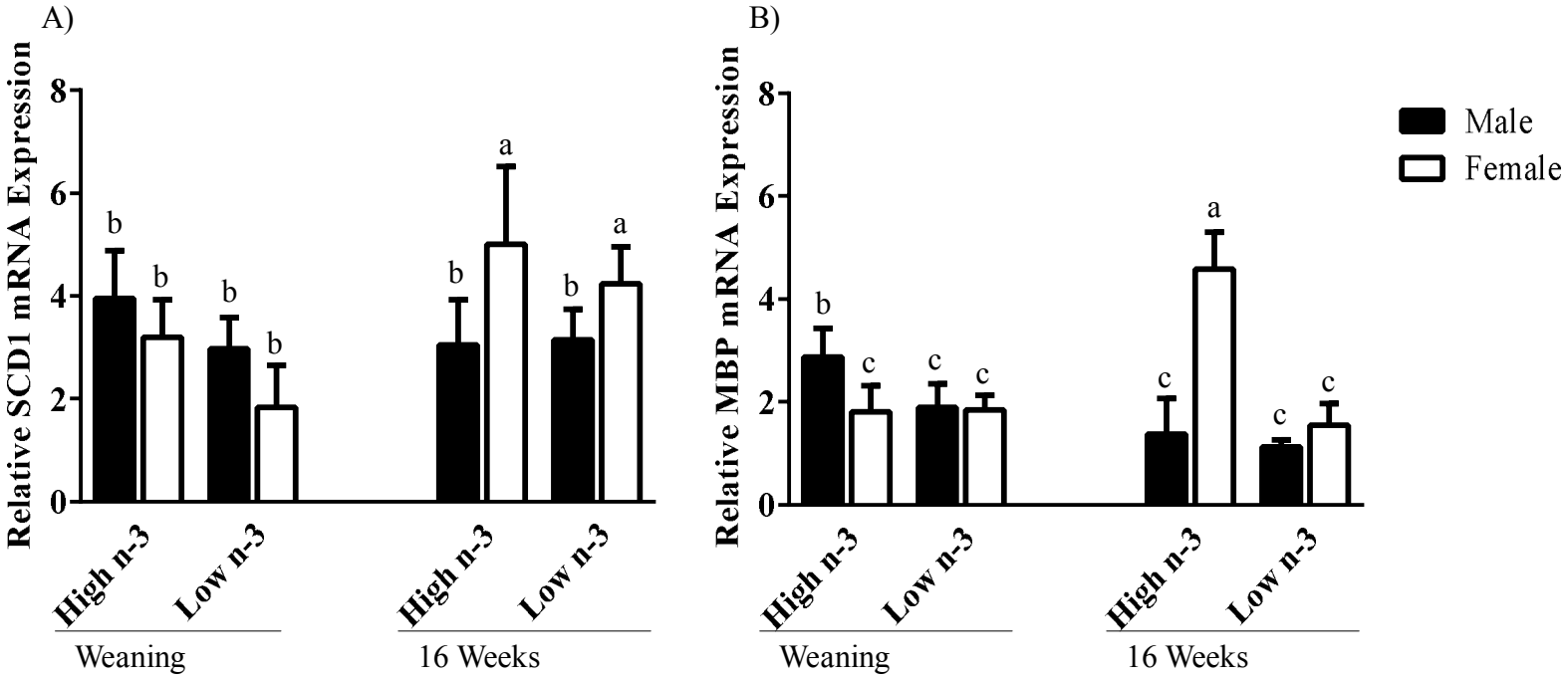
(Figure 3.1A). There was a trend towards higher mRNA expression of BDNF in the females, compared to the males (P=0.068) (Figure 3.1A).

There was no effect of diet on TrkB mRNA expression (Figure 3.1B), however, there was a significant effect of age (P<0.05) and sex (P<0.05) (Figure 3.1B). There was a decrease in the mRNA expression of TrkB in both male and female offspring from weaning to 16 weeks, (P<.0.05) (Figure 3.1B); however, males had a higher cerebellar TrkB mRNA expression compared to females, at both weaning and 16 weeks (P<0.05) (Figure 3.1B).

3.3 Effect of a diet high or low in n-3 PUFA on the mRNA expression of SCD1 and MBP of the cerebellum

The effects of a perinatal and postweaning diet high or low in n-3 PUFA on cerebellar myelin sheath formation related genes (SCD1 and MBP) was assessed using real-time qPCR analysis. There was an independent effect of diet on the mRNA expression of SCD1 (P<0.05) (Figure 3.2A). A diet high in n-3 PUFA significantly increased the mRNA expression of SCD1 compared to the low n-3 PUFA diet (P<0.05) (Figure 3.2A). There was a significant interaction between age and sex on the mRNA expression of SCD1 (P<0.05) (Figure 3.2A); female mice had an increase in the mRNA expression of SCD1 from weaning to 16 weeks (P<0.05); however, this effect of age was not observed in the male offspring. A significant interaction was observed between sex, age, and diet on the mRNA expression of MBP (P<0.05) (Figure 3.2B). Moreover, the effect of diet on the mRNA expression of MBP was sex and age dependent in both male

Figure 3.2: Effects of a diet high or low in n-3 PUFA on the mRNA expression of stearoyl-CoA desaturase-1 and myelin basic protein in the cerebellum: *Data represents female and male cerebellar mRNA expression of stearoyl-CoA desaturase-1 (SCD1) (A) and myelin basic protein (MBP) (B) at weaning and 16 weeks, normalized with β-actin as the house-keeping gene. Main effects and interactions were determined by three-way ANOVA; pairwise comparisons using Bonferroni correction was used to determine differences when there was an observed statistical significant interaction. Statistically significant values are marked with unlike letters (a,b,c) when a significant interaction was observed ($P<0.05$) between age and sex. Mean ($n=8$) ± SD. NS = non-significant.*



Diet: P<0.0001
Age: P<0.05
Sex: NS
Diet*Age: NS
Diet*Sex: NS
Age*Sex: P<0.001
Age*Sex*Diet: NS

Diet: P<0.0001
Age: P<0.05
Sex: NS
Diet*Age: P<0.0001
Diet*Sex: P<0.001
Age*Sex: P<0.0001
Age*Sex*Diet: P<0.0001

and female cerebellums at weaning and 16 weeks ($P<0.05$) (Figure 3.2B). There was also a significant interaction between age and diet ($P<0.05$) (Figure 3.2B). Females fed a diet high in n-3 PUFA revealed an increase in MBP mRNA expression from weaning to 16 weeks, compared to females fed a low n-3 PUFA diet ($P<0.05$) (Figure 3.2B). On the other hand, male offspring fed a diet high in n-3 PUFA showed higher MBP mRNA expression compared to males fed a low n-3 PUFA diet at weaning ($P<0.05$) (Figure 3.2B), while no effect of the diet was observed at 16 weeks.

3.4 Effects of a diet high or low in n-3 PUFA on the phospholipid fatty acid composition of the brainstem

The brainstem PL SFA composition of the offspring, obtained from mothers fed a diet high or low in n-3 PUFA, at weaning and 16 weeks postweaning is given in **Table 3.5.** There was a significant independent effect of diet on total SFA and C14:0 ($P<0.05$). The high n-3 PUFA diet group showed an increase in the percentage of cerebellar PL C14:0 and total SFA in the male and female offspring, compared to low n-3 PUFA diet group ($P<0.05$). Interestingly, there was a significant interaction observed between sex and age for C14:0, C16:0, C18:0, and total SFA ($P<0.05$). At weaning, female offspring had a higher percentage of individual and total SFA compared to male ($P<0.05$); however, both female and male offspring had a decrease in the percentage of individual and total SFA from weaning to 16 weeks ($P<0.05$). Moreover, the effect of sex disappeared at 16 weeks, where male and female offspring had similar percentages of individual and total SFA. Finally, there was an observed interaction between age and diet

Table 3.5: Effects of a diet high or low in n-3 PUFA on the phospholipid saturated fatty acid composition of the brainstem from male and female offspring at weaning and 16 weeks postweaning

			SFA (%w/w)			
			C14:0	**C16:0**	**C18:0**	**ΣSFA**
Male	Weaning	High n-3	0.61 ± 0.06^{b}	22.87 ± 1.02^{Ab}	23.25 ± 1.89^{b}	46.73 ± 2.77^{b}
		Low n-3	0.55 ± 0.13^{b}	20.42 ± 0.89^{Bb}	22.67 ± 1.26^{b}	43.64 ± 1.88^{b}
	16 Weeks	High n-3	0.36 ± 0.11^{c}	18.12 ± 1.70^{Cc}	21.70 ± 0.80^{c}	40.18 ± 1.98^{c}
		Low n-3	0.29 ± 0.05^{c}	18.23 ± 1.38^{Cc}	22.09 ± 1.13^{c}	40.61 ± 2.44^{c}
Female	Weaning	High n-3	0.81 ± 0.11^{a}	26.91 ± 1.71^{Aa}	26.82 ± 1.44^{a}	54.54 ± 3.07^{a}
		Low n-3	0.76 ± 0.09^{a}	25.07 ± 1.30^{Ba}	26.90 ± 1.52^{a}	52.73 ± 2.55^{a}
	16 Weeks	High n-3	0.38 ± 0.20^{c}	18.94 ± 1.03^{Cc}	23.70 ± 4.94^{c}	43.02 ± 4.45^{c}
		Low n-3	0.22 ± 0.08^{c}	18.64 ± 0.81^{Cc}	22.49 ± 0.48^{c}	41.36 ± 1.06^{c}
Sex			$P < 0.05$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Diet			$P < 0.005$	$P < 0.005$	NS	$P < 0.05$
Age			$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Sex*Diet			NS	NS	NS	NS
Sex*Age			$P < 0.0001$	$P < 0.0001$	$P < 0.05$	$P < 0.05$
Diet*Age			NS	$P < 0.05$	NS	NS
Sex*Diet*Age			NS	NS	NS	NS

Data are expressed as weight percentage of the total extracted phospholipid fatty acids. Mean ± SD, n=8. Main effects and interactions were determined by three-way ANOVA; pairwise comparisons using Bonferroni correction was used to determine differences when there was an observed statistical significant interaction. Within a column, statistically significant values are marked with unlike superscripts when a significant interaction was observed (P<0.05) between sex and age (a,b,c) and diet and age (A,B,C). NS= non-significant; ΣSFA = sum of saturated fatty acids.

on C16:0 ($P<0.05$). At weaning, both male and female offspring fed a diet high in n-3 PUFA had an increase in C16:0, compared to the low n-3 PUFA diet group ($P<0.05$).

The brainstem PL MUFA composition of the offspring, obtained from mothers fed a diet high or low in n-3 PUFA, at weaning and 16 weeks postweaning is given in **Table 3.6.** There was a significant independent effect of sex ($P<0.05$) and age ($P<0.001$) on C20:1n-9. Male offspring had a higher percentage of C20:1n-9 than females ($P<0.05$); however, both male and female offspring had a decrease in C20:1n-9 from weaning to 16 weeks ($P<0.0001$). There was a significant interaction between diet and age on brainstem PL C16:1n-7 ($P<0.05$). Interestingly, male and female offspring fed a diet high in n-3 PUFA showed higher levels of C16:1n-7 ($P<0.05$), compared to the low n-3 PUFA diet at weaning. Finally, brainstem PL C18:1 and total MUFA in both male and female offspring increased from weaning to 16 weeks ($P<0.0001$).

