THE IMPACT OF MEMBRANE POLYUNSATURATED FATTY ACID COMPOSITION ON NEURONAL GROWTH AND DEVELOPMENT

by

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For my loving, supportive parents

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LIST OF ABBREVIATIONS

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	MTT
Acceptable Macronutrient Distribution Range	AMDR
Acyl-CoA Synthetase	ASCL
Adequate Intake	AI
α-Linolenic Acid	ALA
Arachidonic Acid	ARA
Blood Brain Barrier	BBB
Bovine Serum Albumin	BSA
Calcium-Dependent cytosolic PLA_2	cPLA2
Calcium-Independent PLA_2	iPLA2
Cardiovascular Disease	CVD
Central Nervous System	CNS
Dihomo-γ-linolenic acid	DGLA
Docosapentaenoic acid	DPA
Eicosapentaenoic Acid	EPA
Essential Fatty Acid	EFA
Dietary Guidelines for Americans	DGA
Docosahexaenoic Acid	DHA

Fatty Acid Binding Protein	FABP
Fatty Acid Transport Protein	FATP
Gas Chromatography	GC
Ischemic Heart Disease	IHD
Lipoprotein Lipase	LPL
Linoleic Acid	LA
Long Chain Polyunsaturated Fatty Acid	PUFA
Lysophospholipid acyltransferase	LPAT
Lysophosphatidylcholine	LPC
Monounsaturated Fatty Acid	MUFA
Non-Esterified Fatty Acid	NEFA
Phospholipase A2	PLA2
Plasma membrane fatty acid-binding protein	pFABPpm
Polyunsaturated Fatty Acid	PUFA
Randomized Control Trail	RCT
Saturated Fatty Acid	SFA
Vesicular Monoamine Transporter	VMAT

ABSTRACT

PUFAs serve many important biological and physiological functions within the body and are key for the structure and function of the brain. Omega-6 and omega-3 PUFAs are found in abundance in phospholipids of neuronal membranes that impart structure and function of neurons. Omega-6 PUFAs are instrumental for neurotransmission, neuronal elongation, and neuritogenesis; whereas, omega-3 PUFAs promote neuronal maturation through synaptogenesis. The types of PUFAs incorporated into neuronal membranes is especially important in determining the progression of development. The processes of neurogenesis, neuritogenesis and elongation require large amounts of PUFAs to be incorporated into the membrane phospholipids. To accommodate for the high PUFA needs, maternal dietary PUFA, especially EPA and DHA, recommendations, mobilization of fatty acids into maternal circulation increases, and the accretion rate of PUFA are increased. If maternal nutritional inadequacy of PUFAs occurs during gestation, this can result in impaired cognition, behavioral abnormalities, reduced number of neurons, decreased dendric arborization, altered myelin sheath, and a reduction in brain size.

Even though the essentiality of PUFAs in neuronal development is widely accepted, the mechanism is not well understood. There is a lack of consensus in the current literature on the effects of individual PUFAs on each stage of neuronal development and the molecular pathways involved. Despite the inconsistent evidence, the results of numerous studies have consistently suggested that neuronal membrane PUFA composition is associated with neuronal development outcomes, such as number of neurons and neurites, neurite length, and neurotransmitter release. The varying results may be the result of methodological discrepancies with PUFA composition and concentrations, as well as the models used for neuronal development. Additionally, very few studies have taken into consideration the competitive relationship of omega-6 and omega-3 PUFAs in the body when assessing neurodevelopment.

This thesis was focused on addressing the role of PUFAs in neuronal development and to address some of the inconsistencies in the literature. attempt to elucidate the individual roles of ALA, ARA, and EPA on neuronal membrane composition and neuronal development. The aim of the thesis research project was to assess the impact of individual PUFAs on neuronal membrane PUFA composition, the membrane n-6:n-3 ratio, and the morphology of SH-SY5Y cells during differentiation. The results of this study demonstrated that supplementation of individual PUFAs

alters membrane PUFA composition and the n-6:n-3 ratio. However, there wasn't a significant effect on neurite number with ALA, ARA, and EPA treatment. Lastly, ARA treatment decreased cell viability compared to the other treatments and the BSA control. Furthermore, additional research needs to be conducted to address other morphological measures and functional outcomes, such as neurotransmitter production and release.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

1.1.a. Polyunsaturated fatty acids are important for our overall health and well-being

Over the last several decades, there has been increasing emphasis on incorporating more polyunsaturated fatty acids (PUFAs) and reducing saturated fats in the diet to better our health. In the American diet, saturated fat is the predominant source of dietary fat, which is attributed to an increased risk for chronic diseases such as coronary heart disease and obesity [1]. Comparatively, PUFAs have been regarded to impart health benefits with increased intake. Inadequate consumption of PUFAs has been implicated in the development of many chronic diseases including Alzheimer's disease and other neurological disorders, cardiovascular disease, rheumatoid arthritis[2], immunologic and inflammatory diseases, diabetes, and obesity[3]. In a controlled feeding study conducted in the 1950s, assessing blood cholesterol was the first demonstration that replacing saturated fat for PUFAs has a positive impact on health[1]. Since the 1970s, PUFAs have been recommended to replace dietary saturated fats, the major fat in the American diet, to improve health and address the growing concern for coronary heart disease[1]. As a result, the consumption of LA in the American diet increased from 3-4% to 6-7% of calories by the 1980s[1]. However, in the 1990s, the LA recommendation was contested by the implication that increased LA intake causes more negative health consequences than beneficial[1]. Higher levels of omega-6 PUFAs, specifically Linoleic Acid (LA), in bodily tissue are thought to contribute to the development of atherosclerosis, increase risk for cardiovascular disease[1], and insulin resistance[4]. However, there are inconsistent results on the contribution of LA to cardiovascular-related outcomes[1].

Around the same time that increased LA intake was recommended for health benefits, there was developing interest in omega-3 PUFAs and their impact on health. The observational studies conducted in the 1970s with the Greenland Inuit population were some of first studies to spark interest in omega-3s and demonstrate an association between omega-3 PUFAs and improved health[5]. The Inuit diet is a prime example of a dietary pattern high in omega-3 PUFAs. In this population, there is a low incidence of cardiovascular-renal diseases, improved visual acuity, and improved neurodevelopment in children[6–11]. Since the 1990s, the focus has shifted from

increasing total PUFAs to omega-3 PUFAs in the diet. Many studies have demonstrated the detrimental effects of low omega-3 PUFA consumption on health, including increased risk for metabolic syndrome, coronary heart disease, and type 2 diabetes[4]. With the potential adverse health effects from high omega-6 and low omega-3 consumption, omega-3 fatty acids have become the primary PUFA of concern in dietary recommendations due to the attribution of more extensive health benefits.

With the attention on the beneficial effects of omega-3 PUFAs, the perception of omega-6s has shifted to the negative contributions to chronic diseases and no longer on their usefulness. However, omega-6 and omega-3 PUFAs are both important dietary constituents as they serve a multitude of functions and are found in all tissues of our bodies[12]. PUFAs are required for growth and development, cognition, inflammation and immune response, and regulation of plasma lipids[12–16]. PUFAs are key in various physiological and biological functions in the body including membrane structure and function, neurotransmission, neurogenesis, and signaling molecules[15,17]. Because of their extensive involvement in many essential functions in the body, it is important to consume adequate amounts of both omega-6 and omega-3 PUFAs in the diet.

The Western dietary pattern is associated with many health consequences, such as increased risk of cardiovascular disease (CVD), CVD-related events, type 2 diabetes, and mental disorders[18]. Opposingly, omega-3 fatty acids have long been implicated in optimal health, with some of the most well-known benefits on cardiovascular health, neurocognition, inflammatory diseases such as rheumatoid arthritis, and fetal brain development[2,18,19]. Despite the common attribution of omega-3 PUFAs to health benefits, high omega-3 intake is associated with no reduction of CVD incidence[20], increased risk of Ischemic Heart Disease (IHD), increased risk of atrial fibrillation, impaired immune response[21,22], increased bleeding, higher risk of hemorrhagic stroke, and gastrointestinal distress[18].

1.1.b. Essential fatty acids and PUFA metabolism

PUFAs contain at least 2 double bonds and can be classified into 2 main categories: omega-6s (*n*-6) and omega-3s (*n*-3). The distinction between omega-6 and omega-3 PUFAs is the position of the first double bond in the carbon chain from the methyl end, omega (ω) or *n*. The number in the name denotes the carbon in the chain with the first double bond[15,19]. The first double bond in n-3 PUFAs is found at the 3rd carbon in the chain from methyl carbon[23]. In n-6 PUFAs, the first

double bond is at the 6th carbon position from the methyl carbon[24]. PUFAs can further be distinguished by the carbon chain length and the total number of double bonds in the chain[23,24]. Long chain polyunsaturated fatty acids (LCPUFAs) are synthesized from ALA and LA and have at least 20 carbons[23,24].

PUFAs cannot be intrinsically synthesized as humans lack the necessary enzymes and therefore need to be consumed in the diet. The 18-carbon omega-6 PUFA, LA, and omega-3 PUFA, ALA, are considered essential fatty acids (EFAs) as they must be obtained through the diet. Once consumed, LA and ALA are precursor PUFAs that can be converted into long chain PUFAs (LCPUFAs), which contain at least 20 carbons. This conversion process consists of a series of desaturation and elongation steps that occurs primarily in the liver[15,25] (Figure 1). ALA can be metabolized into its subsequent LCPUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)[23]. LA can be metabolized into its subsequent LCPUFA arachidonic acid (ARA)[24]. Even though humans possess the enzymes, we are not reliant on the metabolism of EFAs to LCPUFAs as preformed LCPUFAs can also be obtained from the diet but are found in much smaller amounts than EFAs[19].

The metabolism of precursor EFAs into their respective LCPUFAs is highly competitive. Once consumed in the diet, the EFAs LA and ALA can be elongated and desaturated to form their subsequent downstream LCPUFAs; however, LA and ALA utilize the same enzymes for LCPUFA synthesis and act as a competitive inhibitor towards each other when there is a surplus of one[26]. Since the efficiency of converting ALA to EPA and DHA in the body is slow and inefficient[19,26], the conversion of n-3 PUFAs is especially dependent on the concentration of LA and total PUFA in the diet[27]. An imbalance in the concentration of each class of PUFA can be disruptive to normal cellular functions[26]. In the Western diet, the increasing intake of LA causes a severe imbalance in the fatty acid composition of cellular membranes, favoring ARA and reducing *n*-3 PUFAs concentrations[15]. With the high concentration of omega-6 PUFAs in the Western diet, there is an increasing concern with the ratio of n-6 to n-3 PUFAs and the associated health consequences.

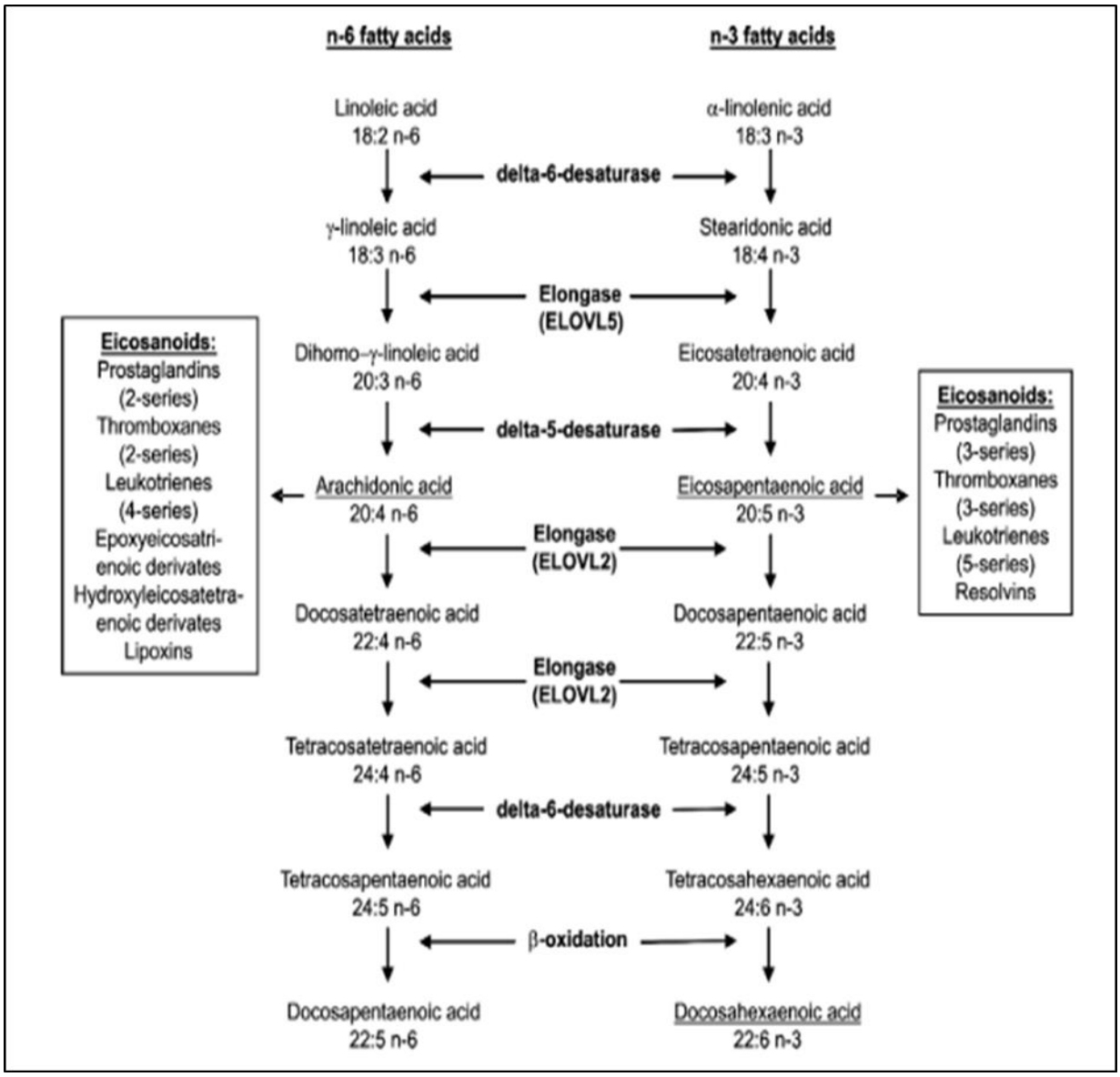

Figure 1. Conversion of n-6 and n-3 PUFAs to LCPUFAs.[26]

1.1.c. Dietary sources and recommended intakes of PUFAs

Both omega-6 and omega-3 PUFAs can be found in plant and animal sources. The most common sources of omega-6 PUFAs are vegetable oils, such as corn and sunflower oil, cereal grains, and saturated animal fats[28]. Omega-3 PUFAs can be found in foods such as oils, nuts, and seafood; following the best sources of alpha-linolenic acid (ALA) are flaxseeds, chia seeds, walnuts, canola oil, fish[15,29–31], and eggs[11]. Preformed long chain omega-3 PUFAs, EPA and

DHA, are found in larger amounts in seafood such as salmon, sardines, herring, mackerel, trout, and oysters[31], as well as in fish and krill oil supplements[2].

The current recommended Acceptable Macronutrient Distribution Range (AMDR) for total omega-6 PUFAs is 2.5 – 9% of calories[32], which is approximately 8g for males and 5.5g for females per day. The AMDR minimum of 2.5% is the Adequate Intake (AI) established for LA to prevent deficiency[6,32]. The recommendation is to not exceed 11% daily calories from total PUFAs, with no more than 9% of total PUFAs from LA to reduce risk of negatively impacting health[32]. The AMDR recommendation for total omega-3 PUFAs in adult males and non-lactating females is 0.5 – 2% of daily calories and 250mg – 2g of EPA+DHA per day[32,33]. The minimum of 0.5% daily calories, or 1.6g for males and 1.1g for females per day[2], is the AI established for ALA to prevent deficiency[32]. To meet the recommended dietary intake of omega-3 PUFAs, especially the 250mg/day of EPA+DHA, the Dietary Guidelines for Americans (DGA) recommends 8 oz. of seafood per week[31,33]. The average American adult meets the AI for ALA but significantly fails to meet the 250mg/day recommendation for EPA+DHA[33]; thus, LA is the prominent PUFA in the recommended total PUFAs AMDR 6 – 11% of daily calories[32].

Omega-3 PUFAs have become the predominant focus in dietary fat and seafood recommendations for chronic disease prevention and improved health outcomes [2,34]. As a result, health organizations such as FAO/WHO and the American Heart Association, as well as the DGA starting with the 2005-2010 edition, recommend at least 2 servings of seafood/fish per week to achieve adequate omega-3 PUFA intake, EPA and DHA specifically[2,4,31,32,34,35]. Even though dietary recommendations from various health organizations consistently suggest increasing dietary intakes for omega-3 PUFAs, the recommended range is large and there is a lack of consensus among health organizations as to the optimal dietary recommendation for omega-3 PUFAs, especially preformed EPA and DHA.

Despite the emphasis on the consumption of omega-3s for health benefits, omega-6 fatty acids remain the most abundant PUFA in the American diet. The increased production and use of vegetable oils, such as corn and soybean oils, and shortening in the 1960s[1] in addition to large amount of high saturated fat foods such as meat in the American/Western diet[4] resulted in an increase in LA intake. The percentage of energy per day derived from LA has consequently increased from 3-4% to 6-7% since the 1980s[1] and has continued this upward trend to the present. The increasing consumption of LA is reflected in the higher concentration of LA in adipose tissue

from 8% to 18%, currently[1,36]. Conversely, omega-3 PUFA consumption has minimally changed over the years[4,15].

1.1.d. The n-6:n-3 ratio is an important consideration for health

The generally perception of PUFAs is that omega-6 PUFAs are "bad" and omega-3 PUFAs are "good" for health. Meeting recommended dietary intake of omega-3 PUFAs is especially important in balancing n-6 to n-3 PUFA intake as n-6 PUFAs are consumed in excess in the American diet. The conflicting nature of the effects of omega-6 and omega-3 PUFAs have on health illustrates the importance of balancing omega-6 and omega-3 PUFA intake to maintain health and prevent chronic health problems. The Western dietary pattern, which is notoriously high in omega-6 PUFAs and low in omega-3, has an exceeding large ratio of omega-6 to omega-3 ranging from 10:1[15] up to 20:1[4,9,11,37–39], with an average of 15 – 16.7:1[11]. The high consumption of omega-6 PUFA in the Western diet has drastically altered the omega-6 to omega-3 ratio from the 1:1 ratio[4,11] in the diet of our ancestors during the Paleolithic period in the evolution of human beings[39]. The large discrepancy with the n-6:n-3 ratio in today's society compared to the paleolithic diet has been attributed to industrialization and grains becoming a prominent component of our diets[9,37,39].

Currently, there is not a consensus on the optimal n-6 to n-3 fatty acid ratio. Intervention studies investigating the n-6:n-3 fatty acid ratio on chronic diseases have demonstrated beneficial effects of ratios ranging from 2:1 to 5:1. These studies demonstrated that a 2.5:1 ratio for colorectal cancer[40,41], a 3:1 ratio for rheumatoid arthritis 3:1[42], and a 5:1 ratio for asthma[43]. It has been proposed that an omega-6 to omega-3 ratio of 4:1 helps to prevent the development of chronic diseases, but the ideal ratio is 1-2:1 to promote optimum health[11,15].

1.2 LCPUFAs are Critical in Brain Development

1.2.a. The importance of omega-3 and omega-6 PUFAs for fetal outcomes

Omega-3 PUFAs are important for every stage of life mainly because of the extensive involvement in essential physiological processes in the body. Women who are pregnant and breastfeeding should be most concerned about achieving adequate intake of omega-3 PUFAs; consequently, these women are instrumental in the health of their child. In the 1980s, the importance of omega-3s in pregnancy was brought to light in an epidemiological study, of a Danish population, which suggested that increasing blood omega-3s compared to omega-6s correlated with improved birth outcomes[44]. Additionally, several studies have assessed fish consumption and LCPUFA supplementation during pregnancy; thus, higher fish consumption by mothers is associated with improved neurodevelopmental outcomes[2,45]. Maternal dietary PUFA intake during pregnancy is implicated in numerous fetal outcomes including gestational weight, premature birth, visual acuity[46], cognitive development, mental disorders[47], metabolic syndrome, and chronic immune disorders[15]. Diets with higher n-3 PUFA consumption during pregnancy is associated with reduced risk of pre-term birth, longer gestation, and higher birth weight [16,48]. Several observational studies and randomized clinical trials (RCTs) propose a relationship between lower DHA levels in blood and increased risk of visual and neurological development complications in infants and children[15]. Despite the numerous benefits, too much omega-3 PUFA consumption can cause problems. High n-3 PUFA intake is associated with increased risk of health issues for both the mother and child because of prolonged gestation and higher birth weight of the infant[48]. The associated health risks include longer duration of labor, excessive bleeding for the mother, increased risk for fetal distress, and perinatal death due to diminished function of the placenta, obesity, diabetes, and metabolic syndrome for the child[48].

The omega-6 LCPUFA, ARA, is also vital in fetal outcomes even though omega-3 LCPUFAs are the predominant focus. ARA is crucial for neurogenesis[49], neurotransmission, visual functions, auditory functions, cognitive development, epilepsy, inflammation, immune response, blood clotting, and initiation of labor[16,48,50]. Additionally, ARA serves an important role in anandamide biosynthesis in the endocannabinoid system in the brain that causes cell proliferation and modulation of neurological functions including emotions[27], motivation and

reward systems, stress, pain, and sleep[51,52]. ARA is speculated to be important for normal growth of the body and brain[15,53], but the results are inconsistent[54].

The intake of n-6 fatty acids needs to be taken into consideration with recommended n-3 intakes during pregnancy. Higher intakes of LA and ARA alter the proportion of n-6 to n-3 fatty acids in tissue in favor of n-6 fatty acids[55]. Excessive concentrations of n-6 PUFAs compared to n-3 PUFAs are disruptive to proper growth and development. Diets with higher n-6 to n-3 ratios are associated with lower birth weight, shorter gestation length[16], and restricted growth[55]. Several animal studies have demonstrated that insufficient omega-3 PUFAs in pregnancy result in impairments in cognition and behavior of offspring[56]. Also, several studies investigating the effects of the composition of DHA and ARA in baby formula on cognitive outcomes during postnatal brain development, illustrate the importance of both classes of LCPUFAs in neurodevelopment. In a study conducted in neonate baboons feed baby formula with approximately equivalent amounts of DHA and ARA, fatty acid composition of brain tissue in several regions had diminished levels of ARA in comparison to the neonates fed formula with a lower amount of DHA. In a study conducted in rats, pregnant rats fed diets with high n-3 LCPUFA and little ARA led to a severely reduced concentration of ARA in brain phospholipids in offspring.[50] Additionally, the DIAMOND (DHA Intake and Measurement of Neural Development) study demonstrates the negative effects of high n-3 LCPUFA, specifically DHA, on cognitive outcomes. Infants given the highest dosage of DHA-supplemented formula, 51mg/100kcal of DHA, from birth to 12 months, had reduced sustained attention at 12 months of age, mental adaptability at 42 months, and verbal IQ at 72 months. LCPUFA composition of red blood cells was determined from samples at 4 month and 12months and yielded that infants fed the highest dosage of DHA had reduced ARA compared to the low and intermediate dosage groups.[57] A study conducted in preterm infants comparing formulas with 2 different doses of ARA with constant DHA showed that preterm infants fed formula with higher levels of ARA had significantly higher ARA concentrations in plasma and higher psychomotor development[50].

1.2.b. Dietary recommendations and usual intakes for pregnancy and lactation

To support fetal requirements for proper growth and development, pregnant women have higher dietary needs for *n*-3 PUFAs. Dietary recommendations for omega-3 LCPUFA consumption during pregnancy vary greatly worldwide. The recommendations range from 115mg

– 1.8g/day of total n-3 PUFA and 200mg – 1000mg/day of DHA [58,59]. In the US, the 2015-2020 DGA provides the recommendation to consume at least 8-12oz of seafood per week for women that are pregnant and lactating[31]. The 2010-2015 DGA is the first edition of the DGA to include the importance of consuming omega-3 fatty acids during pregnancy and lactation for visual and cognitive development[34]. This recommendation ensures the EPA+DHA recommendation of 250 - 300mg/day is met for adequate EPA+DHA needs of the fetus and infant[31,59]. In pregnant and lactating women, the AI for ALA increases to 1.4g and 1.3g, respectively, to meet the needs of fetal and infant development[2]. Additionally, a minimum of 200-300mg/day of DHA is recommended for pregnant and lactating females[59]. The 2015-2020 DGA recommends at least 8 oz. up to 12 oz. of seafood per week for pregnant and lactating women to meet the omega-3 recommended intake[2,31]. Additionally, the American Academy of Pediatrics recommends women who are breastfeeding to consume 200-300mg of DHA, approximately 1-2 servings of fish per week, in order to obtain an adequate amount of DHA for breast milk[2,60].

Even though omega-3 PUFAs are important for proper fetal development, pregnant and lactating women are not meeting the dietary recommendations for seafood and omega-3 PUFAs. In several recent epidemiology studies assessing usual intakes for seafood and EPA+DHA, nearly all women of childbearing age and pregnant women were significantly below seafood and EPA+DHA recommendations[61–64]. Also, there was no difference in intake of n-3 fatty acids in pregnant women vs. non-pregnant women[63,64], even though the AI for total omega-3 PUFAs increases in pregnancy and lactation[2]. The approximate dietary intakes of EPA+DHA from food and food combined with supplements in pregnant women is 78.7 +/- 6.8 mg/day and 97.7 +/- 8 mg/day, respectively[65]. With pregnant women in the US are failing to meet daily requirements of EPA and DHA, there should be more concern for how inadequate omega-3 PUFAs can negatively affect their child. It has been suggested that the detrimental effects on cognition and physiological functions that result from insufficient maternal n-3 PUFA consumption in pregnancy is irreversible after birth even with postnatal supplementation of PUFAs[48]. Nutrition during critical developmental periods can irreversibly affect fetal physiology resulting in increased risk of developing chronic diseases as an adult[66]. Maternal consumptions and transfer of LCPUFAs impacts fetal growth and development as well as disease susceptibility later in life[67].

1.2.c. Omega-3 PUFA intake higher than the AI does not impart additional benefits

Several systematic reviews and meta-analyses assessed the impact of maternal omega-3 LC-PUFA supplementation during pregnancy on neurodevelopment. Many RCTs demonstrated that a high maternal n-3 status does not improve neurodevelopmental outcomes, such as visual acuity and cognition, in infants[68–71]. Conversely, a more recent prospective Norwegian cohort study conducted by Braarud et al. (2018) demonstrated a positive association of maternal DHA status with infant problem-solving abilities at 12 months of age[72]. It has also been shown that mothers taking n-3 PUFA supplements during pregnancy and lactation is associated with increased IQ values in offspring at 4 years of age[73]. Other pregnancy outcomes, such as a short gestation and low birth weight babies, are strongly associated with low maternal LC-PUFA status during pregnancy[74]. In RCTs with breastfed preterm infants, when the lactating mothers were supplemented with n-3 PUFAs, the preterm infants had improved performance on global neurodevelopment tests[75]. Despite the inconsistent results, all the researchers concluded that meeting recommended DHA dietary intake is important for pregnant and lactating women[72,73,75], even if supplementation provides no clear benefit beyond the AI[68–70].

1.2.d. Omega-6 and omega-3 PUFAs are required for neurodevelopment

LCPUFAs are critical during each stage of a fetus's development of the central nervous system (CNS) and the brain. The brain has the most abundant proportion of lipids in comparison to the other organs in the body. Approximately 50 – 60%[76] of the dry weight of the brain is lipids, of which LCPUFAs constitute 20-25%[56]. The brain is largely composed of LC-PUFAs that are essential for structure and function. The brain is largely composed of LC-PUFAs, predominantly ARA and DHA[76], which are found mainly esterified in the sn-2 position of phospholipids in cellular membranes. In the brain, DHA constitutes 10-15% [48,77] and 8-11% for AA of total fatty acid weight[77]. Out of total brain fatty acids, DHA comprises upwards of 40% of total brain LCPUFAs[78]. Polyunsaturated fatty acids are important for optimal neurodevelopment and functioning, as they are highly enriched in the cellular membranes of neurons. Both ARA and DHA are key components in cellular membranes and play essential roles in membrane flexibility, fluidity, and permeability[15,51]. Alterations to FA composition in the brain influences a large

variety of essential functions such as maintenance of axons & dendrites, cell shape, neuronal plasticity, dopamine storage, vesicle formation and transport[15].

DHA is abundantly found in neuronal tissues, especially synaptic membranes and vesicles[78,79]. Synaptic membranes and lipid rafts are abundantly composed of DHA and is required for proper neuronal function. Changes in membrane DHA composition can affect neurotransmission and signaling. Incorporation of DHA in synaptic membranes of developing neurons promotes synaptogenesis [80–84]. In developing neurons, as demonstrated through cell and animal studies, DHA is required for facilitating formation of synaptic proteins for vesicle fusion and incorporation into the membrane elongates and increases branching[74]. Through animal studies, DHA has been linked to dopamine production, receptor activity, and secondary signaling to facilitate neurotransmission[85–87]. DHA also enhances signal transduction[74] and plays a key role in modulation of dopamine synaptic neurotransmission[74]. Human studies have supported this with demonstrating that infants deficient of n-3 PUFAs have a decreased dopamine production[88]. ARA is also involved in neurotransmission via regulation of voltage-gated channels involved in the propagation of action potentials through neurons[51].

n-3 LC-PUFAs are the major determinant of membrane PUFA composition; following, it has been shown in cases of dietary deficiency of n-3 PUFAs, that n-3 PUFAs are replaced with an n-6 PUFA equivalent to be esterified in phospholipids[70]. Since n-3 PUFA is the key determinant of membrane composition, increased turnover of n-3 PUFAs without adequate supply for replacement, equivalent n-6 PUFA will instead be incorporated. The increased incorporation of n-6 PUFA into neuronal membranes, especially in the synapse, alters membrane function because DHA is a key mediator of neurotransmission[74]. LC-PUFAs are mainly found in phospholipid membranes and lipid peroxidation results in loss of membrane fluidity, reduced membrane potential, and increased permeability[89].

Given DHA and AA's critical role in the brain, during pregnancy and lactation adequate dietary intake of LCPUFAs, especially omega-3 fatty acids, is required for optimal fetal brain development. Low dietary supply of DHA "[impairs] neurogenesis, altered gene expression and neurotransmitters"[55]. Animal studies have demonstrated disruption to proper brain development in offspring with DHA deficiency in pregnancy[55]. Studies that have fed animals n-3 PUFA deficient diets yielded reduced DHA concentrations in the brain, neuronal size, dopamine and serotonin, and impaired visual function, memory and learning[75].

1.2.e. PUFAs are required throughout gestation and postnatally

Fetal demands for omega-6 and omega-3 LCPUFAs are high throughout gestation and even after birth for proper growth and development. During gestation, the fetus is dependent on adequate maternal supply of LCPUFAs to meet requirements. To provide sufficient LCPUFA concentrations to the growing and developing fetus, maternal metabolism adapts to accommodate increased needs, which is seen through the high maternal plasma concentration of LCPUFAs. The plasma concentration of LCPUFAs, found in phospholipids, increases by 50% during gestation[90]. The maternal dietary supply of n-6 and n-3 LCPUFAs is important to support increased LCPUFA fetal needs[3,48,91] but is not nearly enough[68]. The increased plasma LCPUFA concentration is also due to increased mobilization of fatty acids from maternal adipose tissues[68,90]. This is supported by the increase in lipolytic activity in adipose, as seen with increased expression of hormone sensitive lipase, highest in the last several weeks of gestation[66,92]. Utilization of maternal adipose tissue stores of LCPUAs also emphasizes the effect of maternal diet prior to conception in addition to prenatal diet[90]. Additionally, it is suggested that women naturally are slightly more efficient at the conversion of EFAs to LCPUFAs than men due to estrogen[3,48]. The conversion rate of ALA to EPA is 21% in women and 8% in men[48]. Also, the conversion rate of ALA to DHA is 9% in women and 0-4% in men[48]. The already higher efficiency in conversion of EFAs to LCPUFAs in women only increases during pregnancy to meet fetal LCPUFA requirements[48].