The brainstem PL n-6 PUFA composition of the offspring, obtained from mothers fed a diet high or low in n-3 PUFA, at weaning and 16 weeks postweaning is given in **Table 3.7.** There was a significant interaction between sex and age ($P<0.05$), and diet and age ($P<0.0001$) for brainstem PL LA and AA. At weaning, diet had no effect on the male and female offspring LA and AA; however, there was a decrease in LA and AA in the offspring fed a high n-3 PUFA diet, compared to the low n-3 PUFA group ($P<0.05$). In addition, there was an observed effect of sex ($P< 0.0001$), with male offspring having a higher percentage of AA than females at weaning; however this effect disappeared at 16 weeks. There was a significant independent effect of diet ($P<0.0001$) on ADA, where a diet high in n-3 PUFA showed lower levels of ADA compared to the low n-3 PUFA diet. Total n-6 PUFA revealed a significant interaction between diet and age ($P<0.01$), again

Table 3.6: Effects of a diet high or low in n-3 PUFA on the phospholipid monounsaturated fatty acid composition of the brainstem from male and female offspring at weaning and 16 weeks postweaning

			MUFA (%w/w)			
			C16:1n-7	**C18:1**	**C20:1n-9**	**ΣMUFA**
Male	Weaning	High n-3	1.16 ± 0.09^{Aa}	22.02 ± 2.30^{B}	2.79 ± 0.76	25.97 ± 3.01^{B}
		Low n-3	0.93 ± 0.29^{Ba}	24.46 ± 2.17^{B}	3.12 ± 1.20	28.51 ± 3.41^{B}
	16 Weeks	High n-3	0.68 ± 0.14^{Bb}	32.69 ± 1.59^{A}	7.60 ± 0.73	40.97 ± 2.11^{A}
		Low n-3	0.65 ± 0.14^{BCb}	29.85 ± 1.67^{A}	6.78 ± 1.31	37.27 ± 2.85^{A}
Female	Weaning	High n-3	0.84 ± 0.10^{Ab}	22.79 ± 3.32^{B}	1.94 ± 0.84	25.57 ± 4.17^{B}
		Low n-3	0.65 ± 0.09^{Bb}	22.91 ± 2.26^{B}	2.86 ± 0.97	25.57 ± 4.17^{B}
	16 Weeks	High n-3	0.63 ± 0.09^{Bb}	30.15 ± 2.51^{A}	6.57 ± 1.11	37.36 ± 3.55^{A}
		Low n-3	0.59 ± 0.10^{BCb}	30.48 ± 1.60^{A}	6.66 ± 1.22	37.36 ± 3.55^{A}
Sex			$P < 0.0001$	NS	$P < 0.05$	NS
Diet			$P < 0.005$	NS	NS	NS
Age			$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Sex*Diet			NS	NS	NS	NS
Sex*Age			$P < 0.005$	NS	NS	NS
Diet*Age			$P < 0.05$	$P < 0.05$	NS	$P < 0.05$
Sex*Diet*Age			NS	$P < 0.05$	NS	NS

Data are expressed as weight percentage of the total extracted phospholipid fatty acids. Mean ± SD, n=8. Main effects and interactions were determined by three-way ANOVA; pairwise comparisons using Bonferroni correction was used to determine differences when there was an observed statistical significant interaction. Within a column, statistically significant values are marked with unlike superscripts when a significant interaction was observed (P<0.05) between sex and age (a,b) and diet and age (A,B,C). NS= non-significant; ΣMUFA = sum of monounsaturated fatty acid.

Table 3.7: Effects of a diet high or low in n-3 PUFA on the phospholipid n-6 polyunsaturated fatty acid composition of the brainstem from male and female offspring at weaning and 16 weeks postweaning

			N-6 PUFA (% w/w)			
			C18:2n-6 (LA)	**C20:4n-6 (AA)**	**C22:4n-6 (ADA)**	**Σn-6PUFA**
Male	Weaning	High n-3	1.63 ± 0.20^{Aa}	8.48 ± 1.33^{Aa}	2.44 ± 0.60^{a}	12.55 ± 1.60^{ACa}
		Low n-3	1.63 ± 0.20^{Aa}	9.34 ± 0.89^{Aa}	3.84 ± 0.33^{a}	14.72 ± 0.59^{Aa}
	16 Weeks	High n-3	0.73 ± 0.14^{Cb}	4.71 ± 0.71^{Cb}	2.34 ± 0.59^{a}	7.78 ± 1.13^{Ccbd}
		Low n-3	0.97 ± 0.16^{Bb}	6.34 ± 0.65^{Bb}	3.17 ± 0.79^{a}	10.48 ± 1.15^{Bcbd}
Female	Weaning	High n-3	1.44 ± 0.19^{Aa}	6.97 ± 1.23^{Ac}	1.64 ± 0.29^{b}	10.05 ± 1.29^{Abc}
		Low n-3	1.31 ± 0.18^{Aa}	7.10 ± 0.75^{Ac}	2.42 ± 0.29^{b}	10.83 ± 0.84^{Ab}
	16 Weeks	High n-3	0.72 ± 0.12^{Cb}	4.80 ± 0.47^{Cb}	2.12 ± 0.52^{ab}	7.64 ± 0.56^{Cd}
		Low n-3	1.08 ± 0.20^{Bb}	6.31 ± 0.51^{Bb}	2.94 ± 0.33^{ab}	10.33 ± 0.65^{Bd}
Sex			NS	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Diet			$P < 0.05$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Age			$P < 0.0001$	$P < 0.0001$	NS	$P < 0.0001$
Sex*Diet			NS	NS	NS	NS
Sex*Age			$P < 0.05$	$P < 0.0001$	$P < 0.005$	$P < 0.0001$
Diet*Age			$P < 0.0001$	$P < 0.01$	NS	$P < 0.01$
Sex*Diet*Age			NS	NS	NS	NS

Data are expressed as weight percentage of the total extracted phospholipid fatty acids. Mean ± SD, n=8. Main effects and interactions were determined by three-way ANOVA; pairwise comparisons using Bonferroni correction was used to determine differences when there was an observed statistical significant interaction. Within a column, statistically significant values are marked with unlike superscripts when a significant interaction was observed (P<0.05) between sex and age (a,b,c,d) and diet and age (A,B,C). LA = linoleic acid; AA = arachidonic acid; ADA = adrenic acid; NS= non-significant; Σn-6 PUFA = sum of n-6 polyunsaturated fatty acids.

showing that offspring fed a diet high in n-3 PUFA had significantly lower amount of total n-6 PUFA compared to the low n-3 PUFA diet group at 16 weeks.

The brainstem PL n-3 PUFA composition of the offspring, obtained from mothers fed a diet high or low in n-3 PUFA, at weaning and 16 weeks postweaning is given in **Table 3.8.** In both male and female offspring, the n-3 PUFA ALA, EPA and C22:5n-3 were not detected in brainstem PL at weaning and 16 weeks. Interestingly, DHA showed a sex and age dependent ($P<0.0001$). At weaning, male offspring had a higher percentage of DHA than female offspring ($P<0.05$); however, from weaning to 16 weeks, male DHA decreased to the levels similar to the females. There was no effect of diet on the PL DHA composition at weaning or 16 weeks in the male and female offspring.

3.5 Effect of diets high or low in n-3 PUFA on the mRNA expression of BDNF and TrkB of the brainstem

The effects of perinatal and postweaning diets high or low in n-3 PUFA on brainstem mRNA expression of BDNF and TrkB were determined using real-time qPCR. There was no independent significant effect of diet, age, and sex on brainstem BDNF mRNA expression (Figure 3.3A). Interestingly, there was a significant interaction between sex and age on BDNF mRNA expression ($P<0.05$) (Figure 3.3A). At weaning, females had a higher mRNA expression of BDNF compared to males ($P<0.05$) (Figure 3.3A); however, the expression of BDNF decreased from weaning to 16 weeks postweaning in the females ($P<0.05$) (Figure 3.3A). There was no significant independent effect of sex, age, and diet on brainstem TrkB mRNA expression (Figure 3.3B).

Table 3.8: Effects of a diet high or low in n-3 PUFA on the phospholipid n-3 polyunsaturated fatty acid composition of the brainstem from male and female offspring at weaning and 16 weeks postweaning

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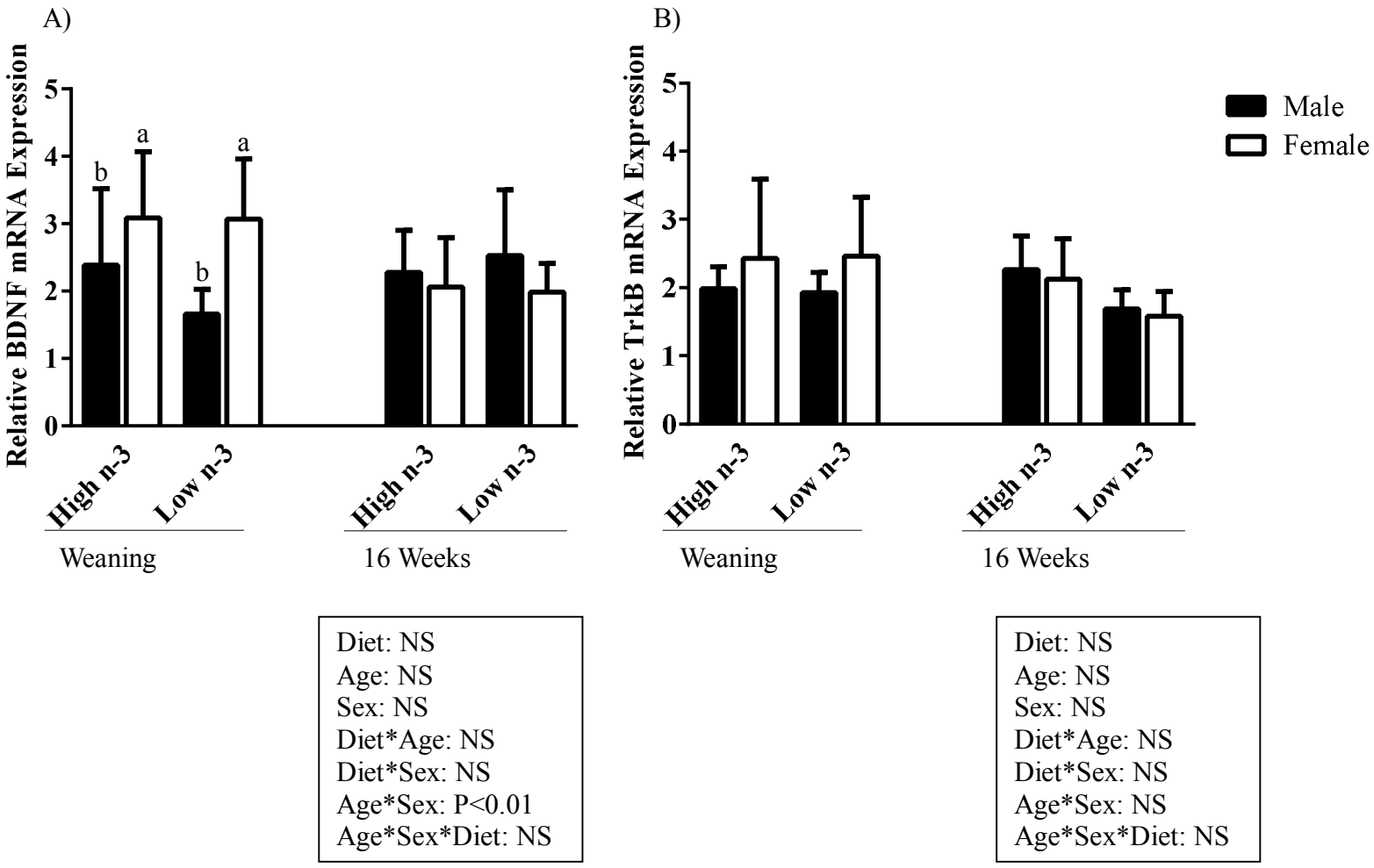
			N-3 PUFA (%w/w)			
			C18:3n-3 (ALA)	**C20:5n-3 (EPA)**	**C22:5n-3**	**C22:6n-3 (DHA)**
Male	Weaning	High n-3	ND	ND	ND	14.73 ± 1.67[a]
		Low n-3	ND	ND	ND	13.13 ± 1.10[a]
	16 Weeks	High n-3	ND	ND	ND	11.07 ± 1.29[b]
		Low n-3	ND	ND	ND	11.63 ± 1.87[b]
Female	Weaning	High n-3	ND	ND	ND	9.84 ± 1.26[b]
		Low n-3	ND	ND	ND	10.01 ± 0.68[b]
	16 Weeks	High n-3	ND	ND	ND	11.98 ± 0.68[b]
		Low n-3	ND	ND	ND	10.58 ± 1.32[b]
Sex						P < 0.0001
Diet						NS
Age						NS
Sex*Diet						NS
Sex*Age						P < 0.0001
Diet*Age						NS
Sex*Diet*Age						P < 0.05

Data are expressed as weight percentage of the total extracted phospholipid fatty acids. Mean ± SD, n=8. Main effects and interactions were determined by three-way ANOVA; pairwise comparisons using Bonferroni correction was used to determine differences when there was an observed statistical significant interaction. Within a column, statistically significant values are marked with unlike superscripts when a significant interaction was observed (P<0.05) between sex and age (a,b). ALA = alpha-linolenic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; NS= non-significant; ND = not detected.