In the first 7 weeks of pregnancy, the embryonic phase, the brain forms, continues to grow, and undergoes functional development until the 8th week of pregnancy, the fetal period[47]. Maternal LCPUFA mobilization and utilization in the fetus begins in early gestion prior to the closure of the neural tube[48], which is approximately 9 weeks. The later stages of pregnancy, especially the third trimester, LCPUFA accretion increases in the fetus; consequently, at this time, LCPUFAs are highly incorporated into the fetus's brain[47] and adipose tissue[90,91,93]. In the first and second trimesters, ARA is the primary LCPUFA that is accumulating in the fetal brain; following, it is the most abundant LCPUFA at birth[56]. Nonetheless, DHA accumulation in the grey matter of the brain[90] and in neuronal synapses[94] begins in the third trimester[47]. The highest accumulation of DHA in the fetal brain, up to 70 mg/day, and white adipose tissue is during the third trimester[48]. Assessment of the PUFA contents of fetal organs has revealed that adipose tissue contains the largest stores of LA, ARA, and DHA at birth; it is estimated that 50% of DHA is in adipose while 23% is found in the brain[93]. When infants are born with higher serum DHA and

ARA, this can impart a continued increased accumulation into body tissues into the first couple postnatal weeks[91]. Adipose tissue has been proposed to be an important supply of DHA for infant brain development; Thus, an adequate PUFA supply during gestation is beneficial for postnatal brain development.[91,93]

DHA is instrumental in the proliferation and differentiation of neuronal cells[56]. During gestation, DHA is part of signaling and synaptogenesis; thus, collecting in neuronal growth cone membranes[95]. High dietary ω-6 fatty acids contribute to reduced docosahexaenoic acid in the developing brain and inhibit secondary neurite growth[96]. Interestingly, in the last trimester DHA is stored in the brain at a rate of 3mg/d; whereas the rate increases to 5mg/d in the post-natal period[3]. During the 3rd trimester, DHA is increasingly incorporated into fetal tissues leading to a substantially lower n-6 to n-3 ratio.[55] In the third trimester and after birth, the brain develops with great speed; thus, the proper level of DHA is critical in neurogenesis. Insufficient DHA in early gestation is more detrimental than deficiencies further in development.

Dendritic arbor growth, the rapid formation of synapses connections, and the accumulation of DHA in the brain continues after birth until two years of age[91]. From birth until the age of 2, the brain is actively developing because a child's brain is only at 25% of the final volume when the child is born[47]. Sufficient supply of DHA and ARA during the last trimester of pregnancy to 2 years of age are required to meet the demands of the high rate of accretion into the brain[70]. During this prenatal and postnatal period, the brain is still undergoing major structural changes[78]. Because DHA continues to collect in the brain up to the age of 2, adequate LCPUFA is extremely important[47]. Brain growth postnatally consists of the growth and expansion of axons and dendrites and the formation of myelin sheath around neuronal axons[47]. DHA gradually increases postnatally and eventually exceeds that of ARA to become the primary LCPUFA in the adult brain[56]. Moreover, adequate DHA levels are both important for fetus gestation and during the first two years of a child's development.

1.3 Mechanisms of Fetal Utilization of PUFAs in Neurodevelopment

1.3.a. Placental Transfer of PUFAs

During pregnancy, the fetus is reliant on maternal LCPUFAs via placental transfer for growth and development[70,73,97]. The placenta is pertinent to supply the fetus since the fetus has limited capability to desaturate and elongate PUFAs [55,90]. Starting in the third trimester, the fetus can convert ALA into DHA and LA into ARA; however, this process is limited[92,98] and cannot supply adequate LCPUFA to meet the high requirements. It is not until 16 weeks after birth that infants can more successfully convert EFAs into their respective downstream LCPUFAs[56]. Even though EFA conversion is low, the fetus is more capable of synthesizing ARA than DHA[90]. For n-3 PUFAs in particular, increased maternal metabolic capacity for DHA synthesis and rate of placental transfer regulate availability of DHA for fetal use[48]. Also, the placenta has a low rate of conversion of EFAs to their subsequent LCPUFAs.[55] Therefore, pre- and perinatal LCPUFA supply is highly dependent on dietary DHA and ARA and preformed DHA and AA are considered particularly important. Preformed LCPUFAs are preferential sources for the developing brain and incorporation into phospholipids[3] as they are more efficiently used. This is supported by the higher plasma concentrations of ARA and DHA in the fetus and neonate[70]. It is suggested that this occurs by selective placenta transport of preformed LCPUFAs and proposed to do so through individual PUFAs being compartmentalized in the placenta[3,99]. ARA has a higher accumulation in the placenta than DHA, but DHA is transported through the placenta into the fetal blood stream at a much higher rate compared to ARA[70].

LCPUFA concentration is higher in fetal circulation than maternal circulation due to its increased needs for growth and development[48]. Within plasma, LCPUFAs can be found esterified in triglycerides and phospholipids and non-esterified fatty acids (NEFAs) bound to albumin. The increase concentration of plasma LCPUFA is important for placental transfer. Triglycerides cannot be transported across the placenta[92]. Triglycerides hydrolyzed by lipoprotein lipase to release esterified fatty acids[99] and non-esterified fatty acids (NEFAs) are the primary maternal fatty acid sources used in placental transfer[55]. Transfer is also dependent on the activity of placental lipoprotein lipase (LPL), phospholipase A2 (PLA2), intracellular lipases, and triacylglycerol hydroxylase[48]. LPL and PLA2 hydrolyze maternal plasma lipoproteins and phospholipids to

liberate LCPUFA. The transport of PUFAs across the placenta is based on a concentration gradient, with the fetus having very low PUFA in comparison to the mother[70,100].

PUFAs, especially DHA and ARA, are transferred across the placenta from maternal circulation into fetal circulation[48]. Once ARA and DHA, as NEFAs,[70] are thought to be transferred through the placental syncytiotrophoblast with by passive diffusion or membrane transport proteins that have a high specificity to LCPUFAs[3,55]. The membrane transport proteins in the syncytiotrophoblast include plasma membrane fatty acid-binding protein (pFABPpm), fatty acid transport protein 4 (FATP-4) [3,55], fatty acid translocase (FATP) CD36 [55,99,100], and Mfsd2a, a sodium-dependent lysophosphatidylcholine symporter [99]. FATP-4 in the placenta is speculated to have a high affinity for DHA as the expression of FATP-4 is correlated to umbilical cord blood DHA concentration[55]. PUFAs can also be transferred across the placenta through passive diffusion in addition to membrane protein-mediated mechanisms[55]. Once NEFAs are taken up into the syncytiotrophoblast, NEFAs can be esterified in phospholipids and triglycerides that are stored in lipid droplets or bind to cytoplasmic transport proteins to be released into the placental villous stroma[99,100]. From the syncytiotrophoblast, the non-esterified LCPUFAs travel through the villous stroma and into the fetal capillary endothelium where NEFAs bind to albumin[99,100].

1.3.b. Fatty acid transport from circulation to the brain

LC-PUFAs in plasma are found in esterified fatty acid pools that include lipoproteins, phospholipids and NEFAs. Circulating lipoproteins contain the largest amount of LCPUFAs, but NEFAs and lysophosphatidylcholine (LPC) are the primary sources of plasma LCPUFAs taken up by the brain[101,102]. Studies conducted in rodents have shown that NEFAs, DHA, is rapidly taken up by the brain[103–106]. Plasma NEFA concentration is supplied by hydrolysis of triacylglycerol in chylomicrons by lipoprotein lipase and adipose tissue by hormone sensitive lipase. NEFAs are bound to albumin in circulation while LPC can be found both bound to albumin or within lipoprotein membrane phospholipids, specifically phosphatidylcholine that can be partially hydrolyzed to sequester LPC[78]. Both NEFAs and LPC are efficiently taken up by the brain; whereas NEFAs are more rapidly cleared from circulation by the brain[78]. NEFAs and LPC released from lipoproteins through the hydrolysis activity of lipoprotein lipase and endothelial

lipase, expressed in brain capillary endothelial cells, can also be taken up by the brain, but this uptake is much less efficient[78].

The mechanism of LCPUFA uptake across the blood brain barrier (BBB) into the brain is not currently well understood. Two mechanisms have been proposed for PUFA uptake into the brain from circulation: passive diffusion and via specialized transport proteins[78,107] (Figure 2). To be transported across the BBB, NEFAs and LPC dissociate from albumin or release through the hydrolysis of lipoproteins and associate with the luminal side of the endothelial membrane of the BBB. NEFAs are thought to move across the BBB via passive diffusion. With passive diffusion, NEFAs incorporate into the membrane and translocate to the cytosolic, inner leaflet of the membrane bilayer by flip-flop[78,107]. This process repeats through the layers of the BBB until it reaches the parenchyma basal membrane[78]. During this process, NEFAs may diffuse into the cytosol with aid of cavolein-1 or FABP. NEFAs can also be trapped within the cell by binding FABP or be acyl-CoA activated by acyl-CoA synthetase of FATP to trap the NEFAs. This can aid in trafficking NEFAs across the cytosol[78,107]. Possibly, LPC is taken up by the brain through membrane transporter mediated mechanisms, such as through Mfsd2a, LPC symporter[99,101] and lysophospholipid transporter[78]. For protein-mediated transported across the BB several transport proteins are implicated to be involved, including FAT(CD36), FABP-5 and -7, and FATP-1 and -4., and Mfsd2a[78,107].

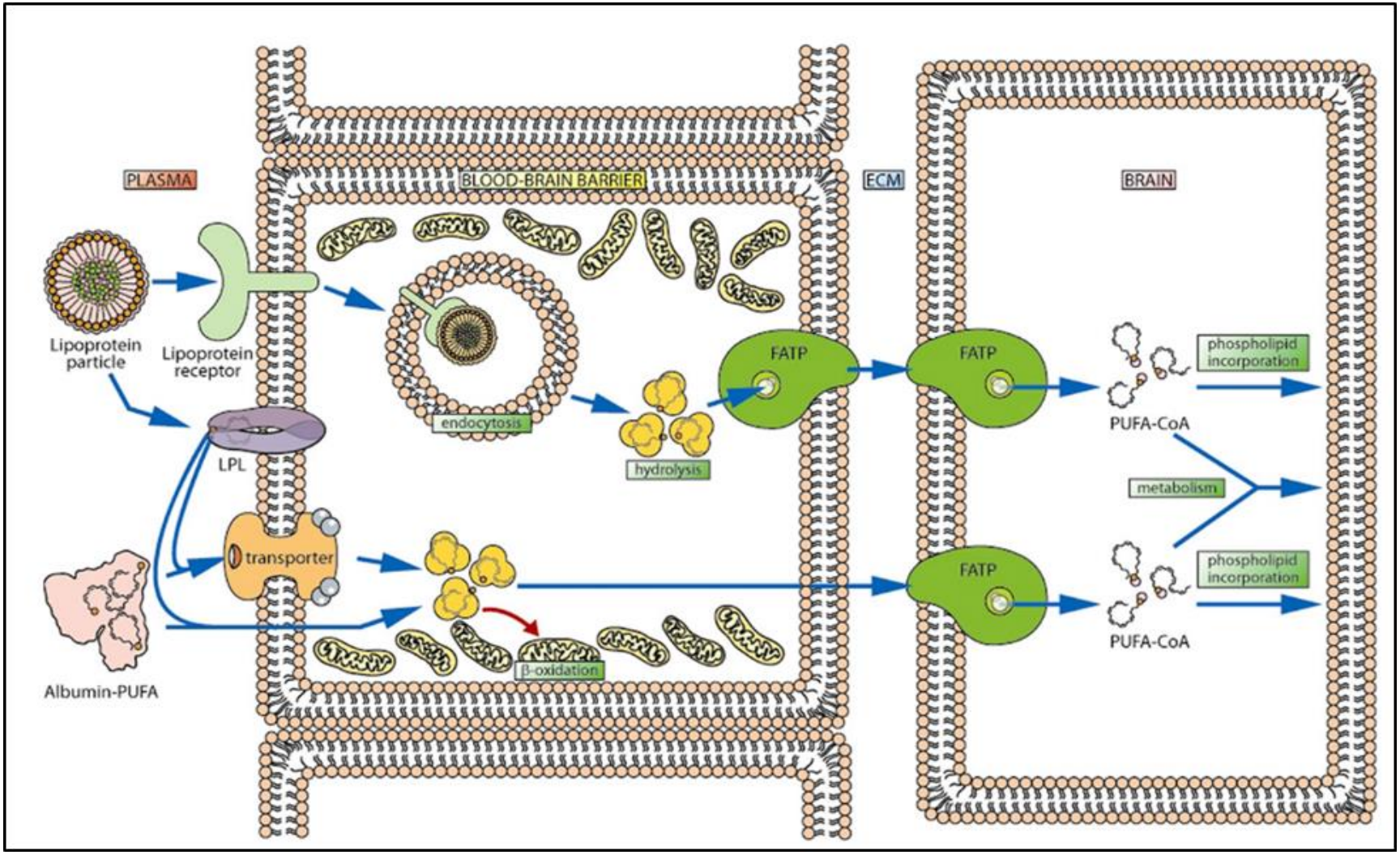

Figure 2. Transport of PUFAs from circulation into the brain.[78]

1.3.c. LCPUFAs in membrane phospholipids

n-6 and *n*-3 LCPUFAs are found throughout the body in cellular membrane phospholipids[91]. In cellular membranes, phospholipids are enriched in and provide a dynamic source of LCPUFAs that influences many functions, such as membrane lipid-protein interactions, continuous membrane turnover via PLA_2 for bioactive lipid synthesis, and act as ligands for transcription factors in gene expression regulation[91]. LCPUFA incorporation into membrane phospholipids is very dependent on the diet. Animal studies have demonstrated that membranes are influences by changes in dietary intake of *n*-3 PUFAs. When *n*-3 PUFA supply is high, *n*-3 PUFAs are preferably incorporated into membrane phospholipids compared to *n*-6 PUFAs[70]; however, this is not the case for *n*-6 PUFAs as incorporation into membrane is independent of diet.[70]

Within the brain, cellular membrane phospholipids undergo a continuous remodeling process of acylation and re-acylation reactions, known as the Lands' cycle [78,108,109] (Figure 3). In the remodeling cycle, LCPUFAs are being incorporated into the sn-2 position of phospholipids and then released by PLA_2 for LCPUFAs to be synthesized into bioactive lipids; LCPUFAs undergo β-oxidation; or LCPUFAs are re-incorporated into membrane phospholipids via the Lands' cycle. The majority of the released non-esterified LCPUFAs from phospholipids are re-esterified into phospholipids, approximately 90%.[78] To be incorporated into phospholipids, acyl-CoA is added to the NEFAs by acyl-CoA synthetase (ASCL) and esterified in the sn-2 position of a lysophospholipid by lysophospholipid acyltransferase (LPAT)[110]. When LCPUFAs are released from membrane phospholipids, PLA_2 hydrolyzes the LCPUFA from the sn-2 position of the phospholipid to yield a NEFA and a lysophospholipid. In the brain, ARA is release by calcium-dependent cytosolic PLA_2 ($cPLA_2$) while DHA is released by calcium-independent PLA_2 ($iPLA_2$). This active metabolism of LCPUFAs in Lands' cycle remodeling in the brain is important for the distribution of LCPUFAs and ensures variation within classes phospholipids[78]. This continuous process also exemplifies the frequent LCPUFA turnover and remodeling in the brain. In rodent studies, dietary LCPUFA intake, especially deprivation of LCPUFAs, is involved in the regulation of enzymes involved in the phospholipid metabolism and the Lands' cycle. The results of these studies show that low *n*-3 intake reduces $iPLA_2$ expression and activity while low *n*-6 PUFA intake has the opposite effect[111,112]. Because the expression and activity of PLA_2 is altered

by dietary intake of LCPUFA, diet contributes to regulating LCPUFA release from the membrane and brain LCPUFA metabolism.

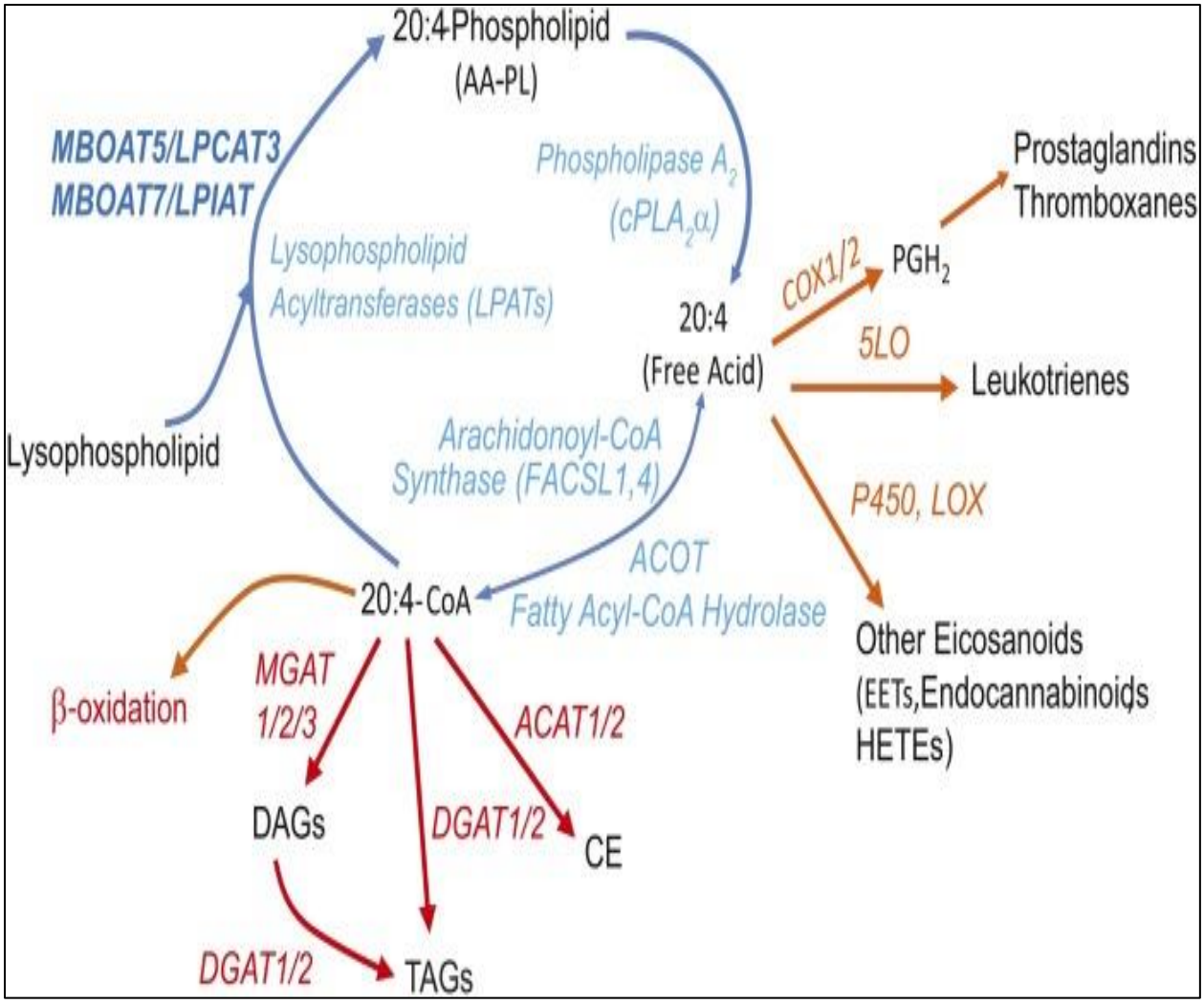

Figure 3. Land's Cycle of Remodeling Cellular Membrane Phospholipids Cycle.[110]

1.4. Incorporation of PUFAs in Neuronal Membranes and Neurodevelopment

1.4.a. Animal models

Membrane phospholipids are important in neurodevelopment are they are required for cellular growth and functioning. The PUFA composition of the phospholipids incorporated into neuronal membranes is especially important in the determining the progression of development. The prenatal accumulation of brain fatty acids is thought to parallel important events in neurogenesis. Numerous animal studies have investigated changes in membrane composition of LCPUFAs during neurodevelopment and maturation of neurons. During neuronal growth, rodent studies have shown that neuronal growth cones have higher concentrations of ARA than DHA. However, DHA concentration increases rapidly in growth cone membranes prior to synaptogenesis and develop into mature synapses[113,114]. Changes in LCPUFA composition of membranes is involved in neurodevelopment, growth cone maturation and synaptogenesis[113]. The offspring of female rats fed ALA deficient diets during gestation led to low DHA and high *n*-6 DPA membrane concentrations in neuronal growth cones but did not affect the membranes of cell bodies. In a rodent study conducted by Bertrand et al. (2006), the embryonic rats whose mothers were fed n-3 deficient diets had smaller brains with altered morphology and reduced neurogenesis[115]. Pregnant mice fed high n-6 to n-3 diets, matching the western diet, yielded impaired neurogenesis in the offspring. Even though the offspring were fed a balanced n-6 to n-3 diet upon birth, they displayed higher anxiety behaviors as adults.[115] This demonstrate a lasting effect of gestational diet on abnormal brain development[116].

Without sufficient DHA concentration in the brain, neurotransmission and neuronal cell functioning is altered. Numerous rodent studies have demonstrated alterations to LC-PUFA metabolism with n-3 and n-6 deficient diets. At birth, offspring of pregnant rats fed high *n*-6 PUFA diets, safflower oil diet, during gestation had significantly less DHA and higher ARA in phospholipids compared to the high DHA fish oil diet. Serotonin levels we also significantly higher in the high *n*-6 safflower oil diet. This is suggestive of maternal diet altering fetal growth cone membrane composition and neurotransmitters involved with neuritogenesis and synaptogenesis[117]. In a study conducted in gerbils, DHA supplementation increased the number of dendritic spines, which eventually form synapses in an adult hippocampus[74,118]. Additionally, there was a substantial increase in phospholipids and synaptic protein levels with DHA and EPA

supplementation[119]. High DHA perinatal diets in pregnant rats led to elevated pre- and post-synaptic proteins and increased number of dendritic spines in the pups[120]. The offspring of female rats fed n-3 deficient diets, ALA deficient, prior to and during gestation had lower syntaxin and increased ternary SNARE complex expression, which are proteins involved in neurotransmission. This is suggestive of a disruption in neurotransmitter vesicle trafficking leading to altered neurotransmission[121]. Studies with infant male rats fed n-3 deficient diets, ALA, had decreased dopamine release, altered vesicular membrane properties and decreased vesicular dopamine storage in frontal cortical neurons[122]. Also, dopamine was significantly lower there is reduced expression of vesicular monoamine transporter2 in the frontal cortex and nucleus accumbens and altered D2 receptor expression in the mesolimbic and mesocortical dopaminergic pathways[123]. A disruption to the DHA membrane composition of a neuron, as demonstrated in studies with n-3 deficient diets, causes dopamine vesicles to be redistributed in presynaptic terminals and decrease extracellular dopamine concentrations[124]. This disruption of dopaminergic neurotransmission in the frontal cortex is associated with hyperactivity[125] and impaired learning[126] in later life.

1.4.b. Cell models

DHA has been repeatedly shown to be involved in neuronal differentiation, neuritogenesis, synaptogenesis, and neurotransmission. Several studies with embryonic neural stem cells from offspring of pregnant rats fed n-3 deficient diets during gestation had decreased neurite length[127] and reduced synaptogenesis[74,128]. In contrast, neuronal stem cells from embryonic rats whose mothers were fed n-3 supplemented diets during gestation had increased neurite length and branching[127], increased neurogenesis, and newly differentiated neurons were morphologically more mature[129]. Additionally, DHA supplementation significantly increased levels of synapsin, a neuron-specific synaptic membrane protein involved in synaptogenesis and synapse maturation, in comparison to ARA supplementation. Consistent with primary neuronal stem cell cultures from prenatal rats, studies conducted with PC12 cells show increased neurogenesis and neurite outgrowth with DHA supplementation[130–132]. PC12 cells have also shown that DHA supplementation led to faster differentiation of the neuronal stem cells, which is demonstrated through morphological changes in neurites and increased mRNA levels of markers of differentiation[131]. Even though n-3 PUFAs, especially DHA, are implicated with increased

neuritogenesis and neurogenesis, some studies have demonstrated similar effects by ARA. In differentiating neuroblastoma cells, the total n-6 PUFA, ARA, and DGLA incorporation into the membrane was increased during the 8-day incubation period; this modification to the phospholipid membrane PUFA composition was associated with neurite extension[133]. It has also been shown in other models of PC12 cells, hippocampal neurons and chick motor neurons that ARA enhances neurite outgrowth[134–136]. Another study in neural stem cells demonstrated that DHA and ARA combined yield enhanced induction of differentiation and a higher population of cells increased neurite length in neural stem cells than each PUFA alone[137]. From these studies, DHA has been consistently demonstrated to improve outcomes during neuronal differentiation while it is still unknown the effects of ARA on neurogenesis, neuritogenesis, and synaptogenesis.

n-3 PUFAs play a fundamental role in the functionality of developing neurons. The synthesis of neurotransmitters, vesicle formation and fusion, and storage are influence by the availability of n-3 PUFAs. In SH-SY5Y neuroblastoma cells, supplementation of DHA yields increased incorporation of DHA in membrane phospholipids compared to ARA[74,138]. Also, DHA enhanced vesicle mobilization and the release of noradrenaline in the SH-SY5Y cells[138]. In PC12 cells, supplementation with *n*-6 PUFAs suppressed neurite outgrowth and decreased uptake of norepinephrine, but increased basal release[130]. In studies with postnatal rats, neural stems from 1-day old pups from mothers fed deficient n-3 diets had slowed proliferation[139]. Also, pups of rats fed ALA deficient diets during gestation demonstrated impaired dopaminergic neurotransmission. This was demonstrated through decreased expression of tyrosine hydroxylase, vesicular monoamine transporter (VMAT-2), reduction of VMAT-associated pre-synaptic vesicles,[124,140,141] and increased dopamine receptors (DA), which is speculated to be a compensatory effect of decreased dopamine[141].

Omega-3 PUFAs also impact plasticity and cell survival, which can impact brain functioning in later life.[140,142–144]. In studies with SH-SY5Y cells, DHA and EPA are neuroprotective and increase cell viability[118,144–147]. DHA also increases dendritic spine density, which is suggestive of increased plasticity and long-term potentiation of neural connections[140,142,148]. The improved cell viability and plasticity with EPA and DHA supplementation has possible implications in neurodevelopmental and neurodegenerative diseases.[148,149]

1.5. PUFAs are Fundamental Nutrients in Neurodevelopment

1.5.a. Omega-6 and omega-3 PUFAs are required for optimal health

Polyunsaturated fatty acids are dietary components that greatly contribute to health at all stages of life. For many decades, omega-3 PUFAs are predominantly attributed to improving health as well as aiding in the prevention and treatment of chronic diseases. Omega-3 PUFAs have long been implicated in a wide variety of chronic health conditions, including cardiovascular disease, coronary heart disease, inflammatory and immunologic diseases, obesity, diabetes, and neurological disorders[1,3]. Numerous studies have demonstrated that inadequate intake of omega-3 fatty acids imparts adverse health consequences, such as increased risk of developing chronic diseases, impeded growth, and impaired neurological functioning[3,4,12–16]. In the US, our dietary pattern has been consistently low in omega-3 PUFAs while omega-6 PUFAs have steadily increased since the 1980s[4,15]. Similar to the effects of inadequate n-3 PUFAs in the diet, high consumption of n-6 PUFAs can have detrimental effects to health by contributing to the development and progression of many chronic diseases such as atherosclerosis, cardiovascular disease[1], and insulin resistance[4]. The negative health consequences associated with the Western dietary pattern should be a major concern for Americans as most adults fail to meet the dietary recommendations for n-3 PUFAs[32,33,61,65]. Pregnant and lactating women should be especially concerned about achieving adequate *n*-3 PUFA intake[59,63,65]. Many studies have demonstrated that omega-3 PUFA intake during pregnancy is associated with fetal outcomes[44] such as, length of gestation, birth weight[16,48,68], intrauterine restricted growth[55], perinatal mortality, visual acuity,[15,68], neurodevelopment[2,15,45,47], and cognitive performance[57]. Maternal consumption of omega-3 PUFAs, especially EPA and DHA, is crucial to meet fetal and neonatal needs.

During the pre- and post-natal periods, the fetus and neonate require large amounts of PUFAs to support optimal growth and development. During the 3rd trimester and up to 2 years of age[47,70], the accretion rate of DHA and ARA is at its highest to support brain growth and neuronal maturation. Even though ARA and DHA are the predominant LCPUFAs in the brain and are both required for neuronal functioning[51,86,87,117], there is a greater focus on the essentiality of omega-3 PUFAs in brain development. Several animal studies have provided evidence that low maternal n-3 PUFA can lead to impaired neurological development. When maternal diets were deficient in n-3 PUFA, the offspring had reduced neuronal connectivity[150,151], decreased plasticity[86], altered

neurotransmission[86,87,122,123,141,152–156], altered brain and neuronal morphology[157–161],and cognitive deficits[86,88,126,153–156,162–166]. Some studies have suggested that these altered neurological outcomes in the offspring can persist into adulthood[155], especially if the offspring consume a prolonged n-3 deficient diet[115].

PUFAs are fundamental components in cellular membranes and the composition imparts functionality. Membrane PUFA composition is especially important in the brain because LCPUFAs comprise 20-25% of the total lipids[56]. LCPUFAs, primarily DHA and ARA, are the most abundant in neuronal membrane phospholipids to support proper neurotransmission. As neurons grow and mature, ARA and DHA are rapidly taken up by the brain and incorporated into the membrane phospholipids. Animal studies and cell models have both demonstrated that PUFA composition in neurons changes with development. During growth, neuronal membranes have a high concentration of ARA; however, the DHA concentration increases rapidly as neurons mature[113,133]. Both ARA and DHA are implicated to be involved in neurite outgrowth[133–136]. Several animal studies manipulated PUFA availability for neuronal development through n-3 deficient diets in gestation. The offspring from mothers fed n-3 deficient diets had a higher concentration of n-6 PUFA in growth cones[113,117], altered brain morphology, impaired neurogenesis,[157] and altered neurotransmission[121,122,124]. Studies using neuronal cells supplemented with DHA, demonstrated enhanced neurite outgrowth[127], synaptogenesis[74,127,128], neurogenesis[129], and neurotransmission[138,148]. The results of the various animal and cellular models that manipulated the availability of n-6 and n-3 PUFAs for incorporation into neuronal membranes illustrate the developmental consequences of altering the membrane PUFA composition.

1.5.b. The role of PUFAs in neurodevelopment is not well understood

The cruciality of adequate intake of omega-3 PUFAs for proper brain development is consistently supported across the literature. Numerous studies in both humans and animals have demonstrated that n-3 deficiency during gestation pose unfavorable neurodevelopmental effects in offspring, including cognitive deficits and a predisposition for psychopathologies[166,167]. Even though there is ample evidence that adequate intake of omega-3 PUFAs are required for offspring brain development, the role of PUFAs in brain development is unclear. Most notably, there is a

limited understanding of the role of the n-6:n-3 PUFA in neurodevelopment. Our understanding is currently constricted by contradictory results in all disciplinary facets of research from observational human studies to cellular models; thus, further investigation of the relationship between n-6:n-3 PUFA in neurodevelopment is required.

Currently, there is a lack of consensus on an optimal n-6:n-3 for neurodevelopment, the mechanism of action for individual PUFAs, and how the interaction between nutrients contribute to neurodevelopment. This discord has been attributed to several discrepancies throughout the literature. One of the biggest contributing issues is that the n-6:n-3 is used as a generalized measure that encompasses various combinations of PUFAs that could constitute the ratio[168]. The more commonly used comparisons of n-6 and n-3 PUFAs are LA/ALA[126,169] and DHA/ARA[170,171], while other studies use a combination of EFAs and LCPUFAs. Methodological inconsistencies throughout the literature also contribute to the conflicting results pertaining to PUFAs and neurodevelopment. Most of the available human studies focused on the relationship between omega-3 PUFAs and brain development and use whole food sources for omega-3 PUFAs, such as fish, seafood, and plant oils. These PUFA sources are comprised of other nutrient constituents that aren't considered. Therefore, this demonstrates a lack of consideration for the interplay between n-3 PUFAs and other nutrients, such as n-6 PUFAs, contributing to neurodevelopmental outcomes[172]. To overcome limitations in human studies, animal models have used varying n-6:n-3 ratios in diets fed during gestation[169]. However, these studies often fail to keep the quantities of all PUFAs, SFAs, and MUFAs consistent across the different diets and utilize different sources, including oils, of the PUFAs for each diet[168]. Studies that alter the n-6:n-3 in diets often overlook the individual PUFAs that vary across diets and attention is on the total omega-3 and omega-6 PUFAs. Individual PUFAs have unique biological and physiological functions and should be studied independently[173]. In vitro studies using neuronal cell cultures have been conducted in attempt to elucidate the role of individual PUFAs in neurodevelopment, but there many limitations in the available evidence. Even though the cell studies controlled PUFA concentrations across treatments in comparison to human and animal studies, there are apparent methodological inconsistencies. Across the literature, the concentrations of PUFAs used in the treatment are in the medium range from 0.1 μM to 200μM[174]. Also, the majority of the previously conducted studies utilize cells derived from rodents and use undifferentiated neural progenitor cells to investigate the impact of PUFA supplementation on neuronal morphology and function. Additionally, there is a

limited understanding of the underlying mechanism[175–177], since the proposed pathways vary widely, such as involvement of Hes-1[178] or endocannabinoid signaling[175] in the differentiation process. Due to the inconsistencies across previously conducted studies, it is difficult to compare results and provide conclusive evidence on the role of the n-6:n-3 ratio in neurodevelopment.