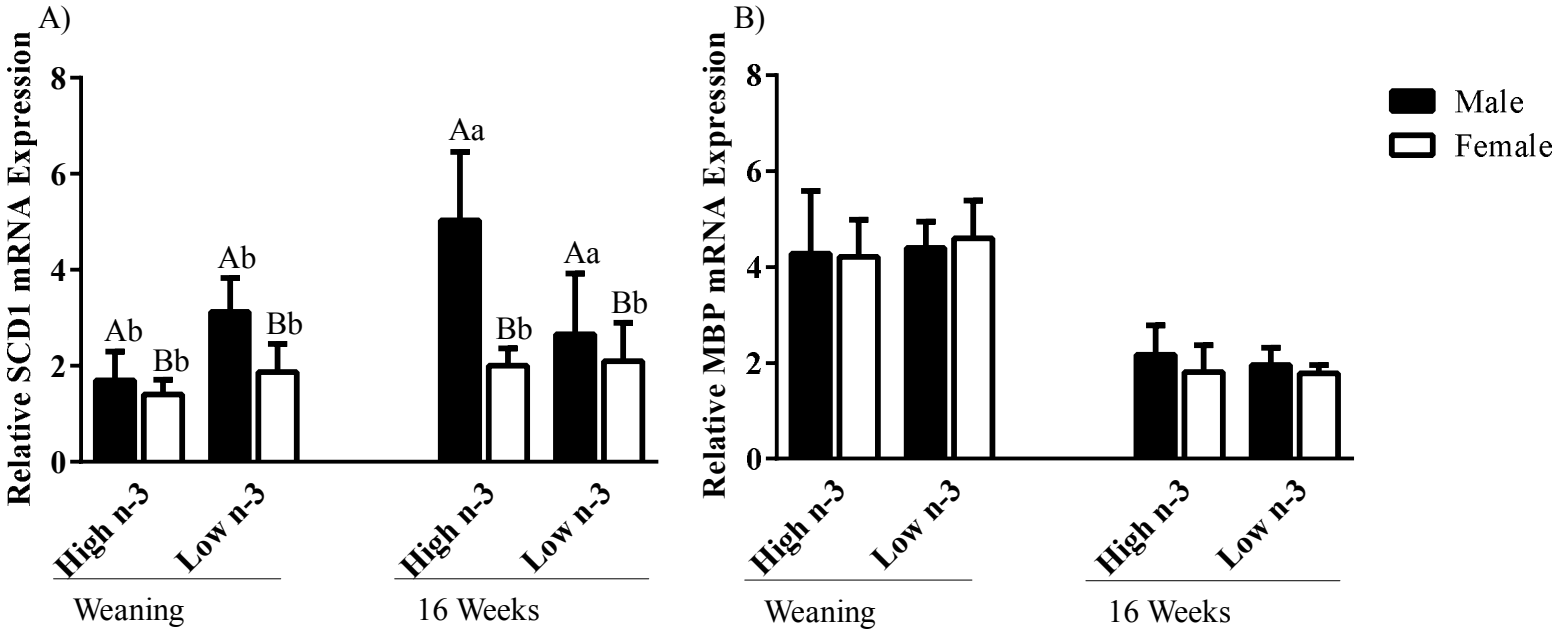
Figure 3.3: Effects of a diet high or low in n-3 PUFA on the mRNA expression of brain-derived neurotrophic factor and tropomyosin-receptor kinase B in the brainstem: *Data represents female and male cerebellar mRNA expression of brain-derived neurotrophic factor (BDNF) (A) and tropomyosin-receptor kinase B (TrkB) (B) at weaning and 16 weeks, normalized with β-actin as the house-keeping gene. Main effects and interactions were determined by three-way ANOVA; pairwise comparisons using Bonferroni correction was used to determine differences when there was an observed statistical significant interaction. Statistically significant values are marked with unlike letters (a,b) when a significant interaction was observed ($P<0.05$) between sex and age. Mean (n=8) ± SD. NS = non-significant.*


A)
Relative BDNF mRNA Expression
5
4
3
2
1
0
a
a
b
b
High n-3
Low n-3
High n-3
Low n-3
Weaning
16 Weeks
Diet: NS
Age: NS
Sex: NS
Diet*Age: NS
Diet*Sex: NS
Age*Sex: P<0.01
Age*Sex*Diet: NS
B)
Relative TrkB mRNA Expression
5
4
3
2
1
0
Male
Female
High n-3
Low n-3
High n-3
Low n-3
Weaning
16 Weeks
Diet: NS
Age: NS
Sex: NS
Diet*Age: NS
Diet*Sex: NS
Age*Sex: NS
Age*Sex*Diet: NS

3.6 Effects of a diet high or low in n-3 PUFA on the mRNA expressions of SCD1 and MBP of the brainstem

The effects of a perinatal and postweaning diet high or low in n-3 PUFA on myelin-related genes in the brainstem was assessed using real-time qPCR analysis of SCD1 and MBP mRNA expression. There was a significant interaction between sex and age ($P<0.05$) (Figure 3.4A), and diet and age ($P<0.0001$) (Figure 3.4A) on the mRNA expression of SCD1 in the brainstem. Interestingly, brainstem SCD1 mRNA expression increased from weaning to 16 weeks in males only ($P<0.05$) (Figure 3.4A), which was higher compared to females at 16 weeks ($P<0.05$) (Figure 3.4A). Furthermore, at 16 weeks, the male offspring fed a diet high in n-3 PUFA had a higher mRNA expression of SCD1 compared to the low n-3 PUFA diet group ($P<0.05$) (Figure 3.4A); however, this effect was not seen in the female brainstem. There was a significant independent effect of age on the mRNA expression of MBP in the brainstem ($P<0.0001$) (Figure 3.4B). There was a decrease in the brainstem mRNA expression of MBP from weaning to 16 weeks in both the male and female offspring ($P<0.0001$) (Figure 3.4B). There was a significant independent effect of age on the mRNA expression of MBP in the brainstem ($P<0.0001$) (Figure 3.4B). There was a decrease in the brainstem mRNA expression of MBP from weaning to 16 weeks in both the male and female offspring ($P<0.0001$) (Figure 3.4B).

Figure 3.4: Effects of a diet high or low in n-3 PUFA on the mRNA expression of stearoyl-CoA desaturase-1 and myelin basic protein in the brainstem: *Data represents female and male cerebellar mRNA expression of stearoyl-CoA desaturase-1 (SCD1) (A) and myelin basic protein (MBP) (B) at weaning and 16 weeks, normalized with β-actin as the house-keeping gene. Main effects and interactions were determined by three-way ANOVA; pairwise comparisons using Bonferroni correction was used to determine differences when there was an observed statistical significant interaction. Statistically significant values are marked with unlike superscripts when a significant interaction was observed ($P<0.05$) between sex and age (a,b) and diet and age (A,B). Mean (n=8) ± SD. NS = non-significant.*



Diet: NS
Age: P<0.0001
Sex: P<0.0001
Diet*Age: P<0.0001
Diet*Sex: NS
Age*Sex: P<0.05
Age*Sex*Diet: P<0.001

Diet: NS
Age: P<0.0001
Sex: NS
Diet*Age: NS
Diet*Sex: NS
Age*Sex: NS
Age*Sex*Diet: NS

3.7 Effects of a diet high or low in n-3 PUFA on the adult female cortical phospholipid fatty acid composition

The cerebral cortical PL composition of the adult female, obtained from feeding a diet high or low in n-3 PUFA, for one month or two months is given in **Table 3.9.** There was an independent effect of diet on C16:0 and C18:0, where a diet high in n-3 PUFA increased C16:0 and C18:0, compared to a diet low in n-3 PUFA for both one and two month feeding period ($P<0.01$). There was an independent effect of age on the PL C14:0 ($P<0.01$) and total SFA ($P<0.01$). Cortical PL total SFA and C14:0 decreased from the one month feeding to the two month feeding in both the high and low n-3 PUFA diet group.

Diet and age were shown to have no effect on the PL total MUFA, specifically C18:1 and C20:1n-9; however, a diet high in n-3 PUFA increased the accretion of PL C16:1n-7 ($P<0.05$). There was no effect of age on the accretion of PL C16:1n-7 in the female cerebral cortex.

There was a significant independent effect of age and diet on the total PL n-6 PUFA and AA amount in the female cerebral cortex ($P<0.001$). A diet high in n-3 PUFA, fed for either one or two months, reduced the amount of total PL n-6 PUFA and AA, compared to the low n-3 PUFA diet group ($P<0.001$). On the other hand, the amount of PL total n-6 and AA was higher in the cerebral cortex at two months compared to the one month group ($P<0.001$). Diet showed an independent effect on the cortical ADA accretion ($P<0.001$), where females fed a diet high in n-3 PUFA showed lower levels of ADA compared to the low n-3 PUFA group, at both one month and two months ($P<0.001$).

Table 3.9: ***Effects of a diet high or low in n-3 PUFA on the adult female cortical phospholipid fatty acid composition after being fed for one or two months***

	1 Month		2 Month		Main Effects		
Fatty Acids (%w/w)	High n-3	Low n-3	High n-3	Low n-3	Diet	Age	Diet*Age
C14:0	0.3 ± 0.12	0.23 ± 0.05	0.16 ± 0.03	0.13 ± 0.01	NS	P<0.001	NS
C16:0	24.67 ± 1.00	22.70 ± 1.7	22.89 ±0.54	22.71 ± 0.26	P<0.05	NS	NS
C18:0	25.10 ± 1.25	24.50 ± 1.80	23.49 ± 0.40	23.58 ± 0.42	P<0.05	NS	NS
ΣSFA	50.10 ± 1.38	47.47 ± 3.10	46.53 ± 0.66	46.41 ± 0.61	NS	P<0.01	NS
C16:1n-7	0.56 ± 0.18	0.47 ± 0.06	0.60 ± 0.03	0.51 ± 0.03	P<0.05	NS	NS
C18:1	20.32 ± 0.43	19.33 ± 2.62	18.97 ± 1.09	18.52 ± 1.14	NS	NS	NS
C20:1n-9	1.51 ± 0.50	1.51 ± 0.66	1.22 ± 0.32	1.24 ± 0.29	NS	NS	NS
ΣMUFA	22.41 ± 1.66	21.32 ± 3.21	20.80 ± 1.38	20.26 ± 1.46	NS	NS	NS
C18:2n-6 (LA)	1.10 ± 0.20	0.92 ± 0.1	1.00 ± 0.07	0.95 ± 0.07	NS	NS	NS
C20:4n-6 (AA)	8.55 ± 0.77	9.96 ± 0.55	10.27 ± 0.72	11.59 ± 0.96	P<0.001	P<0.0001	NS
C22:4n-6 (ADA)	2.34 ± 0.28	3.05 ± 0.33	2.45 ± 0.14	3.07 ± 0.32	P<0.0001	NS	NS
Σn-6	11.94 ± 1.04	13.80 ± 0.64	13.73 ± 0.80	15.60 ± 1.26	P<0.0005	P<0.0005	NS
C18:3n-3 (ALA)	ND	ND	0.05 ± 0.01	ND			
C20:5n-3 (EPA)	ND	ND	0.49 ± 0.90	ND			
C22:5n-3	0.29 ± 0.02	0.07 ± 0.05	0.33 ± 0.08	ND	P<0.0001	NS	NS
C22:6n-3 (DHA)	15.29 ± 1.61^{b}	16.78 ± 1.00^{ab}	18.18 ± 0.30^{a}	17.72 ± 0.58^{a}	NS	P<0.0005	P<0.05

Expressed as weight percentage of the total extracted phospholipid fatty acids. Mean ± SD, n=6. Main effects and interactions were determined by two-way ANOVA; pairwise comparisons using Bonferroni correction was used to determine differences when there was an observed statistical significant interaction. Within a row, statistically significant values are marked with unlike superscripts (a,b) when a significant interaction was observed (P<0.05) between diet and age. LA = linoleic acid; ALA = alpha-linolenic acid; DHA = docosahexaenoic acid; AA = arachidonic acid; EPA = eicosapentaenoic acid; ADA = adrenic acid; ΣSFA = sum of saturated fatty acids; ΣMUFA = sum of monounsaturated fatty acids; Σn-6 PUFA = sum of omega-6 polyunsaturated fatty acids; NS= non-significant; ND = not detected.