The role of omega-6 PUFAs in conjunction with omega-3s in neurodevelopment is not well understood and requires further investigation. In cellular models, there are gaps in knowledge on how PUFAs impact the differentiation of progenitor neural cells to mature neurons. To address these gaps in the literature about PUFAs, n-6:n-3, and neuronal development, my research utilizes SH-SY5Y human neuroblastoma cells that are supplemented with individual PUFAs to assess membrane phospholipid composition and morphology throughout the differentiation process. By using the SH-SY5Y cell line, this increases the translational potential since these cells are human-derived; whereas most of the literature utilizes rodent-derived cells. The previous studies primarily utilize undifferentiated cells to study PUFA membrane composition alterations and functional outcomes, but this does not reflect the neuronal maturation process in development. To provide better insight into the impact of PUFAs on morphological changes that occur during maturation, my research project uses SH-SY5Y cells undergoing differentiation in conjunction to PUFA supplementation. Also, to overcome methodological inconsistencies, my research explicitly defines the constituents of n-6:n-3 and utilizes this ratio in the analysis of the membrane PUFA composition and morphology. Overall, the goal of my research project is to elucidate how individual PUFAs affect membrane composition, the membrane n-6:n-3, and morphology during neuronal differentiation.

1.6. References

1. Sanders, T.A.B. (2019). Omega-6 Fatty Acids and Cardiovascular Disease: Are We Getting Closer to the Truth? *Circulation*, *139*(21), 2437–2439. https://doi.org/10.1161/CIRCULATIONAHA.119.040331

2. Office of Dietary Supplements. *Omega−3 Fatty Acids and Health: Fact Sheet for Health Professionals*. (2020, October 1). US National Institutes of Health. https://ods.od.nih.gov/factsheets/Omega3FattyAcids-HealthProfessional/

3. Guesnet, P., & Alessandri, J.M. (2011). Docosahexaenoic acid (DHA) and the developing central nervous system (CNS) - Implications for dietary recommendations. *Biochimie*, *93*(1), 7–12. https://doi.org/10.1016/j.biochi.2010.05.005

4. Molendi-Coste, O., Legry, V., & Leclercq, I.A. (2011). Why and How Meet n-3 PUFA Dietary Recommendations? *Gastroenterology Research and Practice*, *2011*, 11. https://doi.org/10.1155/2011/364040

5. O'Keefe, J.H., & Harris, W.S. (2000). From Inuit to implementation: Omega-3 fatty acids come of age. *Mayo Clinic Proceedings*, *75*(6), 607–614. https://doi.org/10.4065/75.6.607

6. Lands, B. (2014). Historical perspectives on the impact of n-3 and n-6 nutrients on health. *Progress in Lipid Research*, *55*(1), 17–29. https://doi.org/10.1016/j.plipres.2014.04.002

7. Muskiet, F.A.J. (2010). Pathophysiology and Evolutionary Aspects of Dietary Fats and Long-Chain Polyunsaturated Fatty Acids across the Life Cycle. In J. Montmayeur & J. le Coutre (Eds.), *Fat Detection: Taste, Texture, and Post Ingestive Effects*. CRC Press/Taylor & Francis.

8. Auestad, N., Scott, D.T., Janowsky, J.S., Jacobsen, C., Carroll, R.E., Montalto, M.B., Halter, R., Qiu, W., Jacobs, J.R., Connor, W.E., Connor, S.L., Taylor, J.A., Neuringer, M., Fitzgerald, K.M., Hall, R.T., & Objective, A. (2003). Visual, Cognitive, and Language Assessments at 39 Months: A Follow-up Study of Children Fed Formulas Containing Long-Chain Polyunsaturated Fatty Acids to 1 Year of Age. *Pediatrics*, *112*(3), e177–e183. https://doi.org/10.1542/peds.112.3.e177

9. Simopoulos, A. P. (2010). The omega-6 / omega-3 fatty acid ratio : health implications. *OLC*, *17*(5), 267–275. https://doi.org/10.1684/ocl.2010.0325

10. Jacobsen, J.L., Jacobsen, S.W., Muckle, G., Kaplan-Estrin, M., Ayotte, P., & Dewailly, É. (2008). Beneficial Effects of a Polyunsaturated Fatty Acid on Infant Development: Evidence from the Inuit of Artic Quebec. *Journal of Pediatrics*, *152*(3), 356–364. https://doi.org/10.1016/j.jpeds.2007.07.008

11. Simopoulos, A.P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother*, *56*, 365–379. https://doi.org/10.1002/9781118269336.ch7

12. Jump, D.B. (2002). The biochemistry of n-3 polyunsaturated fatty acids. *Journal of Biological Chemistry*, *277*(11), 8755–8758. https://doi.org/10.1074/jbc.R100062200

13. Abedi, E., & Sahari, M. A. (2014). Long-chain polyunsaturated fatty acid sources and evaluation of their nutritional and functional properties. *Food Science and Nutrition*, *2*(5), 443–463. https://doi.org/10.1002/fsn3.121

14. Hoppenbrouwers, T., Cvejić Hogervorst, J.H., Garssen, J., Wichers, H.J., & Willemsen, L.E.M. (2019). Long chain polyunsaturated fatty acids (LCPUFAs) in the prevention of food allergy. *Frontiers in Immunology*, *10*(MAY), 1–9. https://doi.org/10.3389/fimmu.2019.01118

15. Saini, R.K., & Keum, Y.S. (2018). Omega-3 and omega-6 polyunsaturated fatty acids: Dietary sources, metabolism, and significance - A review. *Life Sciences*, *203*, 255–267. https://doi.org/10.1016/j.lfs.2018.04.049 R

16. Grootendorst-van Mil, N.H., Tiemeier, H., Steenweg-de Graaff, J., Koletzko, B., Demmelmair, H., Jaddoe, V.W.V., Steegers, E. A. P., & Steegers-Theunissen, R.P.M. (2018). Maternal plasma n-3 and n-6 polyunsaturated fatty acids during pregnancy and features of fetal health: Fetal growth velocity, birth weight and duration of pregnancy. *Clinical Nutrition*, *37*, 1367–1374. https://doi.org/10.1016/j.clnu.2017.06.010

17. Hossain, S., Arai, H., Al Mamun, A., Hashimoto, M., & Matsuzaki, K. (2016). Docosahexaenoic acid: one molecule diverse functions. *Critical Reviews in Biotechnology*, *37*(5), 579–597. https://doi.org/10.1080/07388551.2016.1207153

18. Trebatická, J., Dukát, A., Ďuračková, Z., & Muchová, J. (2017). Cardiovascular Diseases, Depression Disorders and Potential Effects of Omega-3 Fatty Acids. *Physiological Research*, *66*(3), 363–382.

19. Calder, P. C., & Yaqoob, P. (2009). Understanding omega-3 polyunsaturated fatty acids. *Postgraduate Medicine*, *121*(6), 148–157. https://doi.org/10.3810/pgm.2009.11.2083

20. Ebbesson, S. O. E., Risica, P. M., Ebbesson, L. O. E., & Kennish, J. M. (2005). Eskimos have CHD despite high consumption of omega-3 fatty acids: the Alaska Siberia project. *International Journal of Circumpolar Health*, *64*(4), 387–395. https://doi.org/10.3402/ijch.v64i4.18015

21. Fenton, J., Hord, N., & Gurzell, E. (2013). Long chain omega-3 fatty acid immunomodulation and the potential for adverse health outcomes. *Prostaglandins Leukot Essent Fatty Acids*, *89*(6), 379–390. https://doi.org/10.1016/j.plefa.2013.09.011.

22. Jones, G. J. B., & Roper, R. L. (2017). The effects of diets enriched in omega-3 polyunsaturated fatty acids on systemic vaccinia virus infection. *Scientific Reports*, *7*(1), 1–6. https://doi.org/10.1038/s41598-017-16098-7

23. Cholewski, M., Tomczykowa, M., & Tomczyk, M. (2018). A comprehensive review of chemistry, sources and bioavailability of omega-3 fatty acids. In *Nutrients* (Vol. 10, Issue 11). https://doi.org/10.3390/nu10111662

24. Catalá, A. (2013). Five Decades with Polyunsaturated Fatty Acids: Chemical Synthesis, Enzymatic Formation, Lipid Peroxidation and Its Biological Effects. *Journal of Lipids*, *2013*, 1–19. https://doi.org/10.1155/2013/710290

25. Lands, B., Bibus, D., & Stark, K.D. (2018). Dynamic interactions of n-3 and n-6 fatty acid nutrients. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *136*, 15–21. https://doi.org/10.1016/j.plefa.2017.01.012

26. Schmitz, G., & Ecker, J. (2008). The opposing effects of n-3 and n-6 fatty acids. *Progress in Lipid Research*, *47*(2), 147–155. https://doi.org/10.1016/j.plipres.2007.12.004

27. Zamberletti, E., Piscitelli, F., De Castro, V., Murru, E., Gabaglio, M., Colucci, P., Fanali, C., Prini, P., Bisogno, T., Maccarrone, M., Campolongo, P., Banni, S., Rubino, T., & Parolaro, D. (2017). Lifelong imbalanced LA/ALA intake impairs emotional and cognitive behavior via changes in brain endocannabinoid system. *Journal of Lipid Research*, *58*(2), 301–316. https://doi.org/10.1194/jlr.M068387

28. Stanton, C., Ross, R. P., Fitzgerald, G. F., Wall, R., & Patterson, E. (2012). Health Implications of High Dietary Omega-6 Polyunsaturated Fatty Acids. *Journal of Nutrition and Metabolism*, *2012*, 1–16. https://doi.org/10.1155/2012/539426

29. Alashmali, S. M., Hopperton, K. E., & Bazinet, R. P. (2016). Lowering dietary n-6 polyunsaturated fatty acids: Interaction with brain arachidonic and docosahexaenoic acids. *Current Opinion in Lipidology*, *27*(1), 54–66. https://doi.org/10.1097/MOL.0000000000000255

30. Barceló-Coblijn, G., & Murphy, E. J. (2009). Alpha-linolenic acid and its conversion to longer chain n-3 fatty acids: Benefits for human health and a role in maintaining tissue n-3 fatty acid levels. *Progress in Lipid Research*, *48*(6), 355–374. https://doi.org/10.1016/j.plipres.2009.07.002

31. U.S. Department of Health and Human Services and U.S. Department of Agriculture. *2015 - 2020 Dietary Guidelines for Americans*.(8th ed.). (2015, December). https://health.gov/our-work/food-and-nutrition/2015-2020-dietary-guidelines

32. Elmadfa, I., & Kornsteiner, M. (2009). Fats and fatty acid requirements for adults. *Annals of Nutrition and Metabolism*, *55*(1–3), 56–75. https://doi.org/10.1159/000228996

33. Papanikolaou, Y., Brooks, J., Reider, C., & Fulgoni, V. L. (2014). U.S. adults are not meeting recommended levels for fish and omega-3 fatty acid intake: Results of an analysis using observational data from NHANES 2003-2008. *Nutrition Journal*, *13*(1), 1–6. https://doi.org/10.1186/1475-2891-13-31

34. U.S. Department of Agriculture and U.S. Department of Health and Human Services. *Dietary guidelines for Americans, 2010*. (7th ed.).(2010, December) U.S. Government Printing Office. https://www.dietaryguidelines.gov/

35. U.S. Department of Health and Human Services and U.S. Department of Agriculture *Dietary Guidelines for Americans, 2005* (6th ed.).(2005, January). U.S. Government Printing Office. http://www.healthierus.gov/dietaryguidelines

36. Carlson, S. E., & Colombo, J. (2016). Docosahexaenoic Acid and Arachidonic Acid Nutrition in Early Development. *Advances in Pediatrics*, *63*(1), 453–471. https://doi.org/10.1016/j.yapd.2016.04.011

37. Simopoulos, A. P. (2016). An increase in the Omega-6/Omega-3 fatty acid ratio increases the risk for obesity. *Nutrients*, *8*(3), 1–17. https://doi.org/10.3390/nu8030128

38. Simopoulos, A. P. (2004). Omega-6/Omega-3 Essential Fatty Acid Ratio and Chronic Diseases. *Food Reviews International*, *20*(1), 77–90. https://doi.org/10.1081/fri-120028831

39. Simopoulos, A. P. (2011). Evolutionary aspects of diet: The omega-6/omega-3 ratio and the brain. *Molecular Neurobiology*, *44*(2), 203–215. https://doi.org/10.1007/s12035-010-8162-0

40. Bartram, H. P., Gostner, A., Scheppach, W., Reddy, B. S., Rao, C. V., Dusel, G., Richter, F., Richter, A., & Kasper, H. (1993). Effects of fish oil on rectal cell proliferation, mucosal fatty acids, and prostaglandin E2 release in healthy subjects. *Gastroenterology*, *105*(5), 1317–1322. https://doi.org/10.1016/0016-5085(93)90135-Y

41. Bartram, H. P., Gostner, A., Reddy, B. S., Rao, C. V., Scheppach, W., Dusel, G., Richter, A., Richter, F., & Kasper, H. (1995). Missing anti-proliferative effect of fish oil on rectal epitelium in healthy volunteers consuming a high-fat diet: potential role of the n-3:n-6 fatty acid ratio. *European Journal of Cancer Prevention*, *4*(3), 231–237. https://doi.org/10.1097/00008469-199506000-00004

42. James, M.J., & Cleland, L.G. (1997). Dietary n-3 fatty acids and therapy for rheumatoid arthritis. *Seminars in Arthritis and Rheumatism*, *27*(2), 85–97. https://doi.org/10.1016/S0049-0172(97)80009-1

43. Broughton, K.S., Johnson, C.S., Pace, B.K., Liebman, M., & Kleppinger, K.M. (1997). Reduced asthma symptoms with n-3 fatty acid ingestion are related to 5- series leukotriene production. *American Journal of Clinical Nutrition*, *65*(4), 1011–1017. https://doi.org/10.1093/ajcn/65.4.1011

44. Greenberg, J.A., Bell, S.J., & Ausdal, W. Van. (2008). Omega-3 Fatty Acid supplementation during pregnancy. *Reviews in Obstetrics & Gynecology*, *1*(4), 162–169. http://www.ncbi.nlm.nih.gov/pubmed/19173020%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2621042

45. Helland, I.B., Smith, L., Saarem, K., Saugstad, O.D., & Drevon, C.A. (2003). Maternal supplementation with very-long-chain n-3 fatty acids during pregnancy and lactation augments children's IQ at 4 years of age. *Pediatrics*, *111*(1). https://doi.org/10.1542/peds.111.1.e39

46. Larque, E., Demmelmair, H., & Koletzko, B. (2002). Perinatal Supply and Metabolism of Long-Chain Polyunsaturated Fatty Acids Importance for the Early Development of the Nervous System. *Annals New York Academy of Science*, *967*, 299–310.