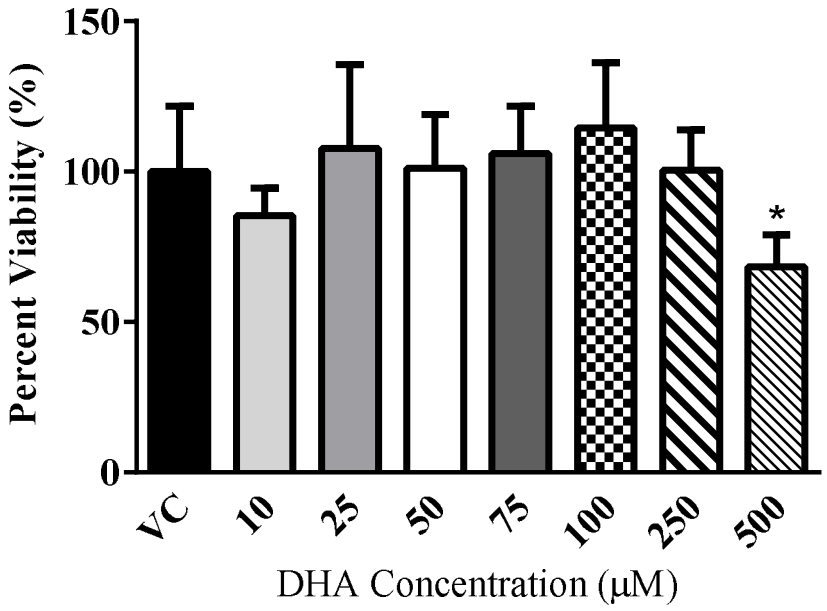
There was a significant interaction observed between diet and age for the cortical PL DHA ($P<0.05$). Independently, diet had no effect on PL DHA at weaning or 16 weeks; however, a diet high in n-3 PUFA caused a higher accretion of PL DHA at two months compared to the one month group ($P<0.05$). Furthermore, there was an increase in the accretion of DHA in the animals at two months compared to the one month group ($P<0.05$). Interestingly, ALA and EPA were not detected in female cortical PL at one month. However, both ALA and EPA were detected at two months after feeding a diet high in n-3 PUFA (statistical analysis could not be performed for these fatty acids due to no detection for some treatments). Finally, a diet high in n-3 PUFA increased cortical PL C22:5n-3 levels at both one month and two month feeding periods, compared to the low n-3 PUFA diet group ($P<0.0001$).

3.8 Investigating the effects of specific fatty acids on the regulation of gene expression of BDNF, TrkB, SCD1 and MBP using primary cerebral cortical cells

3.8.1. Effects of various concentrations of DHA on cell viability of primary cerebral cortical cells

The effect of DHA treatment at varying concentrations on primary cerebral cortical cell viability was assessed using the MTT assay. The percent viability of cerebral cortical cells treated with 10, 25, 50, 75, 100, and 250 μM of DHA was not statistically different from the control cells treated with vehicle alone (Figure 3.5). However, the viability of cerebral cortical cells decreased after treatment with 500 μM DHA compared to vehicle control ($P<0.05$) (Figure 3.5).

Figure 3.5: Effects of various concentrations of docosahexaenoic acid treatments on cell viability of primary cerebral cortical cells: *The viability of cerebral cortical cells were measured using MTT assay after treatment with various concentrations of docosahexaenoic acid (DHA) as explained in the methods section. Data was assessed using one-way ANOVA to determine significance ($P<0.05$). Significant effects were further analyzed using Bonferroni post-hoc test. Cell viability was calculated by comparing DHA treatment groups to vehicle control (VC). Values are expressed as mean ($n=4$) $\pm$ SD. (* = $P<0.05$).*


150
100
50
0
Percent Viability (%)
*
VC
10
25
50
75
100
250
500
DHA Concentration (μM)

3.8.2. Treatment of primary cortical cells with DHA increased the accretion of DHA in the phospholipid fraction

The PL fatty acid composition of cerebral cortical cells is reported in **Table 3.10.** There was no significant effect of various concentrations of DHA (10, 25, 50, 75, and 100 μM) on the PL fatty acid composition of total and individual SFAs. There was a decrease in C16:1n-7 after treatment of cortical cells with 75 and 100 μM DHA compared to the vehicle control ($P<0.05$). However, total MUFA and C18:1 was not changed after DHA treatments compared to the control cells. Interestingly, there was a decrease in AA after treatment with 50 and 100 μM DHA ($P<0.05$); compared to control. Treatment with DHA at higher concentrations (50, 75, and 100 μM) significantly increased the percentage of PL DHA in the cerebral cortical cells compared to vehicle control ($P<0.05$). However a dose of 75 and 100 μM showed no further increase compared to 50 μM, thus for the future experiments, a dose of 50 μM DHA treatment was used.

3.8.3 Effects of DHA and AA treatment on primary cerebral cortical cells BDNF and TrkB mRNA expression

The effect of DHA and AA treatment on cerebral cortical cell BDNF and TrkB mRNA expression was assessed using real-time qPCR analysis. Treatment with DHA significantly increased the mRNA expression of BDNF compared to vehicle control and AA treated cells ($P<0.05$) (Figure 3.6). In contrast, the mRNA expression of BDNF of cells treated with AA was not statistically different from cells treated with the vehicle control (Figure 3.6). There was also a significant increase in the mRNA expression of TrkB for cells treated with DHA, compared to vehicle control ($P<0.05$) (Figure 3.7). AA

Table 3.10: Effects of various concentrations of docosahexaenoic acid treatments on primary cerebral cortical cells phospholipid fatty acid composition

	DHA Treatments					
Fatty Acids (%w/w)	**Vehicle**	**10 µM**	**25 µM**	**50 µM**	**75 µM**	**100 µM**
C14:0	0.73 ± 0.63	1.25 ± 0.28	1.24 ± 0.21	1.04 ± 0.17	0.64 ± 0.57	0.39 ± 0.67
C16:0	29.71 ± 1.63	29.50 ± 0.98	30.16 ± 0.30	29.37± 0.17	29.43 ± 0.33	30.12 ± 0.50
C18:0	22.7 ± 2.19	22.41 ± 0.54	19.29 ± 0.65	19.24 ± 1.18	20.75 ± 1.52	21.56 ± 2.30
ΣSFA	53.13 ± 2.61	53.17 ± 0.91	50.69 ± 0.48	49.65± 1.06	50.82 ± 1.89	52.06 ± 2.64
C16:1n-7	6.09 ± 1.18[a]	5.88 ± 0.16[a]	5.96 ± 0.68[a]	5.77 ± 0.09[a]	4.66 ± 0.23[b]	4.26 ± 0.24[b]
C18:1	27.99 ± 0.78	26.84 ± 0.55	28.89 ± 0.88	29.3± 0.6	28.07± 1.59	27.13 ± 1.99
C20:1n-9	ND	ND	ND	ND	ND	ND
ΣMUFA	34.08 ± 1.76	32.71 ± 0.65	34.85 ± 1.5	35.07± 0.66	32.7± 21.80	31.4 ± 2.21
C18:2n-6 (LA)	ND	ND	ND	ND	ND	ND
C20:4n-6 (AA)	6.27 ± 0.38	5.77 ± 0.39[bc]	6.14 ± 0.23	6.0 ± 0.07[b]	6.73 ± 0.12[a]	5.94 ± 0.31[c]
C22:4n-6 (ADA)	ND	ND	ND	ND	ND	ND
Σn-6 PUFA	6.27 ± 0.38	5.77 ± 0.39	6.14 ± 0.23	6.0 ± 0.07	6.73 ± 0.12	5.94 ± 0.31
C18:3n-3 (ALA)	ND	ND	ND	ND	ND	ND
C20:5n-3 (EPA)	ND	ND	ND	ND	ND	ND
C22:5n-3	ND	ND	ND	ND	ND	ND
C22:6n-3 (DHA)	6.52 ± 0.86	8.34 ± 1.21	8.32 ± 1.21	9.27 ± 1.76[b]	9.72 ± 0.35[b]	10.60 ± 0.43[a]
Σn-3 PUFA	6.52 ± 0.86	8.34 ± 1.21	8.32 ± 1.21	9.27 ± 1.76	9.72 ± 0.35	10.60 ± 0.43

Data are expressed as weight percentage of the total extracted phospholipid fatty acids. Mean ± SD, n=3. Main effects and interactions were determined by one-way ANOVA. Significant effects were further analyzed using Bonferroni post-hoc test. Mean values within a row marked with unlike superscripts (a,b) were significantly different (P<0.05). ALA = alpha-linolenic acid; LA = linoleic acid; ADA = adrenic acid; EPA = eicosapentanoic aicd; AA = arachidonic acid; DHA = docosahexaenoic acid; ΣSFA = sum of saturated fatty acids, ΣMUFA = sum of monounsaturated fatty acids, Σn-6 PUFA = sum of omega-6 polyunsaturated fatty acids, Σn-3 PUFA = sum of omega-3 polyunsaturated fatty acids, ND = not detected.

Figure 3.6: Effect of docosahexaenoic acid and arachidonic acid treatment on primary cerebral cortical cells brain-derived neurotrophic factor mRNA expression: *Cerebral cortical cell brain-derived neurotrophic factor (BDNF) mRNA expression after treatment with* 50 μM *docosahexaenoic acid (DHA) and arachidonic acid (AA) was assessed using real-time qPCR, as explained in the method section. Data were assessed using one-way ANOVA to determine significance ($P<0.05$). Significant effects were further analyzed using Bonferroni post-hoc test. Mean values were marked with unlike letters (a,b) when significantly different ($P<0.05$). BDNF mRNA expression was normalized with β-actin as the house-keeping gene. Values are expressed as mean (n=3) ± SD. VC = Vehicle control.*

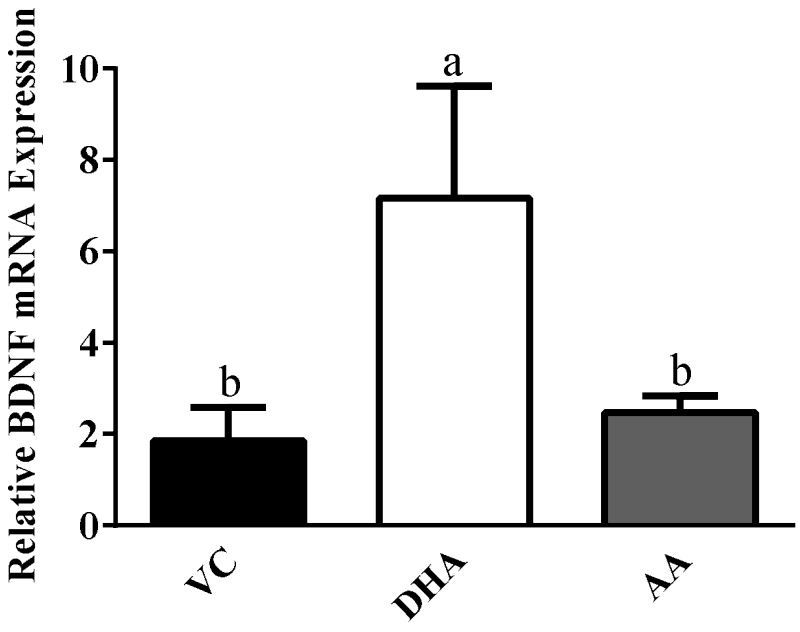
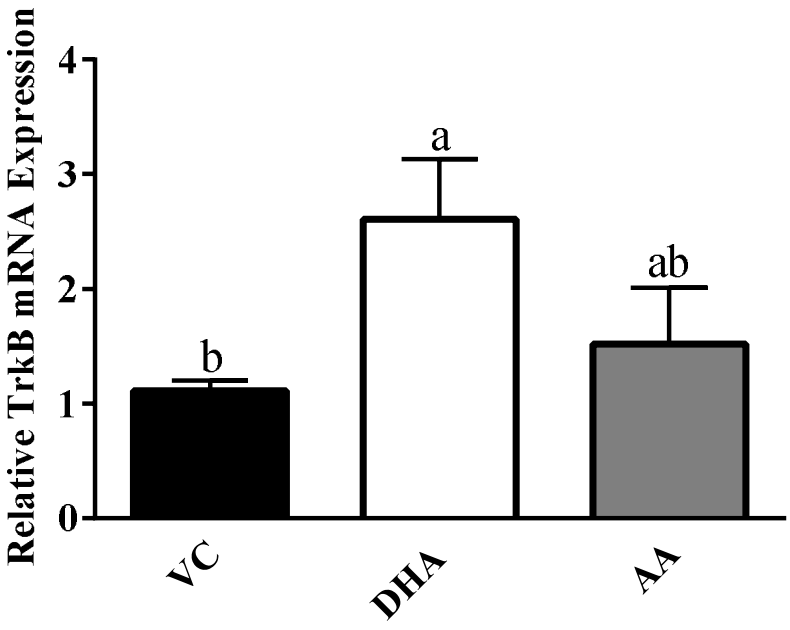

Relative BDNF mRNA Expression
10
8
6
4
2
0
a
b
b
VC
DHA
AA

Figure 3.7: Effect of docosahexaenoic acid and arachidonic acid treatment on primary cerebral cortical cells tropomyosin-receptor kinase B mRNA expression*: Cerebral cortical cell tropomyosin-receptor kinase B (TrkB) mRNA expression after treatment with 50* μM *docosahexaenoic acid (DHA) and arachidonic acid (AA) was assessed using real-time qPCR, as explained in the method section. Data were assessed using one-way ANOVA to determine significance (P<0.05). Significant effects were further analyzed using Bonferroni post-hoc test. Mean values were marked with unlike letters (a,b) when significantly different (P<0.05). TrkB gene expression was normalized with β-actin as the house-keeping gene. Values are expressed as mean (n=3) ± SD. VC = Vehicle control.*


Relative TrkB mRNA Expression
4
3
2
1
0
a
ab
b
VC
DHA
AA

treatment had no significant effect on TrkB mRNA expression compared to DHA treatment and vehicle control (Figure 3.7).

3.8.4 Effects of DHA and AA treatment on primary cerebral cortical cells SCD1 and MBP mRNA expression

The effect of DHA and AA treatment on cerebral cortical cell mRNA expression of SCD1 and MBP was determined using real-time qPCR analysis. Treatment with DHA showed a significant increase in the mRNA expression of SCD1 ($P<0.05$) (Figure 3.8). In addition, DHA treatment resulted in a significantly higher SCD1 mRNA expression in the cerebral cortical neurons compared to AA treatment ($P<0.05$) (Figure 3.8). However, the mRNA expression of SCD1 after AA treatment was not significantly different from the vehicle control (Figure 3.8). DHA treatment also significantly increased the mRNA expression MBP ($P<0.05$) (Figure 3.9) compared to cells treated with the vehicle control. In addition, the mRNA expression of MBP was not significantly different between DHA and AA treatment (Figure 3.9).

Figure 3.8: Effect of docosahexaenoic acid and arachidonic acid treatment on primary cerebral cortical cells stearoyl-CoA desaturase-1 mRNA expression*: Cerebral cortical cell stearoyl-CoA desaturase-1 (SCD1) mRNA expression after treatment with 50 μM docosahexaenoic acid (DHA) and arachidonic acid (AA) was assessed using real-time qPCR, as explained in the method section. Data were assessed using one-way ANOVA to determine significance (P<0.05). Significant effects were further analyzed using Bonferroni post-hoc test. Mean values were marked with unlike letters (a,b) when significantly different (P<0.05). SCD1 gene expression was normalized with β-actin as the house-keeping gene. Values are expressed as mean (n=3) ± SD. VC = Vehicle control.*

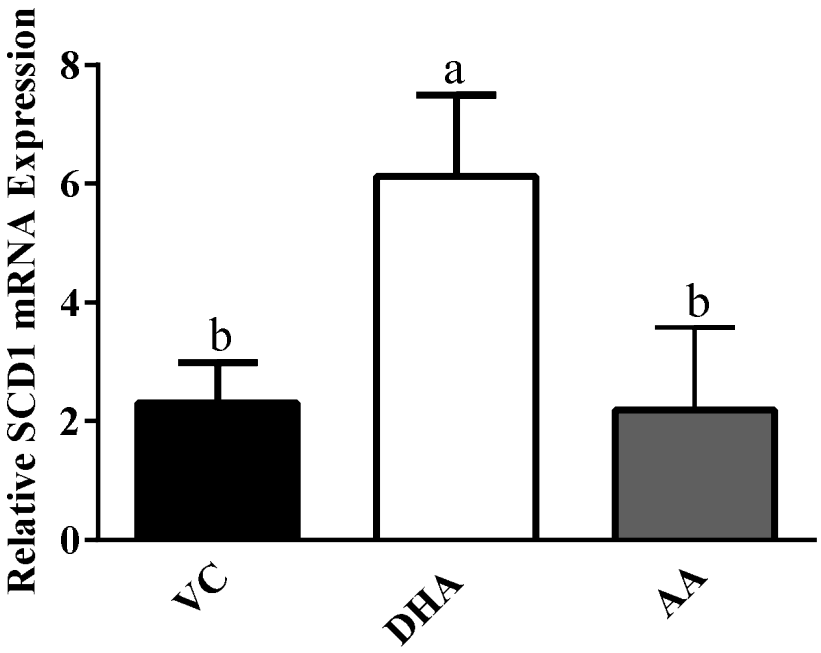
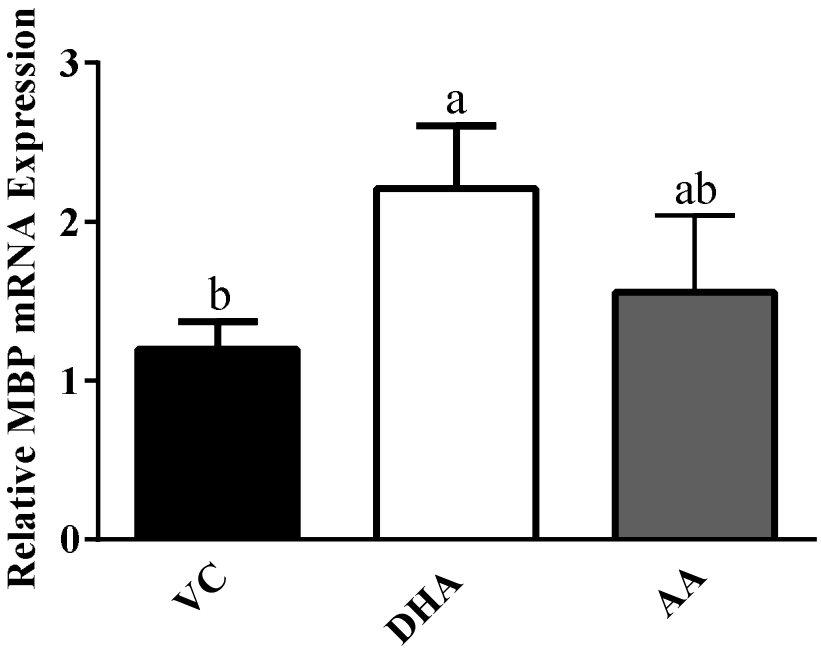

Relative SCD1 mRNA Expression
8
6
4
2
0
a
b
b
VC
DHA
AA

Figure 3.9: Effect of docosahexaenoic acid and arachidonic acid treatment on primary cerebral cortical cells myelin basic protein mRNA expression: *Cerebral cortical cell myelin basic protein (MBP) mRNA expression after treatment with 50* μM *docosahexaenoic acid (DHA) and arachidonic acid (AA) was assessed using real-time qPCR, as explained in the method section. Data were assessed using one-way ANOVA to determine significance (P<0.05). Significant effects were further analyzed using Bonferroni post-hoc test. Mean values were marked with unlike letters (a,b) when significantly different (P<0.05). MBP gene expression was normalized with β-actin as the house-keeping gene. Values are expressed as mean (n=3) ± SD. VC = Vehicle control.*


Relative MBP mRNA Expression
3
2
1
0
a
ab
b
VC
DHA
AA

Chapter 4: Discussion

4.1 Effects of a diet high or low in n-3 PUFA on the phospholipid fatty acid composition of various regions of the brain

The adequate consumption of PUFA is important for the proper development of the neuronal membranes (Clandinin *et al.*, 1980). Furthermore, there is a high accretion of AA and DHA during the last trimester of pregnancy and early postnatal period, a time-point corresponding to the rapid development of the brain (Arbuckle & Innis, 1992; Wainwright, 2002). In rodents, this period corresponds to the last three days of gestation and up to the first prenatal week (Clandinin *et al.*, 1980; Green & Yavin, 1996). Our laboratory has previously shown that a perinatal and postweaning diet high in n-3 PUFA increased the accretion of DHA in the cerebral cortex of male offspring, compared to male mice fed a low n-3 PUFA diet (Balogun & Cheema, 2014). Specific regions of the brain have a specific fatty acid composition (Carrié *et al.*, 2000), which may be important for their function. Although several studies have focused on the effect of diets high in n-3 PUFA on cortical fatty acid compositions, there are no studies to date to show the effects of diets high in n-3 PUFA on the fatty acid composition of other regions of the brain. This thesis focused on the effects of diets high or low in n-3 PUFA on the fatty acid composition of cerebellum and brainstem.

4.1.1 Effects of a diet high or low in n-3 PUFA on the phospholipid fatty acid composition of the cerebellum

We investigated the effects of a perinatal and postweaning diet high in n-3 PUFA on the cerebellar PL fatty acid composition in both male and female offspring to delineate sex-specific effects at weaning and 16 weeks postnatal.

4.1.1.1 Effect of diets high or low in n-3 PUFA on SFA and MUFA composition of the cerebellum

Patients diagnosed with a neuropsychiatric disease show changes in the SFA and MUFA PL fatty acid composition of the brain (Hamazaki *et al.*, 2012; McNamara *et al.*, 2014). We observed a decrease in total SFA, and an increase in total MUFA, particularly C18:1 in male and female cerebellum from weaning to 16 weeks. Multiple rodent and human studies have also observed the same effect of age on the whole brain and cerebellum PL SFA and MUFA content (Carrié *et al.*, 2000; Xiao *et al.*, 2005; Martinez & Mougan, 1998). However, we are the first to assess the sex-specific differences in SFA and MUFA PL fatty acid composition of the cerebellum.

The accumulation of SFA and MUFA in the developing brain has been attributed to *de novo* synthesis rather than transport from the peripheral pool (Edmond *et al.*, 1998). We made novel observations showing that a longer exposure to a diet high in n-3 PUFA increased cerebellar PL C18:1 in both male and females, compared to mice fed a low n-3 PUFA diet. An increase in C18:1 may be due to an increase in the expression or activity of the enzymes responsible for the desaturation and elongation of SFAs into MUFAs in the cerebellum, particularly C18:1. A possible explanation is the association of n-3 PUFAs with the rate-limiting enzyme SCD1. It has previously been shown that feeding a

diet high in ALA increased the mRNA expression of SCD1 in male rats compared to the standard chow diet (Cintra *et al.*, 2012), which is similar to our findings.

Interestingly, the amygdala from post-mortem MDD patients had higher amounts of C16:0, and lower amounts of C18:1, compared to control subjects (Hamazaki *et al.*, 2012). Furthermore, McNamara & Liu (2011) found a reduction in human SCD mRNA expression in the prefrontal cortex of patients with MDD, compared to control. Our results suggest that one of the underlying mechanisms by which n-3 PUFA may promote beneficial health effects on neuropsychiatric illnesses due to the regulation of SFA and MUFA metabolism via SCD1 gene expression, which is discussed further in **Section 4.1.2.1**.

4.1.1.2 Effect of diets high or low in n-3 PUFA on the n-6 and n-3 PUFA composition of the cerebellum

A perinatal and sustained postweaning diet high in n-3 PUFA increased the accretion of DHA in both male and female cerebellum, compared to the low n-3 PUFA diet group, similar to our previously published findings in the cortex (Balogun & Cheema, 2014). Xiao *et al.* (2005) also reported similar findings where a perinatal and postweaning diet supplemented with n-3 PUFA increased the accretion of DHA in the five-week-old male rat offspring cerebellum, compared to a diet deficient in n-3 PUFA. However, these authors only studied the male offspring, and the diet adequate with n-3 PUFA was comprised of only 3.5 % ALA, which is comparable to the levels in a standard chow diet (Xiao *et al.*, 2005). Furthermore, we report for the first time an age and sex-specific effect of DHA accretion in the cerebellum PLs. At weaning, female offspring had lower accretion of PL DHA compared to the male offspring; however, as the females

aged from weaning to 16 weeks, the accretion of DHA increased, and was similar to that of the males. A plausible explanation for the sex specific difference may be due to an increase in the desaturation and elongation of ALA to DHA in young adult female mice, which is regulated by estrogens (Childs *et al.*, 2008; Alessandri *et al.*, 2008).