47. Janssen, C.I.F., & Kiliaan, A.J. (2014). Long-chain polyunsaturated fatty acids (LCPUFA) from genesis to senescence: The influence of LCPUFA on neural development, aging, and neurodegeneration. *Progress in Lipid Research*, *53*(1), 1–17. https://doi.org/10.1016/j.plipres.2013.10.002

48. Akerele, O.A., & Cheema, S.K. (2016). A balance of omega-3 and omega-6 polyunsaturated fatty acids is important in pregnancy. *Journal of Nutrition and Intermediary Metabolism*, *5*, 23–33. https://doi.org/10.1016/j.jnim.2016.04.008

49. Maekawa, M., Takashima, N., Matsumata, M., Ikegami, S., Kontani, M., Hara, Y., Kawashima, H., Owada, Y., Kiso, Y., Yoshikawa, T., Inokuchi, K., & Osumi, N. (2009). Arachidonic acid drives postnatal neurogenesis and elicits a beneficial effect on prepulse inhibition, a biological trait of psychiatric illnesses. *PLoS ONE*, *4*(4), 1–9. https://doi.org/10.1371/journal.pone.0005085

50. Brenna, J.T. (2016). Long-chain polyunsaturated fatty acids and the preterm infant: A case study in developmentally sensitive nutrient needs in the United States. *American Journal of Clinical Nutrition*, *103*(2), 606S-615S. https://doi.org/10.3945/ajcn.114.103994

51. Tallima, H., & Ridi, R.E. (2018). Arachidonic acid: Physiological roles and potential health benefits – A review. *Journal of Advanced Research*, *11*, 33–41. https://doi.org/10.1016/j.jare.2017.11.004

52. Hanna, V. S., & Hafez, E.A.A. (2018). Synopsis of arachidonic acid metabolism: A review. *Journal of Advanced Research*, *11*, 23–32. https://doi.org/10.1016/j.jare.2018.03.005

53. Harauma, A., Hatanaka, E., Yasuda, H., Nakamura, M.T., Salem, N., & Moriguchi, T. (2017). Effects of arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid on brain development using artificial rearing of delta-6-desaturase knockout mice. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *127*(October), 32–39. https://doi.org/10.1016/j.plefa.2017.10.001

54. Lauritzen, L., Fewtrell, M., & Agostoni, C. (2015). Dietary arachidonic acid in perinatal nutrition: A commentary. *Pediatric Research*, *77*(1), 263–269. https://doi.org/10.1038/pr.2014.166

55. Cetin, I., & Koletzko, B. (2008). Long-chain v-3 fatty acid supply in pregnancy and lactation. *Current Opinion in Clinical Nutrition and Metabolic Care*, *11*, 297–302. https://doi.org/10.1111/j.1477-8947.1982.tb01029.x

56. Hadders-Algra, M. (2008). Prenatal long-chain polyunsaturated fatty acid status: The importance of a balanced intake of docosahexaenoic acid and arachidonic acid. *Journal of Perinatal Medicine*, *36*(2), 101–109. https://doi.org/10.1515/JPM.2008.029

57. Colombo, J., Jill Shaddy, D., Kerling, E. H., Gustafson, K. M., & Carlson, S. E. (2017). Docosahexaenoic acid (DHA) and arachidonic acid (ARA) balance in developmental outcomes☆. *Prostaglandins Leukotrienes & Essential Fatty Acids*, *121*, 52–56. https://doi.org/10.1016/j.plefa.2017.05.005.

58. *Global Recommendations for EPA and DHA Intake*. (2014). Global Organization for EPA and DHA Omega-3. https://goedomega3.com/intake-recommendations

59. Brenna, J. T., & Lapillonne, A. (2009). Background paper on fat and fatty acid requirements during pregnancy and lactation. *Annals of Nutrition and Metabolism*, *55*(1–3), 97–122. https://doi.org/10.1159/000228998

60. Eidelman, A. I., & Schanler, R. J. (2012). Breastfeeding and the use of human milk. *Pediatrics*, *129*(3), e827–e841. https://doi.org/10.1542/peds.2011-3552

61. Richter, C. K., Bowen, K. J., Mozaffarian, D., Kris-Etherton, P. M., & Skulas-Ray, A. C. (2017). Total Long-Chain n-3 Fatty Acid Intake and Food Sources in the United States Compared to Recommended Intakes: NHANES 2003–2008. *Lipids*, *52*(11), 917–927. https://doi.org/10.1007/s11745-017-4297-3

62. Tang, M., Zhang, M., Wang, L., Li, H., Cai, H., Dang, R., Jiang, P., Liu, Y., Xue, Y., & Wu, Y. (2018). Maternal dietary of n-3 polyunsaturated fatty acids affects the neurogenesis and neurochemical in female rat at weaning. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *128*(January 2017), 11–20. https://doi.org/10.1016/j.plefa.2017.11.001

63. Thompson, M., Hein, N., Hanson, C., Smith, L. M., Anderson-Berry, A., Richter, C. K., Bisselou, K. S., Appiah, A. K., Kris-Etherton, P., Skulas-Ray, A. C., & Nordgren, T. M. (2019). Omega-3 fatty acid intake by age, gender, and pregnancy status in the United States: National health and nutrition examination survey 2003–2014. *Nutrients*, *11*(1), 177–189. https://doi.org/10.3390/nu11010177

64. Nordgren, T. M., Lyden, E., Anderson-Berry, A., & Hanson, C. (2017). Omega-3 fatty acid intake of pregnant women and women of childbearing age in the united states: Potential for deficiency? *Nutrients*, *9*(3). https://doi.org/10.3390/nu9030197

65. Zhang, Z., Fulgoni, V. L., Kris-Etherton, P. M., & Mitmesser, S. H. (2018). Dietary intakes of EPA and DHA omega-3 fatty acids among US childbearing-age and pregnant women: An analysis of NHANES 2001–2014. *Nutrients*, *10*(4). https://doi.org/10.3390/nu10040416

66. Khaire, A. A., Kale, A. A., & Joshi, S. R. (2015). Maternal omega-3 fatty acids and micronutrients modulate fetal lipid metabolism: A review. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *98*, 49–55. https://doi.org/10.1016/j.plefa.2015.04.007

67. Lauritzen, L., Fewtrell, M., & Agostoni, C. (2015). Dietary arachidonic acid in perinatal nutrition: A commentary. *Pediatric Research*, *77*(1), 263–269. https://doi.org/10.1038/pr.2014.166

68. Larqué, E., Gil-Sánchez, A., Prieto-Sánchez, M. T., & Koletzko, B. (2012). Omega 3 fatty acids, gestation and pregnancy outcomes. *British Journal of Nutrition*, *107*(SUPPL. 2). https://doi.org/10.1017/S0007114512001481

69. Rogers, L. K., Valentine, C. J., & Keim, S. A. (2013). DHA supplementation: Current implications in pregnancy and childhood. *Pharmacological Research*, *70*(1), 13–19. https://doi.org/10.1016/j.phrs.2012.12.003

70. Lauritzen, L., Brambilla, P., Mazzocchi, A., Harsløf, L. B. S., Ciappolino, V., & Agostoni, C. (2016). DHA effects in brain development and function. *Nutrients*, *8*(1), 1–17. https://doi.org/10.3390/nu8010006

71. Colombo, J., Jill Shaddy, D., Gustafson, K., Gajewski, B. J., Thodosoff, J. M., Kerling, E., & Carlson, S. E. (2019). The Kansas University DHA Outcomes Study (KUDOS) clinical trial: Long-term behavioral follow-up of the effects of prenatal DHA supplementation. *American Journal of Clinical Nutrition*, *109*, 1380–1392. https://doi.org/10.1093/ajcn/nqz018

72. Braarud, H. C., Markhus, M. W., Skotheim, S., Stormark, K. M., Frøyland, L., Graff, I. E., & Kjellevold, M. (2018). Maternal DHA status during pregnancy has a positive impact on infant problem solving: A Norwegian prospective observation study. *Nutrients*, *10*(5). https://doi.org/10.3390/nu10050529

73. Koletzko, B., Agostoni, C., Carlson, S., Clandinin, T., Hornstra, G., Neuringer, M., Uauy, R., Yamashiro, Y., & Willatts, P. (2007). Long chain polyunsaturated fatty acids (LC-PUFA) and perinatal development. *Acta Paediatrica*, *90*(4), 460–464. https://doi.org/10.1111/j.1651-2227.2001.tb00452.x

74. Tanaka, K., Farooqui, A. A., Siddiqi, N. J., Alhomida, A. S., & Ong, W. Y. (2012). Effects of docosahexaenoic acid on neurotransmission. *Biomolecules and Therapeutics*, *20*(2), 152–157. https://doi.org/10.4062/biomolther.2012.20.2.152

75. Makrides, M., Collins, C. T., & Gibson, R. A. (2011). Impact of fatty acid status on growth and neurobehavioural development in humans. *Maternal and Child Nutrition*, *7*(SUPPL. 2), 80–88. https://doi.org/10.1111/j.1740-8709.2011.00304.x

76. Hadders-Algra, M., Bouwstra, H., Van Goor, S. A., Dijck-Brouwer, D. A. J., & Muskiet, F. A. J. (2007). Prenatal and early postnatal fatty acid status and neurodevelopmental outcome. *Journal of Perinatal Medicine*, *35*(SUPPL. 1), 28–34. https://doi.org/10.1515/JPM.2007.034

77. Healy-Stoffel, M., & Levant, B. (2018). N-3 (Omega-3) Fatty Acids: Effects on Brain Dopamine Systems and Potential Role in the Etiology and Treatment of Neuropsychiatric Disorders. *CNS & Neurological Disorders - Drug Targets*, *17*(3), 216–232. https://doi.org/10.2174/1871527317666180412153612

78. Lacombe, R. J. S., Chouinard-Watkins, R., & Bazinet, R. P. (2018). Brain docosahexaenoic acid uptake and metabolism. *Molecular Aspects of Medicine*. https://doi.org/10.1016/j.mam.2017.12.004

79. Kan, I., Melamed, E., Offen, D., & Green, P. (2007). Docosahexaenoic acid and arachidonic acid are fundamental supplements for the induction of neuronal differentiation. *Journal of Lipid Research*, *48*(3), 513–517. https://doi.org/10.1194/jlr.C600022-JLR200

80. Kidd, P. M. (2007). Omega-3 DHA and EPA for cognition, behavior, and mood: Clinical findings and structural-functional synergies with cell membrane phospholipids. *Alternative Medicine Review*, *12*(3), 207–227. https://doi.org/10.1155/2013/824563

81. Tian, C., Fan, C., Liu, X., Xu, F., & Qi, K. (2011). Brain histological changes in young mice submitted to diets with different ratios of n-6/n-3 polyunsaturated fatty acids during maternal pregnancy and lactation. *Clinical Nutrition*, *30*(5), 659–667. https://doi.org/10.1016/j.clnu.2011.03.002

82. Horrocks, L. A., & Farooqui, A. A. (2004). Docosahexaenoic acid in the diet: Its importance in maintenance and restoration of neural membrane function. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *70*(4), 361–372. https://doi.org/10.1016/j.plefa.2003.12.011

83. Haag, M. (2003). Essential fatty acids and the brain. *Can J Psychiatry*, *48*(3), 195–203. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12728744

84. McNamara, R. K. (2015). Role of perinatal long-chain omega-3 fatty acids in cortical circuit maturation: Mechanisms and implications for psychopathology. *World Journal of Psychiatry*, *5*(1), 15. https://doi.org/10.5498/wjp.v5.i1.15

85. Innis, S. M., & de La Presa Owens, S. (2001). Dietary fatty acid composition in pregnancy alters neurite membrane fatty acids and dopamine in newborn rat brain. *The Journal of Nutrition*, *131*(1), 118–122. https://doi.org/10.1074/jbc.M710037200

86. Bhatia, H. S., Agrawal, R., Sharma, S., Huo, Y. X., Ying, Z., & Gomez-Pinilla, F. (2011). Omega-3 fatty acid deficiency during brain maturation reduces neuronal and behavioral plasticity in adulthood. *PLoS ONE*, *6*(12). https://doi.org/10.1371/journal.pone.0028451

87. Delion, S., Chalon, S., Hérault, J., Guilloteau, D., Besnard, J.-C., & Durand, G. (1994). Chronic Dietary α-Linolenic Acid Deficiency Alters Dopaminergic and Serotoninergic Neurotransmission in Rats. *The Journal of Nutrition*, *124*(12). https://doi.org/10.1093/jn/124.12.2466

88. Wainwright, P. E. (2002). Dietary essential fatty acids and brain function: a developmental perspective on mechanisms. *Proceedings of the Nutrition Society*, *61*(01), 61–69. https://doi.org/10.1079/pns2001130

89. A. Farooqui, A. (2012). n-3 Fatty Acid-Derived Lipid Mediators in the Brain: New Weapons Against Oxidative Stress and Inflammation. *Current Medicinal Chemistry*, *19*(4), 532–543. https://doi.org/10.2174/092986712798918851

90. Hadders-Algra, M. (2011). Prenatal and early postnatal supplementation with long-chain polyunsaturated fatty acids: Neurodevelopmental considerations. *American Journal of Clinical Nutrition*, *94*(6), 1874–1879. https://doi.org/10.3945/ajcn.110.001065

91. Innis, S. M. (2007). Fatty acids and early human development. *Early Human Development*, *83*(12), 761–766. https://doi.org/10.1016/j.earlhumdev.2007.09.004

92. Herrera, E. (2002). Lipid metabolism in pregnancy and its consequences in the fetus and newborn. *Endocrine*, *19*(1), 43–55. https://doi.org/10.1385/ENDO:19:1:43

93. Kuipers, R. S., Luxwolda, M. F., Offringa, P. J., Rudi Boersma, E., Dijck-Brouwer, D. A. J., & Muskiet, F. A. J. (2012). Fetal intrauterine whole body linoleic, arachidonic and docosahexaenoic acid contents and accretion rates. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *86*(1–2), 13–20. https://doi.org/10.1016/j.plefa.2011.10.012

94. Cardoso, C., Afonso, C., & Bandarra, N. M. (2018). Dietary DHA, bioaccessibility, and neurobehavioral development in children. *Critical Reviews in Food Science and Nutrition*, *58*(15), 2617–2631. https://doi.org/10.1080/10408398.2017.1338245

95. McNamara, R. K., & Carlson, S. E. (2006). Role of omega-3 fatty acids in brain development and function: Potential implications for the pathogenesis and prevention of psychopathology. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *75*(4–5), 329–349. https://doi.org/10.1016/j.plefa.2006.07.010

96. Novak, E. M., Dyer, R. A., & Innis, S. M. (2008). High dietary ω-6 fatty acids contribute to reduced docosahexaenoic acid in the developing brain and inhibit secondary neurite growth. *Brain Research*, *1237*, 136–145. https://doi.org/10.1016/j.brainres.2008.07.107

97. Larqué, E., Pagán, A., Prieto, M. T., Blanco, J. E., Gil-Sánchez, A., Zornoza-Moreno, M., Ruiz-Palacios, M., Gázquez, A., Demmelmair, H., Parrilla, J. J., & Koletzko, B. (2014). Placental fatty acid transfer: A key factor in fetal growth. *Annals of Nutrition and Metabolism*, *64*(3–4), 247–253. https://doi.org/10.1159/000365028

98. Innis, S. (2003). Perinatal biochemistry and physiology of long-chain polyunsaturated fatty acids. *The Journal of Pediatrics*, *143*(4 Suppl), S1-8. https://doi.org/10.1067/S0022-3476(03)00396-2

99. Lewis, R. M., Wadsack, C., & Desoye, G. (2018). Placental fatty acid transfer. *Current Opinion in Clinical Nutrition and Metabolic Care*, *21*(2), 78–82. https://doi.org/10.1097/MCO.0000000000000443

100. Lewis, R. M., Childs, C. E., & Calder, P. C. (2018). New perspectives on placental fatty acid transfer. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *138*(October), 24–29. https://doi.org/10.1016/j.plefa.2018.10.001

101. Liu, J. J., Green, P., John Mann, J., Rapoport, S. I., & Sublette, M. E. (2015). Pathways of polyunsaturated fatty acid utilization: Implications for brain function in neuropsychiatric health and disease. *Brain Research*, *1597*(646), 220–246. https://doi.org/10.1016/j.brainres.2014.11.059

102. Picq, M., Chen, P., Perez, M., Michaud, M., Véricel, E., Guichardant, M., & Lagarde, M. (2010). DHA metabolism: Targeting the brain and lipoxygenation. *Molecular Neurobiology*, *42*(1), 48–51. https://doi.org/10.1007/s12035-010-8131-7

103. Chen, C. T., Kitson, A. P., Hopperton, K. E., Domenichiello, A. F., Trépanier, M. O., Lin, L. E., Ermini, L., Post, M., Thies, F., & Bazinet, R. P. (2015). Plasma non-esterified docosahexaenoic acid is the major pool supplying the brain. *Scientific Reports*, *5*, 1–12. https://doi.org/10.1038/srep15791

104. Domenichiello, A. F., Chen, C. T., Trepanier, M.-O., Stavro, P. M., & Bazinet, R. P. (2014). Whole body synthesis rates of DHA from α-linolenic acid are greater than brain DHA accretion and uptake rates in adult rats. *Journal of Lipid Research*, *55*, 62–74. https://doi.org/10.1194/jlr.M042275

105. Igarashi, M., Kim, H. W., Gao, F., Chang, L., Ma, K., & Rapoport, S. I. (2012). Fifteen weeks of dietary n-3 polyunsaturated fatty acid deprivation increase turnover of n-6 docosapentaenoic acid in rat-brain phospholipids. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, *1821*(9), 1235–1243. https://doi.org/10.1016/j.bbalip.2011.11.002

106. Spector, A. A. (2001). Plasma free fatty acid and lipoproteins as sources of polyunsaturated fatty acid for the brain. *Journal of Molecular Neuroscience*, *16*(2–3), 159–165; discussion 215-21. https://doi.org/10.1385/JMN:16:2-3:159

107. Zhang, W., Li, P., Hu, X., Zhang, F., Chen, J., & Gao, Y. (2011). Omega-3 polyunsaturated fatty acids in the brain: metabolism and neuroprotection. *Fronteirs in Bioscience*, *16*, 2653–2670.