It was obvious that a diet high in n-3 PUFA caused a decrease in AA in the cerebellum PLs. There was an independent effect of diet and age on the cerebellar PL n-6 PUFA AA and ADA, where a diet high in n-3 PUFA caused a decrease in cerebellar AA and ADA in both male and female offspring, compared to the low n-3 PUFA diet group. Moreover, both male and female offspring showed a decrease in AA and ADA from weaning to 16 weeks, which is similar to our previous results on the PL fatty acid profile of the male cerebral cortex (Balogun & Cheema, 2014). Demar *et al.* (2006) found a decrease in LA elongation and desaturation in adult rodent brains, and most of the LA utilized by the brain was β-oxidized into downstream aqueous products. Thus, a decrease in AA and ADA from weaning to 16 weeks postweaning in our study is likely due to a decrease in the desaturation and elongation process during ageing.

Elongation and desaturation of n-3 and n-6 PUFAs are carried out by fatty acid desaturases (FADS) and elongases (ELOVL). Interestingly, postmortem studies have observed altered expression of these genes in the prefrontal cortex of patients with schizophrenia (Liu *et al.*, 2009) and MDD (McNamara and Liu, 2011). Therefore, alterations in n-3 and n-6 PUFA fatty acid synthesis and composition of the brain have been linked to neuropsychiatric illnesses. Although we did not measure the gene expression of FADS and ELOVL, we postulate the sex and age-specific differences are likely due to differential regulation of these enzymes. The gene expression of FADS and

ELOVL is regulated by estradiol (Marks *et al*., 2013), which may explain the sex-specific effects we observed. Whether the gene expression of FADS and ELOVL are age and brain region specific is unknown and requires future study.

4.1.2 Effects of a diet high or low in n-3 PUFA on the phospholipid fatty acid composition of the brainstem

The brainstem is a region of the brain that comprises the pons, medulla oblongata, and the mid-brain. Brainstem-related symptoms, such as gait and balance impairment (Golbe, 2001), as well as depression (Rub *et al.*, 2000) in Alzheimer's patients suggests the brainstem to play a role in the pathogenesis of Alzheimer's disease (Morcinek *et al.*, 2013). Moreover, studies have shown an association between n-3 PUFA supplementation and Alzheimer's disease (Green *et al.* 2006; Luchtman & Song, 2013; Kiso, 2011). However, no study to date has determined the effects of a diet high and low in n-3 PUFA on the alterations in the fatty acid composition of the brainstem. We determined the PL fatty acid composition of the brainstem of male and female offspring fed a diet high or low in n-3 PUFA at weaning and 16 weeks postweaning.

4.1.2.1 Effect of diets high or low in n-3 PUFA on SFA and MUFA composition of the brainstem

Total SFA decreased, while C18:1 increased from weaning to 16 weeks in the brainstem in both male and female offspring. However, unlike the cerebellum, there was no effect of a diet high in n-3 PUFA on MUFA and SFA content of the brainstem at both weaning and 16 weeks in both sexes. This may be due to the specific PL fatty acid composition of each region of the brain (Chavko *et al.*, 1993; Svennerholm, 1968). In the current study, we assessed the fatty acid composition of total extracted PL classes.

However, the PLs that primarily make-up the neuronal membrane are PC, PI, PS, PE and Sph (Svennerholm, 1968). Moreover, the most abundant PL in the brain is PE and PC (Sun & Horrocks, 1967). Interestingly, compared to gray matter, adult white matter PE and PC are sparse in DHA, but are highly enriched with MUFA and SFA (Kishimoto *et al.*, 1969; Svennerholm, 1968). The brainstem has the highest amount of white matter, followed by the cerebellum, thus having less PC and PE and less DHA (Chavko *et al*., 1993; Svennerholm, 1968). Therefore, a plausible explanation for the region-specific differences seen with a perinatal and postweaning diet high n-3 PUFA may be due to the enrichment of neuronal membrane PL DHA in the brainstem, compared to the cerebellum. This is further discussed when assessing the brainstem PL n-6 and n-3 PUFA in **section 4.1.2.2**.

4.1.2.2 Effect of diets high or low in n-3 PUFA on the n-6 and n-3 PUFA composition of the brainstem

Our laboratory has previously established that the accretion of cortical PL total n-6 PUFA decreases with age (Balogun & Cheema, 2014). In the current study, we found that the amount of PL total n-6 PUFA, AA and LA in the brainstem decreased with age in both male and females. Our findings are similar to the observations of Xiao *et al.* (2005), who reported a reduction in LA and AA in the medulla oblongata and midbrain regions of the brainstem after feeding female rats a standard chow diet from 3 days post-birth, 21 days, 10 weeks, and up until 18 months of age. The most notable finding was that a high n-3 PUFA diet decreased PL ADA in both male and female offspring at weaning and 16 weeks, compared to the low n-3 PUFA diet group; however, a high n-3 PUFA diet was only shown to have an effect on LA and AA at 16 weeks in both the male and female

brainstem. A plausible explanation for this may be due to the changes in PL classes during brain development (Svennerholm, 1968). The brainstem consists of mostly white matter, whereas the cerebellum has a gray matter outer core, and an inner region composed of white matter (Chavko *et al.*, 1993). Furthermore, gray matter and white matter are comprised of different PL classes, whose fatty acid compostion changes with development (Svennerholm, 1968). Specifically, as the fetal brain matures, the DHA esterified to the PL classes PS and PE, embedded in the white matter, are reduced in the white matter (Svennerholm, 1968). In our study, we isolated and assessed total PL of the brainstem neuronal membrane, which is comprised of mostly white matter, thus having only limited amounts of PL classes esterified with DHA. Therefore, the effects of a diet high in n-3 PUFA may be diluted when measuring the fatty acid composition of total PLs from the brainstem due to the low amount of specific PL with esterfied DHA.

The PL DHA composition of the brainstem was not affected by a diet high in n-3 PUFA; however, there was an effect of sex and age on brainstem DHA accretion. The amount of DHA did not change from weaning to 16 weeks in the females, while it decreased in the males. In contrast to our results, Xiao *et al.* (2005) reported that the medulla oblongata and midbrain from female rats fed a standard chow diet from weaning to 10 weeks had a reduction in DHA. The differences in the results may be due to the isolation of the particular regions of the brainstem, whereas we isolated the full brainstem. No study to date has assessed the whole or specfic-region of the brainstem in an age and sex-specfic manner, thus, we are the first to show the sex-specfic differences in brainstem DHA. At this time, we speculate these age and sex-specific differences seen on DHA accretion in the brainstem are due to the male and female sex steroids (Giltay *et*

al., 2004). A human study assessing hormone replacement therapy in tran-sexual patients found a reduction in the conversion of ALA to DHA in testosterone treated patients; however, patients treated with estradiol had a higher conversion of ALA to DHA (Giltay *et al*., 2004). This study speculated that the n-3 PUFA conversion differences elicited by the hormones was due to the hormones upregulating the conversion of ALA to DHA (Giltay *et al.*, 2004). Therefore, as the males age into puberty, they have a reduction in the conversion of ALA to DHA, which may explain the decrease in the accretion of DHA in the male brainstem from weaning to 16 weeks.

4.2 Effects of a diet high or low in n-3 PUFA on the mRNA expression of BDNF and TrkB in different regions of the brain

The neurotrophic factor, BDNF, plays a major role in the regulation of synaptic plasticity, neuronal survival, and mediates neuronal cell differentiation (Lewin & Barde, 1996; Huang & Reichardt, 2001). In addition, PUFAs play a critical role in neuronal signalling, particularly in the intracellular pathways tightly regulated by the neurotrophic factor BDNF (Yehunda *et al.*, 2002). Our laboratory has previously reported an increase in the mRNA expression of BDNF in the cerebral cortex of male offspring fed a perinatal and postweaning diet high in n-3 PUFA (Balogun & Cheema, 2014). However, no study to date has investigated the effects of a perinatal and postweaning diet high in n-3 PUFA on the mRNA expression of BDNF, and its high affinity receptor TrkB, in the cerebellum and brainstem. In the current study, we determined the effects of a perinatal and postweaning diet high or low in n-3 PUFA on the mRNA expression of BDNF and TrkB in the brainstem and cerebellum.

4.2.1 Effects of a diet high or low in n-3 PUFA on the mRNA expression of BDNF and TrkB in the cerebellum

Alterations in the expression of BDNF and its high-affinity receptor TrkB are associated with the underlying pathogenesis of several neuropsychiatric diseases (Li *et al*., 2015). Although studies have shown that a diet high in n-3 PUFA increases the mRNA expression of BDNF in the cerebral cortex (Balogun & Cheema, 2014) and hippocampus (Rathod *et al.*, 2014), it was interesting to note that a diet high in n-3 PUFA had no effect on the mRNA expression of BDNF and TrkB in the cerebellum. As a diet high in n-3 PUFA led to an increase in PL DHA in both the cerebral cortex (Balogun & Cheema, 2014) and cerebellum, we postulated that this increase in PL DHA would also increase the expression of BDNF and TrkB as it did in the cerebral cortex. The cerebellum, compared to the cerebral cortex, contains less gray matter and more white matter, which is deficient in PE and PS containing DHA (Chavko *et al.*, 1993; Svennerholm, 1968). However, the neuronal membrane of the cerebral cortex is highly enriched with PE and PC containing DHA (Chavko *et al.*, 1993; Svennerholm, 1968). Therefore, the effects of a diet high in n-3 PUFA diet on the expression of BDNF and TrkB in the cerebellum may be reduced due to specific fatty acid composition of the PL in the neuronal membrane.

4.2.1.1. Effects of age on the mRNA expression of BDNF and TrkB in the cerebellum

The mRNA expression of BDNF in the brain decreases with age; however, each region of the brain has a specific age-dependent BDNF mRNA expression (Das *et al.*, 2001). In our study, we found an increase in cerebellar BDNF mRNA expression from weaning to 16 weeks in both male and female offspring. Diet had no effect on cerebellar

TrkB and BDNF mRNA expression, which was contrary to our previous findings where cortical BDNF mRNA expression increased in male offspring fed a diet high in n-3 PUFA (Balogun & Cheema, 2014). The cerebral cortex is highly enriched with DHA containing PS and PE, compared to the cerebellum (Chavko *et al.*, 1993; Svennerholm, 1968); thus we suspect the observed region-specific differences in the BDNF and TrkB mRNA expression are likely due to the specific PL composition of the cerebral cortex, compared to the cerebellum. Therefore, future studies should isolate the specific PL classes of each region and quantify the specific fatty acid moieties esterified to specific PL species.

Interestingly, Das *et al.* (2001) has previously reported a decrease in BDNF mRNA expression in the cerebral cortex with age, while there was an increase in BDNF mRNA expression with age in the cerebellum, which is consistent with our findings. The age and region-specific regulation of BDNF mRNA expression was suggested to be due to different time points of development for each specific region (Das *et al.*, 2001; Altman, 1972). For example, in the cerebellum there is an extensive amount of neurogenesis and neuronal differentiation during the first three weeks postnatal, compared to other regions of the brain (Altman, 1966). Furthermore, this increase in neuronal growth and development has been shown to coincide with an increase in the mRNA expression of BDNF (Maisonpierre *et al.*, 1990). Our findings have thus confirmed that the regulation of BDNF gene expression is age and region specific.

Although we found an increase in the mRNA expression of BDNF with age, there was a decrease in the mRNA expression of TrkB from weaning to 16 weeks, in both males and females. Others have also shown a decrease in the mRNA expression of TrkB

from postnatal day zero and adult brains (Masana *et al.*, 1993). The reasoning for this downregulation of TrkB mRNA expression may be due to the cellular and structural morphology of the cerebellum (Segal *et al.*, 1991). Studies assessing the developing cerebellum have found TrkB to be highly expressed (Klein *et al.*, 1989; Middlemas *et al.*, 1991); however, the inner granular cell layer of the mature cerebellum was previously shown to express low levels of TrkB (Lamballe *et al.*, 1991). Therefore, it is possible that immature granule cells express TrkB, but as the granular cells mature, the TrkB receptor expression decreases (Segal *et al.*, 1991).

4.2.1.2. Sex-specific effects on the mRNA expression of BDNF and TrkB in the cerebellum

Sex differences have been shown to affect the expression of BDNF and TrkB in the forebrain and hippocampal regions of mice (Hill *et al*., 2012); however, it is not known if BDNF and TrkB mRNA expression has sex-specific regulation in the cerebellum. There was no difference in BDNF mRNA expression between males and females. However, there was an effect of sex on the mRNA expression of TrkB, where males had a higher expression than the females at both weaning and 16 weeks. Hill *et al.* (2012) have also reported similar results in the cortex, where the protein expression of BDNF increased, while TrkB decreased with age in males, but there was no change in the females. The sex-specific effects were due to the circulating androgens in the males. Patients diagnosed with bipolar disorder have a reduction in the cerebellar protein expression of TrkB, compared to healthy patients (Soontornniyomkij *et al*., 2011). Interestingly, females have a higher incidence of mood disorders, such as bipolar disorder, compared to males (Nolen-Hoeksema, 1987). Thus, we postulate that a lower

gene expression of TrkB in the female cerebellum may be associated with a higher incidence of bipolar disorder.