108. Chen, C. T., Green, J. T., Orr, S. K., & Bazinet, R. P. (2008). Regulation of brain polyunsaturated fatty acid uptake and turnover. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *79*, 85–91. https://doi.org/10.1016/j.plefa.2008.09.003

109. Green, J. T., Orr, S. K., Bazinet, R. P., & Green, J.T.; Orr, S.K.; Bazinet, R. P. (2008). The emerging role of group VI calcium-independent phospholipase A2 in releasing docosahexaenoic acid from brain phospholipids. *The Journal of Lipid Research*, *49*, 939–944. https://doi.org/10.1194/jlr.R700017-JLR200

110. Murphy, R. C., & Folco, G. (2019). Lysophospholipid acyltransferases and leukotriene biosynthesis: Intersection of the Lands cycle and the arachidonate PI cycle. *Journal of Lipid Research*, *60*(2), 219–226. https://doi.org/10.1194/jlr.S091371

111. Kim, H. W., Rao, J. S., Rapoport, S. I., & Igarashi, M. (2011). Regulation of rat brain polyunsaturated fatty acid (PUFA) metabolism during graded dietary n-3 PUFA deprivation. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *85*(6), 361–368. https://doi.org/10.1016/j.plefa.2011.08.002

112. Kim, H. W., Rao, J. S., Rapoport, S. I., & Igarashi, M. (2011). Dietary n-6 PUFA deprivation downregulates arachidonate but upregulates docosahexaenoate metabolizing enzymes in rat brain. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, *1811*(2), 111–117. https://doi.org/10.1016/j.bbalip.2010.10.005

113. Martin, R. E., & Bazan, N. G. (1992). Changing Fatty Acid Content of Growth Cone Lipids Prior to Synaptogenesis. *Journal of Neurochemistry*, *59*(1), 318–325. https://doi.org/10.1111/j.1471-4159.1992.tb08906.x

114. Green, P., Glozman, S., Kamensky, B., & Yavin, E. (1999). Developmental changes in rat brain membrane lipids and fatty acids: The preferential prenatal accumulation of docosahexaenoic acid. *Journal of Lipid Research*, *40*(5), 960–966.

115. Bertrand, N., & Dahmane, N. (2006). Sonic hedgehog signaling in forebrain development and its interactions with pathways that modify its effects. *Trends in Cell Biology*, *16*(11), 597–605. https://doi.org/10.1016/j.tcb.2006.09.007

116. Sakayori, N., Tokuda, H., Yoshizaki, K., Kawashima, H., Innis, S. M., Shibata, H., & Osumi, N. (2016). Maternal Nutritional Imbalance between Linoleic Acid and Alpha-Linolenic Acid Increases Offspring's Anxious Behavior with Sex-Dependent Manner in Mice. *Tohoku J Exp Med*, *240*(1), 31–37. https://doi.org/10.1620/tjem.240.31.

117. Innis, S. M., De, S., & Owens, P. (2001). Dietary Fatty Acid Composition in Pregnancy Alters Neurite Membrane Fatty Acids and Dopamine in Newborn Rat Brain. *J Nutr*, *131*, 118–122.

118. Sakamoto, T., Cansev, M., & Wurtman, R. J. (2007). Oral Supplementation with Docosahexaenoic Acid and Uridine-5'- Monophosphate Increases Dendritic Spine Density in Adult Gerbil Hippocampus. *Brain Research*, *1182*, 50–59. https://doi.org/10.1016/j.brainres.2007.08.089

119. Cansev, M., & Wurtman, R. J. (2007). Chronic administration of docosahexaenoic acid or eicosapentaenoic acid, but not arachidonic acid, alone or in combination with uridine, increases brain phosphatide and synaptic protein levels in gerbils. *Neuroscience*, *148*(2), 421–431. https://doi.org/10.1038/jid.2014.371

120. Wurtman, R. J. (2008). Synapse Formation and Cognitive Brain Development: effect of docosahexaenoic (DHA) and other dietary constituents. *Metabolism*, *57*(Suppl 2), S6-10. https://doi.org/10.1038/jid.2014.371

121. Pongrac, J. L., Slack, P. J., & Innis, S. M. (2007). Dietary polyunsaturated fat that is low in (n-3) and high in (n-6) fatty acids alters the SNARE protein complex and nitrosylation in rat hippocampus. *The Journal of Nutrition*, *137*(8), 1852–1856. https://doi.org/10.1093/jn/137.8.1852

122. Zimmer, L., Hembert, S., Durand, G., Breton, P., Guilloteau, D., Besnard, J. C., & Chalon, S. (1998). Chronic n-3 polyunsaturated fatty acid diet-deficiency acts on dopamine metabolism in the rat frontal cortex: A microdialysis study. *Neuroscience Letters*, *240*(3), 177–181. https://doi.org/10.1016/S0304-3940(97)00938-5

123. Zimmer, L., Delion-Vancassel, S., Cantagrel, S., Breton, P., Delamanche, S., Guilloteau, D., Durand, G., & Chalon, S. (2002). The dopamine mesocorticolimbic pathway is affected by deficiency in n-3 polyunsaturated fatty acids. *AM J Clin Nutr*, *75*, 662–667.

124. Chalon, S. (2006). Omega-3 fatty acids and monoamine neurotransmission. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *75*(4–5), 259–269. https://doi.org/10.1016/j.plefa.2006.07.005

125. Vancassel, S., Blondeau, C., Lallemand, S., Cador, M., Linard, A., Lavialle, M., & Dellu-Hagedorn, F. (2007). Hyperactivity in the rat is associated with spontaneous low level of n-3 polyunsaturated fatty acids in the frontal cortex. *Behavioural Brain Research*, *180*(2), 119–126. https://doi.org/10.1016/j.bbr.2007.02.032

126. Brenna, J. T. (2011). Animal studies of the functional consequences of suboptimal polyunsaturated fatty acid status during pregnancy, lactation and early post-natal life. *Maternal and Child Nutrition*, *7*(SUPPL. 2), 59–79. https://doi.org/10.1111/j.1740-8709.2011.00301.x

127. Calderon, F., & Kim, H. Y. (2004). Docosahexaenoic acid promotes neurite growth in hippocampal neurons. *Journal of Neurochemistry*, *90*(4), 979–988. https://doi.org/10.1111/j.1471-4159.2004.02520.x

128. Cao, D., Kevala, K., Kim, J., Moon, H.-S., Jun, S. B., Lovinger, D., & Kim, H.-Y. (2009). Docosahexaenoic acid promotes hippocampal neuronal development and synaptic function. *Journal of Neurochemistry*, *111*(2), 510–521. https://doi.org/10.1111/j.1471-4159.2009.06335.x

129. Kawakita, E., Hashimoto, M., & Shido, O. (2006). Docosahexaenoic acid promotes neurogenesis in vitro and in vivo. *Neuroscience*, *139*(3), 991–997. https://doi.org/10.1016/j.neuroscience.2006.01.021

130. Ikemoto, A., Kobayashi, T., Watanabe, S., & Okuyama, H. (1997). Membrane fatty acid modifications of PC12 cells by arachidonate or docosahexaenoate affect neurite outgrowth but not norepinephrine release. *Neurochemical Research*, *22*(6), 671–678. https://doi.org/10.1023/A:1027393724676

131. Dagai, L., Peri-Naor, R., & Birk, R. Z. (2009). Docosahexaenoic acid significantly stimulates immediate early response genes and neurite outgrowth. *Neurochemical Research*, *34*(5), 867–875. https://doi.org/10.1007/s11064-008-9845-z

132. Martin, R. E., Wickham, J. Q., Om, A.-S., Sanders, J., & Ceballos, N. (2000). Uptake and Incorporation of Docosahexaenoic Acid (DHA) into Neuronal Cell Body and Neurite/Nerve Growth Cone Lipids: Evidence of Compartmental DHA Metabolism in Nerve Growth Factor-Differentiated PC12 Cells. *Neurochemical Research*, *25*(5), 715–723.

133. Petroni, A., Blasevich, M., Papini, N., La Spada, P., & Galli, C. (1996). Changes in Arachidonic Acid Levels and Formation and in Lipid Synthesis in the Human Neuroblastoma SK-N-BE During Retinoic Acid-Induced Differentiation. *Journal of Neurochemistry*, *67*, 549–556.

134. Dehaut, F., Bertrand, I., Miltaud, T., Pouplard-Barthelaix, A., & Maingault, M. (1993). N-6 polyunsaturated fatty acids increase the neurite length of PC12 cells and embryonic chick motoneurons. *Neuroscience Letters*, *161*(2), 133–136. https://doi.org/10.1016/0304-3940(93)90277-R

135. Williams, E. J., Walsh, F. S., & Doherty, P. (1994). The Production of Arachidonic Acid Can Account for Calcium Channel Activation in the Second Messenger Pathway Underlying Neurite Outgrowth Stimulated by NCAM, N-Cadherin, and L1. *Journal of Neurochemistry*, *62*(3), 1231–1234. https://doi.org/10.1046/j.1471-4159.1994.62031231.x

136. Robson, L. G., Dyall, S., Sidloff, D., & Michael-Titus, A. T. (2010). Omega-3 polyunsaturated fatty acids increase the neurite outgrowth of rat sensory neurones throughout development and in aged animals. *Neurobiology of Aging*, *31*(4), 678–687. https://doi.org/10.1016/j.neurobiolaging.2008.05.027

137. Kan, I., Melamed, E., Offen, D., & Green, P. (2007). Docosahexaenoic acid and arachidonic acid are fundamental supplements for the induction of neuronal differentiation. *Journal of Lipid Research*, *48*(3), 513–517. https://doi.org/10.1194/jlr.C600022-JLR200

138. Géraldine, M., Stéphanie, D., Bénédicte, L., Isabelle, D., Monique, L., & Sylvie, V. (2010). DHA enhances the noradrenaline release by SH-SY5Y cells. *Neurochemistry International*, *56*(1), 94–100. https://doi.org/10.1016/j.neuint.2009.09.006

139. Goustard-Langelier, B., Koch, M., Lavialle, M., & Heberden, C. (2013). Rat neural stem cell proliferation and differentiation are durably altered by the in utero polyunsaturated fatty acid supply. *Journal of Nutritional Biochemistry*, *24*(1), 380–387. https://doi.org/10.1016/j.jnutbio.2012.08.001

140. Luchtman, D. W., & Song, C. (2013). Cognitive enhancement by omega-3 fatty acids from child-hood to old age: Findings from animal and clinical studies. *Neuropharmacology*, *64*, 550–565. https://doi.org/10.1016/j.neuropharm.2012.07.019

141. Kuperstein, F., Eilam, R., & Yavin, E. (2008). Altered expression of key dopaminergic regulatory proteins in the postnatal brain following perinatal n-3 fatty acid dietary deficiency. *Journal of Neurochemistry*, *106*(2), 662–671. https://doi.org/10.1111/j.1471-4159.2008.05418.x

142. Dyall, S. C. (2015). Long-chain omega-3 fatty acids and the brain: A review of the independent and shared effects of EPA, DPA and DHA. *Frontiers in Aging Neuroscience*, *7*(APR), 1–15. https://doi.org/10.3389/fnagi.2015.00052

143. Bazan, N. G., Musto, A. E., & Knott, E. J. (2011). Endogenous signaling by omega-3 docosahexaenoic acid-derived mediators sustains homeostatic synaptic and circuitry integrity. *Molecular Neurobiology*, *44*(2), 216–222. https://doi.org/10.1007/s12035-011-8200-6

144. Akbar, M., Calderon, F., Wen, Z., & Kim, H. Y. (2005). Docosahexaenoic acid: A positive modulator of Akt signaling in neuronal survival. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(31), 10858–10863. https://doi.org/10.1073/pnas.0502903102

145. Kim, S. J., Zhang, Z., Saha, A., Sarkar, C., Zhao, Z., Xu, Y., & Mukherjee, A. B. (2010). Omega-3 and omega-6 fatty acids suppress ER- and oxidative stress in cultured neurons and neuronal progenitor cells from mice lacking PPT1. *Neuroscience Letters*, *479*(3), 292–296. https://doi.org/10.1016/j.neulet.2010.05.083

146. Kou, W., Luchtman, D., & Song, C. (2008). Eicosapentaenoic acid (EPA) increases cell viability and expression of neurotrophin receptors in retinoic acid and brain-derived neurotrophic factor differentiated SH-SY5Y cells. *European Journal of Nutrition*, *47*(2), 104–113. https://doi.org/10.1007/s00394-008-0703-1

147. Zhang, Y. P., Brown, R. E., Zhang, P. C., Zhao, Y. T., Ju, X. H., & Song, C. (2018). DHA, EPA and their combination at various ratios differently modulated Aβ25-35-induced neurotoxicity in SH-SY5Y cells. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *136*(February 2017), 85–94. https://doi.org/10.1016/j.plefa.2017.07.003

148. Blanpied, T. A., & Ehlers, M. D. (2004). Microanatomy of dendritic spines: Emerging principles of synaptic pathology in psychiatric and neurological disease. *Biological Psychiatry*, *55*(12), 1121–1127. https://doi.org/10.1016/j.biopsych.2003.10.006

149. Lukiw, W. J., Cui, J. G., Marcheselli, V. L., Bodker, M., Botkjaer, A., Gotlinger, K., Serhan, C. N., & Bazan, N. G. (2005). A role for docosahexaenoic acid-derived neuroprotectin D1 in neural cell survival and Alzheimer disease. *Journal of Clinical Investigation*, *115*(10), 2774–2783. https://doi.org/10.1172/JCI25420

150. Grayson, D. S., Kroenke, C. D., Neuringer, M., & Fair, D. A. (2014). Dietary omega-3 fatty acids modulate large-scale systems organization in the rhesus macaque brain. *The Journal of Neuroscience*, *34*(6), 2065–2074. https://doi.org/10.1523/JNEUROSCI.3038-13.2014

151. Madore, C., Nadjar, A., Delpech, J. C., Sere, A., Aubert, A., Portal, C., Joffre, C., & Layé, S. (2014). Nutritional n-3 PUFAs deficiency during perinatal periods alters brain innate immune system and neuronal plasticity-associated genes. *Brain, Behavior, and Immunity*, *41*(1), 22–31. https://doi.org/10.1016/j.bbi.2014.03.021

152. Zimmer, L., Delpal, S., Guilloteau, D., Aïoun, J., Durand, G., & Chalon, S. (2000). Chronic n-3 polyunsaturated fatty acid deficiency alters dopamine vesicle density in the rat frontal cortex. *Neuroscience Letters*, *284*(1–2), 25–28. https://doi.org/10.1016/S0304-3940(00)00950-2

153. Yoshida, S., Yasuda, a, Kawazato, H., Sakai, K., Shimada, T., Takeshita, M., Yuasa, S., Kobayashi, T., Watanabe, S., & Okuyama, H. (1997). Synaptic vesicle ultrastructural changes in the rat hippocampus induced by a combination of alpha-linolenate deficiency and a learning task. *Journal of Neurochemistry*, *68*(3), 1261–1268.