4.2.2 Effects of a diet high or low in n-3 PUFA on mRNA expression of BDNF and TrkB in the brainstem

The brainstem is an area of the brain that houses many sensory and motor tracts. Recent studies have implied the importance of brainstem in Alzheimer's disease (Morcinek *et al.*, 2013; Golbe, 2001; Rub *et al.*, 2000). The expression of BDNF is suggested to be high in the brainstem (Tang *et al.*, 2010); however, no studies to date have assessed the effects of a diet high in n-3 PUFA on the mRNA expressed of BDNF and TrkB in the brainstem in a sex and age-specific manner. In this study, we assessed the effects of perinatal and postweaning diets high or low in n-3 PUFA on the mRNA expression of BDNF and TrkB in the brainstem from male and female offspring. There was no effect of diet on the mRNA expression of BDNF and TrkB in both male and female offspring at weaning and 16 weeks postweaning. This was expected, as a diet high in n-3 PUFA had no effect on the accretion of DHA in the male and female brainstem.

There was an effect of sex and age on the mRNA expression of BDNF at weaning, where the BDNF mRNA expression was higher in females than males. The effects of sex and age were not observed in the brainstem TrkB mRNA expression. We are the first to examine the developmental differences of the TrkB receptor in the brainstem; however, one human study found that the various regions of the brainstem from 2-9 month old infants had a higher protein expression of TrkB than 46-60 year old males (Tang *et al.*, 2010). Although different from our results, Tang *et al.* (2010) used aged adults, which

does not correspond to the young adult mice used in our study. We are unable to determine the underlying mechanism for the sex and age-specific effects; however, we postulate that it may be due to the sex hormone estradiol during development. During brain development, estradiol has been shown to be important in neuronal growth and development, which may be due to the upregulation of BDNF via estrogen (Toran-Allerand, 1976). Interestingly, this effect was only found in the developing brain and was lost in the adult brain, which may explain for observing a higher mRNA expression of BDNF in the females at weaning, compared to 16 weeks (Toran-Allerand, 1976).

4.3 Effects of a diet high or low in n-3 PUFA on the mRNA expression of SCD1 and MBP in different regions of the brain

The fatty acid profile of the cerebellum obtained from the male and female offspring fed a diet high in n-3 PUFA revealed a significant interaction between age and diet for C18:1. Furthermore, a diet high in n-3 PUFA has been shown to upregulate the expression of SCD1 in the hypothalamus (Cintra *et al*., 2012), which strengthens our findings with the increased production of cerebellar PL C18:1 after longer exposure to a diet high in n-3 PUFA. This finding was quite interesting, as the brain is fully capable of synthesizing SFA and MUFA *de novo* via the rate limiting enzyme SCD1 (DeWille & Farmer, 1992). One particular study suggests that SCD1 plays an important role in maintaining SFA and MUFA content of the brain (McNamara & Liu, 2011). These authors showed a decrease in the expression of brain SCD1 in patients with neuropsychiatric disorders, compared to healthy control (McNamara & Liu, 2011). A probable explanation for this finding could be the reduction in the conversion of SFA to MUFA via SCD1, which is vitally important for the formation of the myelin sheath

(Marbois *et al.*, 1992). Our finding from the cerebellum PL C18:1 provides a possible relationship between a diet high in n-3 PUFA and the regulation of genes involved in myelin formation. Thus, in the current study, we wanted to determine the effects of a perinatal and postweaning diet high in n-3 PUFA effects on the mRNA expression of SCD1 and MBP, an essential structural protein for myelin formation, of the cerebellum and brainstem from male and female offspring.

4.3.1 Effects of a diet high or low in n-3 PUFA on the mRNA expression of SCD1 and MBP in cerebellum

We are the first to report an effect of a diet high in n-3 PUFA on the mRNA expression of SCD1 in the cerebellum of both male and female mice. A diet high in n-3 PUFA increased the mRNA expression of SCD1 in both male and females at weaning, compared to mice fed a low n-3 PUFA diet. The gene expression of SCD1 at weaning coincides with the need to desaturate SFA into MUFA during myelin sheath development (Marbois *et al.*, 1992). The increase in SCD1 mRNA expression on a high n-3 PUFA, particularly during weaning, may aid in the formation of the developing myelin sheath. It was interesting to note that only females at 16 weeks showed higher expression of SCD1 gene expression compared to females fed the low n-3 PUFA diet. This could have been due to the higher amount of total SFA in the cerebellum PL, thus requiring a higher expression of SCD1 to convert the higher amount of PL total SFA into MUFA. Another plausible explanation for this increase in SCD1 in female offspring fed a diet high in n-3 PUFA 16 weeks postweaning is an increase in the female sex hormones, which may have a synergistic effect on the expression of SCD1 in the high n-3 PUFA diet group. No study to date has assessed the effect of female sex hormones in the brain and the

regulation of SCD1; however, one *in vivo* study assessing rat hepatic SCD1 found that estradiol and progesterone upregulate SCD1 protein expression (Marks *et al.*, 2013). Furthermore, Cintra *et al.* (2012) also reported an increase in the protein expression of SCD1 in the hypothalamus of male rats fed a diet high in n-3 PUFA, thus providing evidence that both a diet high in n-3 PUFA and female sex hormones can alter SCD1 protein expression. During the process of myelination, the brain rapidly converts SFA into MUFA to incorporate MUFA in the developing myelin (Marbois *et al.*, 1992). In addition, myelination also requires structural proteins, such as MBP, which has been shown to be influenced by the intake of dietary fats (DeWille & Farmer, 1992). MBP is the only structural protein found thus far to be essential in the formation of CNS myelin, and has been shown to coincide with myelin formation (Muse *et al.*, 2001; Shiota *et al.*, 1989; Boggs, 2006). Therefore, determining whether a diet high in n-3 PUFA alters the gene expression of MBP would be important.

There was a significant effect of diet, sex, and age on the mRNA expression of MBP in the cerebellum. A diet high in n-3 PUFA increased the mRNA expression of MBP in males at weaning, compared to the animals fed a low n-3 PUFA diet. On the other hand, a diet high in n-3 PUFA increased the mRNA expression of MBP in the females at 16 weeks. It has previously been shown that the protein expression of MBP is upregulated in the cerebral cortex from offspring of mice fed a diet high in n-3 PUFA (Tian *et al.*, 2011). We are unable to fully explain our observations of age, sex, and diet on the MBP and SCD1 mRNA expression; however, we postulate that it may be due to the differences with male and female hormones during these two periods of development (Hill *et al.*, 2012).

Next, we investigated whether a longer exposure to a diet high in n-3 PUFA will increase the mRNA expression of MBP in the cerebellum. A diet high in n-3 PUFA increased the mRNA expression of MBP, which was consistent with an increase in the mRNA expression of SCD1 for males at weaning, and for females at 16 weeks. This is an interesting observation related to sex- and age-dependent regulation of MBP and SCD1, which we believe could be due to the varying levels of sex hormones during development and needs further investigation.

4.3.2 Effects of a diet high or low in n-3 PUFA on mRNA expression of SCD1 and MBP in brainstem

We did not find a significant effect of a diet high in n-3 PUFA on the fatty acid levels of SFA and MUFA in the brainstem of both female and male offspring. A previous study has reported that patients who consume a diet high in n-3 PUFA prior to a traumatic brain injury have a faster recovery after an injury to the brainstem, compared to controls (Bailes & Mills, 2010). The brainstem is a highly myelinated area of the brain (Chavok *et al.*, 1993; Svennerholm, 1968; Marbois *et al.*, 1992), thus we investigated whether a diet high in n-3 PUFA will have an effect on the mRNA expression of SCD1 or MBP in the brainstem.

There was no effect of a diet high in n-3 PUFA on the mRNA expression of SCD1 in the brainstem. However, males showed an increase in the mRNA expression of SCD1 from weaning to 16 weeks. In addition, SCD1 mRNA expression was higher in males compared to the females at 16 weeks. The sex-specific effects on SCD1 mRNA expression are likely due to the male androgens during development as reported previously by Hill *et al. (*2012). Interestingly, unlike the cerebellum, a diet high in n-3

PUFA had no effect on the accretion of DHA in the brainstem, and SCD1 mRNA expression. The brainstem contains a sparse amount of PE enriched with DHA compared to the cerebellum, thus, the region-specific effects of a diet high in n-3 PUFA on the SCD1 mRNA expression is likely due to specific fatty acid composition of PL classes in the cerebellum and brainstem (Chavok *et al.*, 1993; Svennerholm, 1968). There is no study to date to show whether DHA regulates the mRNA expression of SCD1 in different regions of the brain, which is discussed further in **Section 4.5.**

The MBP mRNA expression was affected only by age in our study, where the expression decreased from weaning to 16 weeks in both the males and females. Unlike the cerebellum, the brainstem is the first region of the brain to develop, thus myelination of the rodent brainstem occurs and ends earlier than other regions of the brain, specifically the cerebellum (Smith, 1973). This would explain a decrease in MBP gene expression from weaning to 16 weeks postweaning, compared to the cerebellum.

Diet did not have an effect on the brainstem MBP mRNA expression, which is most likely due to no change in DHA accretion in the brainstem PL because of the sparse amount of DHA enriched in the PL of the brainstem white matter (Chavok *et al.*, 1993; Svennerholm, 1968). The lack of an effect of a diet high in n-3 PUFA on MBP mRNA expression corresponds to no change in the SCD1 mRNA expression in the brainstem.

4.4 Effects of a diet high or low in n-3 PUFA on the adult female cortical phospholipid fatty acid composition

During the development of the brain, there is rapid incorporation of n-3 PUFA into neuronal membrane PLs, where they play important roles in neurodevelopment (Clandinin *et al.*, 1980, Hoffman *et al.*, 1993). However, this rapid accumulation of n-3

PUFA has been shown to taper off in the adult brain (Moriguchi & Harauma, 2013). In addition, it is thought that the adult nervous system is quite resistant to DHA losses due to dietary challenges (Bourre *et al*., 1993). Conversely, studies assessing adult non-human primates have shown that a diet adequate in LA, but very low in ALA, rendered a loss in brain DHA (Pawlosky *et al.*, 2001). Therefore, the effects of a diet high in n-3 PUFA on adult-brain PL fatty acid composition remain controversial. We investigated the effects of feeding a diet low or high in n-3 PUFA for one or two-months on the accretion of DHA in the cerebral cortex of adult female mice. A longer exposure to a diet high in n-3 PUFA increased the accretion of DHA in adult cerebral cortex, compared to that of a low n-3 PUFA diet group. Adult rodent brains are capable of replacing 2-8% of the neuronal membrane DHA daily from the unesterfied DHA plasma pool (Rapoport *et al.*, 2001) supporting our observation that a diet high in n-3 PUFA increases the accretion of DHA in the PL fraction of brain cortex when exposed for a longer time period.

An increase in brain PL DHA is followed by a concomitant decrease in brain PL AA (Wainwright *et al.*, 1991). Our findings show an independent effect of diet and age on cortical PL total n-6 PUFA. A diet high in n-3 PUFA caused a decrease in cortical total n-6 PUFA AA compared to the low n-3 PUFA diet. Marteinsdottir *et al.* (1998) found similar results using male rats fed diets high in n-3 PUFA for two-months; however, these authors did not assess the effects of different time periods. Thus, our findings have established that a longer exposure to a diet high in n-3 PUFA increases the accretion of DHA in the brain.

4.5 Effects of DHA on the fatty acid accretion and mRNA expression of BDNF, TrkB, MBP and SCD1 in primary cortical cells in culture

In a previous study in our laboratory, we found that a perinatal and postweaning diet high in n-3 PUFA increased the accretion of DHA in cerebral cortex PLs, compared to the low n-3 PUFA diet group (Balogun & Cheema, 2014). This increase in cerebral cortex PL DHA was positively correlated with the mRNA expression of BDNF and TrkB. Thus, we speculate that the positive effects elicited by a diet high in n-3 PUFA are due to an increase in the accretion of DHA in the neuronal membrane, which will lead to an increase in the mRNA expression of BDNF/TrkB and genes involved in myelin formation. The cerebral cortex PLs are also enriched in AA; both AA and DHA are required for proper development and function of the brain (Martinez *et al.*, 1992; Stillwell *et al*., 2005). However, AA and DHA have been shown to have different effects on the regulation of various signalling pathways in cortical neurons (Katakura *et al*., 2013). Since our diets contained both DHA and AA, we cannot isolate the effects of DHA and AA. Therefore, we adapted a primary cortical cell culture model to investigate the individual effects of these fatty acids on the mRNA expression of BDNF, TrkB, SCD1 and MBP. The use of primary cortical cells is a valuable technique, which more accurately represents the in vivo environment compared to that of established cell lines (Eide & McMurray, 2005).

The primary cortical neurons treated with DHA (50, 75 and 100 μM DHA) showed an increase in the accretion of DHA, compared to vehicle control. Similar findings have been reported by Cao *et al.* (2005), who showed an increase in DHA accretion in PS and PE after treating rat cortical neurons with 25 μM DHA for 24 hours.

Our findings to show an increase in DHA accretion after treatment with DHA support our *in vivo* observations where C57BL/6N mice fed with a diet high in n-3 PUFA showed accretion of DHA in brain cortex (Balogun & Cheema, 2014). Our *in vitro* studies have also established that the accretion of DHA is dose dependent.

The treatment of cortical cells with DHA increased the mRNA expression of BDNF and TrkB, whereas AA had no effect on the mRNA expression of BDNF or TrkB compared to vehicle control, suggesting that the effect is specific to DHA. Rao *et al.* (2007) has previously shown that DHA treatment (1.5 μM) of rat primary cortical astrocytes increased the mRNA expression of BDNF similar to our observations with DHA. These authors have further reported that the downstream inflammatory metabolites of AA reduce frontal cortex BDNF protein and mRNA expression (Rao *et al.*, 2013). However, we did not observe an inhibition of BDNF or TrkB mRNA expression when cortical neuronal cells were treated with AA. It is possible that the downstream metabolites of AA induce a more potent effect compared to AA. Kou et al. (2008) has previously shown an increase in TrkB mRNA expression upon treatment of SH-SY5Y neuroblastoma cell line with EPA, an n-3 PUFA. Bhatia *et al.* (2011) reported activation of the TrkB receptor after feeding a diet high in DHA to rats for 15 weeks, while AA had no effect, supporting our observation that the regulation of BDNF and TrkB pathway is specific towards DHA. Future studies should focus on investigating the mechanism/s by which DHA regulates the gene expression of BDNF and TrkB.

Our *in vivo* data showed that a diet high in n-3 PUFA increased the accretion of DHA in the cerebellum, and also increased the mRNA expression of SCD1. Thus, we

asked the question whether treatment of primary neuronal cortical cells with n-3 PUFA would have an effect on the mRNA expression of SCD1. Interestingly, DHA treatment increased the mRNA expression of SCD1 in primary cortical cells, whereas AA had no effect, compared to vehicle control. A previous study has reported that treatment of cortical neurons with 100ng/mL BDNF increased the protein expression of SCD1 (Suzuki *et al.*, 2012). Our findings have shown that DHA treatment of primary cortical cells increased the mRNA expression of both BDNF and SCD1, thus it is possible that an increase in BDNF mRNA expression caused an increase in SCD1 mRNA expression, or vice versa.

An increase in SCD1 gene expression is required for myelin sheath formation, thus we also investigated whether DHA treatment will have an effect on the mRNA expression of MBP. We observed that DHA treatment increased the mRNA expression of MBP in primary cortical cells, while AA had no effect, compared to vehicle control. It has previously been shown that the protein expression of MBP is upregulated in the cerebral cortex from offspring of mice fed a diet high in n-3 PUFA (Tian *et al.*, 2011) similar to our *in vivo* studies. Another study using oligodendrocytes showed that AA treatment reduced the phosphorylation of MBP (Takeda & Soliven, 1997). The phosphorylation of MBP is a post-translational modification necessary to promote proper structural and functional properties of the myelin membranes (Moscarello, 1990). Therefore, it would be interesting to compare the phosphorylation of MBP after DHA or AA treatment in primary cortical cells in the future studies.

4.6 Limitations

The findings of this thesis show the importance of a diet high in n-3 PUFA on the expression of BDNF, TrkB and genes related to myelin formation in the brain, and how the effects of the diet are age- and sex-specific; however the underlying cellular mechanisms were not explored and should be undertaken in the future. Furthermore, we have only measured the gene expression of BDNF, TrkB, SCD1 and MBP; all of these genes are also regulated at post-transcriptional and translational level (Moscarello, 1990; Kaplan & Miller, 2000; Bhatia *et al.*, 2011), which will be important to investigate in the future. Lastly, it is well known that ageing has a dramatic effect on the function of the brain, thus it will be important to investigate whether a diet high in n-3 PUFA will have beneficial effects in older animals.

4.7 Overall Conclusion

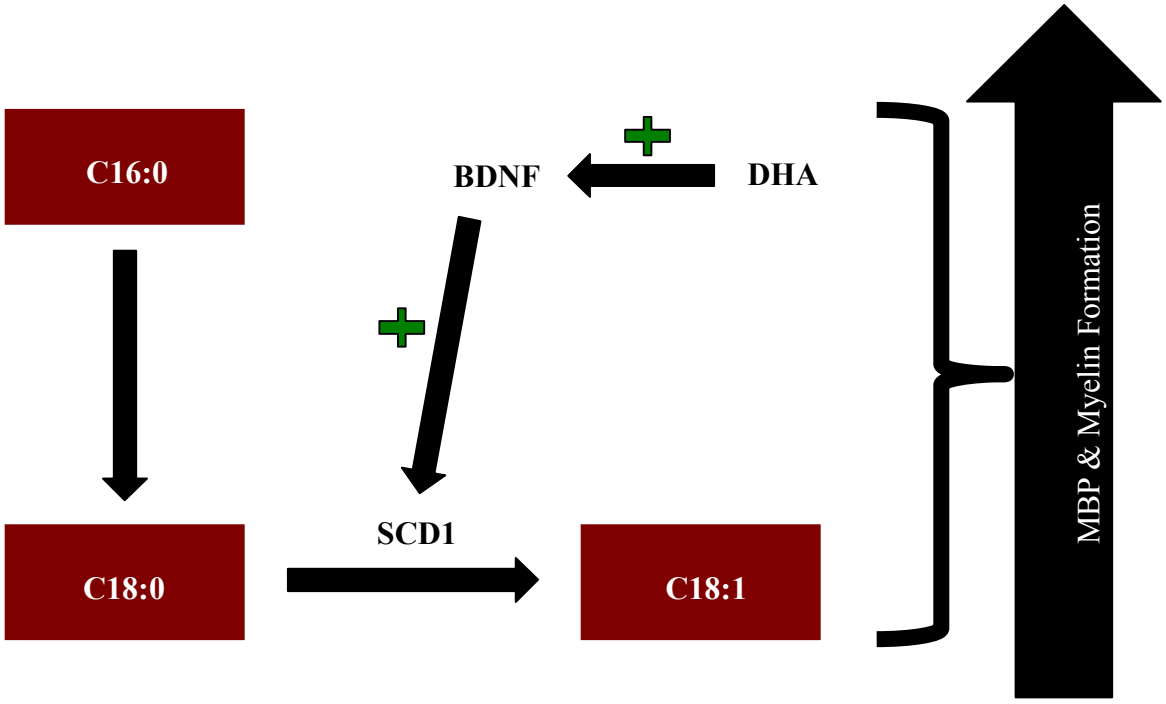
Overall, our findings reveal that the effects of a perinatal and postweaning diet high in n-3 PUFA on the brain are age, sex, and region-specific. Our *in vivo* work, assessing the effects of a diet high in n-3 PUFA showed age and sex-specific changes in different regions of the brain. Our data have established that the cerebellar accretion of n-3 PUFA and the mRNA expression of genes related to myelin sheath formation were increased by a diet high in n-3 PUFA; however, these effects were not observed in the brainstem. In addition, our findings have also established sex and age related effects on the fatty acid composition, BDNF, TrkB, SCD1 and MBP mRNA expression in specific regions of the brain. Our findings have further confirmed that the brain is plastic as the fatty acid composition of the adult cerebral cortex can be altered, where a diet high in n-3 PUFA can increase the accretion of DHA. The *in vitro* studies have established that the

effects observed in the *in vivo* studies are due to accretion of DHA in the brain, which regulates the mRNA expression of BDNF, TrkB, SCD1 and MBP. Lastly, our findings show the complexity of the brain, and that factors such as diet, age, and sex need to be considered in neurological disorders.

4.8 Perspectives

We propose that the DHA-mediated increase in the gene expression of BDNF will lead to an increase in the SCD1 mRNA expression. This will then increase the synthesis of C18:1 rendering an increase in the formation of MBP for myelin sheath formation (Figure 4.1). Thus, future research should focus on elucidating the postulated mechanism.

Figure 4.1: The postulated mechanism by which docosahexaenoic acid (DHA) upregulates myelin formation via brain-derived neurotrophic factor (BDNF). *SCD1= stearoyl-CoA desaturase-1; MBP= myelin basic protein.*


C16:0
BDNF
DHA
SCD1
C18:0
C18:1
MBP & Myelin Formation

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Appendix I

Fatty acid composition of the experimental diets

Fatty Acids	Low n-3	Medium n-3	High n-3
14:0	0.11	0.39	1.26
16:0	6.32	7.43	8.71
18:0	5.35	4.53	2.67
∑ **SFA**	11.77	12.35	12.64
16:1n-7	0.36	0.09	2.41
18:1	27.82	25.81	25.14
20:1n-9	ND	0.54	0.61
∑ **MUFA**	28.18	26.43	28.16
18:2n-6	57.73	57.03	47.86
20:4n-6	0.11	0.14	0.23
18:3n-6	0.04	0.04	0.10
22:4n-6	0.09	ND	0.54
∑ **Omega-6**	57.92	57.18	48.90
18:3n-3	0.55	0.64	0.78
20:5n-3	0.31	1.37	3.64
22:6n-3	0.39	1.16	3.19
18:4n-3	0.15	0.20	0.87
22:5n-3	0.46	0.32	0.63
20:4n-3	0.08	0.12	0.66
∑**Omega-3**	1.93	3.81	9.76

Data are expressed as weight percentage of the total extracted fatty acids. Σ SFA= sum of saturated fatty acids, Σ MUFA= sum of monounsaturated fatty acids, Σ PUFA= sum of polyunsaturated fatty acids, Σ Omega-6= sum of omega-6 fatty acids, Σ Omega-3= sum of omega-3 fatty acids, ND= Not detected.

Appendix II

Composition of the semi-purified diet designed for 20% (w/w) fat level

Ingredients	Semi-Synthetic diet (g/kg)
Casein	200
DL-methionine	3
Sucrose	305
Corn starch	190
Alphacel non-nutritive bulk	50
Vitamin mix$^{\Psi}$	11
Mineral mix*	40
Fat	200

Supplied in quantities adequate to meet NRC requirements (National Research Council, 1995).

$^{\Psi}$Vitamin Mix *(1 kg): Thiamine hydrochloride, 0.6 g; riboflavin, 0.6 g; pyridoxine hydrochloride, 0.7 g; nicotinic acid, 3.0 g; d-calcium pantothenate, 1.6 g; folic acid, 0.2 g; d-biotin, 0.02 g; cyanocobalamin (vitamin B_{12}), 0.001 g; retinyl palmitate (vitamin A) pre-mix (250,000 IU/g), 1.6 g; DL-α-tocopherol acetate (250 IU/g), 20 g; cholecalciferol (vitamin D_3, 400,000 IU/g), 0.25 g; menaquinone (vitamin K_2), 0.005 g; sucrose, finely powdered, 972.9 g*

***Mineral Mix:** Calcium phosphate dibasic, 500.0 g/kg; sodium chloride, 74.0 g/kg; potassium citrate monohydrate, 220.0 g/kg; potassium sulfate, 52.0 g/kg; magnesium oxide, 24.0 g/kg; manganese carbonate (43-48% Mn), 3.50 g/kg; ferric citrate (16-17% Fe), 6.0 g/kg; zinc carbonate (70% ZnO), 1.6 g/kg; cupric carbonate (53-55% Cu), 0.30 g/kg; potassium iodate, 0.01 g/kg; sodium selenite, 0.01 g/kg; chromium potassium sulfate, 0.55 g/kg; sucrose, finely powdered, 118.0 g/kg*