154. Takeuchi, T., Fukumoto, Y., & Harada, E. (2002). Influence of a dietary n-3 fatty acid deficiency on the cerebral catecholamine contents, EEG and learning ability in rat. *Behavioural Brain Research*, *131*(1–2), 193–203. https://doi.org/10.1016/S0166-4328(01)00392-8

155. Moreira, J. D., Knorr, L., Ganzella, M., Thomazi, A. P., de Souza, C. G., de Souza, D. G., Pitta, C. F., e Souza, T. M., Wofchuk, S., Elisabetsky, E., Vinadé, L., Perry, M. L. S., & Souza, D. O. (2010). Omega-3 fatty acids deprivation affects ontogeny of glutamatergic synapses in rats: Relevance for behavior alterations. *Neurochemistry International*, *56*(6–7), 753–759. https://doi.org/10.1016/j.neuint.2010.02.010

156. Lozada, L. E., Desai, A., Kevala, K., Lee, J.-W., & Kim, H.-Y. (2017). Perinatal Brain Docosahexaenoic Acid Concentration Has a Lasting Impact on Cognition in Mice. *The Journal of Nutrition*, *12*, jn254607. https://doi.org/10.3945/jn.117.254607

157. Coti Bertrand, P., O'Kusky, J. R., & Innis, S. M. (2006). Maternal dietary (n-3) fatty acid deficiency alters neurogenesis in the embryonic rat brain. *Journal of Nutrition*, *136*, 1570–1575. https://doi.org/136/6/1570 [pii]

158. Yavin, E., Himovichi, E., & Eilam, R. (2009). Delayed cell migration in the developing rat brain following maternal omega 3 alpha linolenic acid dietary deficiency. *Neuroscience*, *162*(4), 1011–1022. https://doi.org/10.1016/j.neuroscience.2009.05.012

159. Ahmad, A., Moriguchi, T., & Salem, N. (2002). Decrease in neuron size in docosahexaenoic acid-deficient brain. *Pediatric Neurology*, *26*(3), 210–218. https://doi.org/10.1016/S0887-8994(01)00383-6

160. Ahmad, A., Murthy, M., Greiner, R. S., Moriguchi, T., & Salem, N. (2002). A decrease in cell size accompanies a loss of docosahexaenoate in the rat hippocampus. *Nutritional Neuroscience*, *5*(2), 103–113. https://doi.org/10.1080/10284150290018973

161. Wainwright, P. E., Bulman-Fleming, M. B., Lévesque, S., Mutsaers, L., & McCutcheon, D. (1998). A Saturated-Fat Diet during Development Alters Dendritic Growth in Mouse Brain. *Nutritional Neuroscience*, *1*(1), 49–58. https://doi.org/10.1080/1028415X.1998.11747212

162. Moriguchi, T., Greiner, R. S., & Salem, N. (2000). Behavioral deficits associated with dietary induction of decreased brain docosahexaenoic acid concentration. *Journal of Neurochemistry*, *75*(6), 2563–2573. https://doi.org/10.1046/j.1471-4159.2000.0752563.x

163. García-Calatayud, S., Redondo, C., Martín, E., Ruiz, J. I., García-Fuentes, M., & Sanjurjo, P. (2005). Brain docosahexaenoic acid status and learning in young rats submitted to dietary long-chain polyunsaturated fatty acid deficiency and supplementation limited to lactation. *Pediatric Research*, *57*(5 I), 719–723. https://doi.org/10.1203/01.PDR.0000156506.03057.AD

164. Larrieu, T., Madore, C., Joffre, C., & Layé, S. (2012). Nutritional n-3 polyunsaturated fatty acids deficiency alters cannabinoid receptor signaling pathway in the brain and associated anxiety-like behavior in mice. *Journal of Physiology and Biochemistry*, *68*(4), 671–681. https://doi.org/10.1007/s13105-012-0179-

165. Reisbick, S., Neuringer, M., Hasnain, R., & Connor, W. E. (1994). Home cage behavior of rhesus monkeys with long-term deficiency of omega-3 fatty acids. *Physiology and Behavior*, *55*(2), 231–239. https://doi.org/10.1016/0031-9384(94)90128-7

166. Carrié, I., Clément, M., Javel, D. De, Francès, H., Bourre, J.-M., Inserm, U., Neuro-pharmaco-nutrition, U. De, Ponroy, L., & de Javel, D. (2000). Phospholipid supplementation reverses behavioral and biochemical alterations induced by n-3 polyunsaturated fatty acid deficiency in mice. *Journal of Lipid Research*, *41*(3), 473–480. https://doi.org/10.1080/09637480500512930

167. Anderson, G. J., Neuringer, M., Lin, D. S., & Connor, W. E. (2005). Can prenatal N-3 fatty acid deficiency be completely reversed after birth? Effects on retinal and brain biochemistry and visual function in rhesus monkeys. *Pediatric Research*, *58*(5), 865–872. https://doi.org/10.1203/01.pdr.0000182188.31596.5a

168. Harris, W. S. (2018). The Omega-6:Omega-3 ratio: A critical appraisal and possible successor. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *132*(March), 34–40. https://doi.org/10.1016/j.plefa.2018.03.003

169. Shrestha, N., Sleep, S. L., Cuffe, J. S. M., Holland, O. J., Perkins, A. V., Yau, S. Y., McAinch, A. J., & Hryciw, D. H. (2020). Role of omega-6 and omega-3 fatty acids in fetal programming. *Clinical and Experimental Pharmacology and Physiology*, *47*(5), 907–915. https://doi.org/10.1111/1440-1681.13244

170. Davis-Bruno, K., & Tassinari, M. S. (2011). Essential fatty acid supplementation of DHA and ARA and effects on neurodevelopment across animal species: A review of the literature. *Birth Defects Research Part B - Developmental and Reproductive Toxicology*, *92*(3), 240–250. https://doi.org/10.1002/bdrb.20311

171. Yang, B., Li, R., Woo, T., Browning, J. D., Song, H., Gu, Z., Cui, J., Lee, J. C., Fritsche, K. L., Beversdorf, D. Q., Sun, G. Y., & Greenlief, C. M. (2019). Maternal dietary docosahexaenoic acid alters lipid peroxidation products and (n-3)/(n-6) fatty acid balance in offspring mice. *Metabolites*, *9*(3), 40–53. https://doi.org/10.3390/metabo9030040

172. Spiller, P., Hibbeln, J. R., Myers, G., Vannice, G., Golding, J., Crawford, M. A., Strain, J. J., Connor, S. L., Brenna, J. T., Kris-Etherton, P., Holub, B. J., Harris, W. S., Lands, B., McNamara, R. K., Tlusty, M. F., Salem, N., & Carlson, S. E. (2019). An abundance of seafood consumption studies presents new opportunities to evaluate effects on neurocognitive development. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *151*(September), 8–13. https://doi.org/10.1016/j.plefa.2019.10.001

173. Dyall, S. C. (2011). Methodological issues and inconsistencies in the field of omega-3 fatty acids research. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *85*(5), 281–285. https://doi.org/10.1016/j.plefa.2011.04.009

174. Hejr, H., Ghareghani, M., Zibara, K., Ghafari, M., Sadri, F., Salehpour, Z., Hamedi, A., Negintaji, K., Azari, H., & Ghanbari, A. (2017). The ratio of 1/3 linoleic acid to alpha linolenic acid is optimal for oligodendrogenesis of embryonic neural stem cells. *Neuroscience Letters*, *651*, 216–225. https://doi.org/10.1016/j.neulet.2017.05.020

175. Sakayori, N., Kikkawa, T., Tokuda, H., Kiryu, E., Yoshizaki, K., Kawashima, H., Yamada, T., Arai, H., Kang, J. X., Katagiri, H., Shibata, H., Innis, S. M., Arita, M., & Osumi, N. (2016). Maternal Dietary Imbalance between Omega-6 and Omega-3 Polyunsaturated Fatty Acids Impairs Neocortical Development via Epoxy Metabolites. *Stem Cells*, *34*, 470–482. https://doi.org/10.1002/stem.2246

176. Guo, B. Q., Ding, S. Bin, & Li, H. Bin. (2019). Maternal n-3 PUFAs deficiency during pregnancy inhibits neural progenitor cell proliferation in fetal rat cerebral cortex. *International Journal of Developmental Neuroscience*, *76*(June), 72–79. https://doi.org/10.1016/j.ijdevneu.2019.07.001

177. Tang, M., Zhang, M., Cai, H., Li, H., Jiang, P., Dang, R., Liu, Y., He, X., Xue, Y., Cao, L., & Wu, Y. (2016). Maternal diet of polyunsaturated fatty acid altered the cell proliferation in the dentate gyrus of hippocampus and influenced glutamatergic and serotoninergic systems of neonatal female rats. *Lipids in Health and Disease*, *15*(1), 1–10. https://doi.org/10.1186/s12944-016-0236-1

178. Katakura, M., Hashimoto, M., Okui, T., Shahdat, H. M., Matsuzaki, K., & Shido, O. (2013). Omega-3 polyunsaturated fatty acids enhance neuronal differentiation in cultured rat neural stem cells. *Stem Cells International*, 9. https://doi.org/10.1155/2013/490476

CHAPTER 2. EFFECT OF PUFA SUPPLEMENTATION ON MEMBRANE COMPOSITION AND CELL MORPHOLOGY IN SH-SY5Y CELLS

2.1. Introduction

The extent of development in the brain during gestation is an important determinant of the trajectory of neurocognitive functioning in a child. Starting with the third trimester of pregnancy until approximately three years of life, the brain undergoes a period of enormous developmental changes in structure and functionality. During this time, the brain is sensitive to environmental factors, such as nutritional deficiencies, that can adversely affect essential neurological structural components and functions[1]; thus, impacting fetal brain development[2]. It has been proposed that the effects of inadequate nutrition during gestation can persist into adulthood[3]; Some of the long-term health problems that can result from suboptimal brain development include neurological diseases such as Alzheimer's disease[4]; learning disabilities such as dyslexia, autism[5,6], and ADHD[4]; psychiatric disorders such as schizophrenia[4]; and other chronic diseases[7,8]. This sensitive period *in utero* is when the fetal brain is most rapidly developing; thus, it is an important time in gestation to optimize fetal brain growth and development by ensuring proper nutrition.

Polyunsaturated fatty acids (PUFAs) are one of the nutrients that has a substantial impact on fetal neurodevelopment. Polyunsaturated fatty acids (PUFAs) are essential components of the brain, constituting 35% of the dry weight[9], and omega-6 and omega-3 PUFAs are found in abundance in neuronal membrane phospholipids imparting structure and function required in numerous neurological processes. In the third trimester, the brain has a higher susceptibility of being negatively impacted by deficiency in PUFAs, which can be attributed to the high accretion rate of PUFAs, specifically ARA and DHA, into the brain[10–13]. The large accumulation of PUFAs in the brain greatly contributes to the accelerated growth and functionality by supporting the growth of developing neurons. At birth, the brain is at nearly its full size, approximately 80% of the size of the adult brain, and has the largest number of neurons than any other stage of life to ensure optimal connectivity[14]. The accumulation of PUFAs in the fetal brain *in utero* is primarily regulated by maternal PUFA status and placental transport[15]. The adequacy of maternal dietary consumption of PUFAs contributes to PUFA status to allow for sufficient availability of PUFAs

to be transported to the fetus. Adequate intake of omega-3 PUFAs is of concern in the US as most pregnant and lactating women fall short of the dietary recommendations[16–19]. If maternal consumption of PUFAs is inadequate, this can affect the accumulation and incorporation of PUFAs into the brain; thus, adequacy of maternal PUFA intake is a key component for proper fetal brain growth and development.

Overall, it is largely agreed upon that adequate n-3 PUFAs, especially DHA, intake is essential for proper neurodevelopment[20–22]. Inadequate intake of omega-3 PUFAs during gestation has strong implications on adverse outcomes such as premature birth, shorter duration of gestation, low birth weight, perinatal death[15], impaired visual[23], and neurological development[24,25], and increased risk of chronic diseases in later life[24,26,27]. The results of numerous observational studies on fish consumption in pregnant women show an association between low seafood consumption and suboptimal neurodevelopmental outcomes in verbal and performance IQ, behavior, fine motor skills, social skills, and communication skills[28,29]. In support, several rodent studies have provided evidence that maternal n-3 PUFA deficiency can lead to cognitive deficits affecting learning, memory, emotion, behavior, and olfactory senses[30] in offspring[31–40]. It has been suggested that impairments resulting from inadequate maternal n-3 intake during gestation may persist into adulthood[8,41], even if offspring are supplemented with n-3 PUFAs postnatally[15,36]. Recently, there has been increasing emphasis on the importance of balancing the n-6:n-3 ratio in the diet, due to the competitive nature of omega-6 and omega-3 for utilization of the same enzymes to be incorporated into phospholipids and be converted to their subsequent LCPUFA products. Several studies have suggested the essentiality of a balanced n-6:n-3 ratio for neurodevelopment; in rodent studies, offspring from highly unbalanced n-6:n-3 ratio maternal diets during gestation exhibited impaired memory, abnormal social activity and emotional reactivity[39], learning impairments[42], reduced locomotion[43], and anxious behavior[8,30]. Even though the focus of dietary recommendations is to increase the consumption of omega-3 PUFAs, adequate intake of omega-6 is also essential during pregnancy for optimal fetal neurodevelopment.

Despite the widely accepted implications for PUFAs involvement in neurodevelopment, the cellular and molecular mechanism of how PUFAs affect brain development is not well understood. One consistent finding across the literature is that alterations to the availability of omega-6 and omega-3 PUFAs is reflected in the PUFA composition of neuronal membrane

phospholipids. Omega-6 and omega-3 PUFAs are found in abundance in neuronal membrane phospholipids imparting structure and function[44–46]. The incorporation of both n-6 and n-3 PUFAs into neuronal membrane has been shown to be involved in the various processes of neuronal growth and development[12,25,47,48]. The role that the individual PUFAs serve in the growth and developmental processes remains unclear. Rodent studies have yielded that inadequate n-3 PUFAs have adverse effects on offspring brain development, such as smaller brain size[49] , smaller neuron size[50–52], altered brain[49,53] and neuronal morphology[50,54,55], decreased neuron proliferation[49,56], decreased neuronal connectivity[57,58], and altered neurotransmission[8,36,41,59–64]. Several cell culture studies have demonstrated that the high concentration of membrane ARA is associated with increased neurite elongation[65–69]. However, other studies have shown that ARA impairs neurite extension[70–72]; as well as suggest that ARA is needed in combination with DHA for neurite outgrowth[73]. Also, there is inconsistent evidence as to whether ARA enhances or impairs neurogenesis[49,74] and neurotransmission[15,49,60–62,75,76] and synaptogenesis[77,78]. With the inconsistent results of the involvement of ARA, other studies have proposed that the presence of a higher concentration of DHA is necessary for neuronal growth and development. In studies with cells supplemented with DHA resulted in enhanced neuritogenesis[71], synaptogenesis[71,77,78], and neurotransmission[79,80]. Many studies have provided evidence that DHA is important for the maturation of neurons[72,73] as the membrane composition shifts to have a high proportion of DHA than ARA in the neuronal membrane[65,66]. Overall, the literature supports DHA plays an important role in neuronal growth and development, whereas the role of ARA is undetermined.

Despite the numerous studies that attempt address the role of PUFAs in neuronal development, there are many limitations and several methodological discrepancies that could potentially be attributed to overwhelmingly inconsistent results. Across the literature, there is a lack of consistency in the sources of dietary PUFAs used, the various types of PUFAs in the diet composition, and the amount of fatty acids in the diet. The various human studies that have looked at omega-3 fatty acids in association with neurodevelopmental outcomes in offspring did not account for variations in the diet, whole food sources of PUFAs and the consequential differences in lipid composition in the diet. Similarly, animal studies have utilized different sources for PUFAs that often contain a variety of other fatty acids and fatty acid concentrations in the diets were inconsistent across treatment groups[42,43,54,81–84]. The presence of other fatty acids in the PUFA sources and the variation in the fatty acid composition of the diet has the potential to be

confounding. In comparison to human and animal studies, cell culture studies have controlled for consistent PUFA concentrations across treatment groups, but there are inconsistent concentrations of PUFAs used for supplementation between studies; the concentrations utilized in the current research range from 0.1 to 200 μM[85]. Additionally, cell culture studies have utilized primary rodent cultures collected from embryos at different times in the embryonic period and most studies have used undifferentiated cells, which does not simulate the developmental process. Lastly, the proposed mechanisms of how PUFAs impact neuronal development are disparate[40,86–88], making the underlying cellular and molecular pathway more unclear. Contributing to the limited knowledge of the roles of PUFAs in neurodevelopment, very few studies in the current literature have taken into consideration the opposing roles of omega-6 and omega-3 PUFAs in the body. The studies that are currently available have varying definitions of which PUFAs constitute the omega-6 to omega-3 ratio[39,42,81]; some studies solely focus on LA and ALA while other studies use a combination of EFAs and LCPUFAs, making the results difficult to compare. Due to these limitations, the role of PUFAs in neurodevelopment remains unclear and the current research should be cautiously interpreted.

Further research needs to be conducted to provide a better understanding of PUFAs and the n-6:n-3 PUFA ratio in neuronal development. As it stands, there is not enough evidence to elucidate how PUFAs affect brain development; utilizing clearly defined methods and confronting the discrepancies in the current literature can help to progress our knowledge of the mechanism of PUFAs in neurodevelopment. This study aims to address some of the inconsistencies and methodological limitations in the current literature, such as clearly defining the n-6:n-3 ratio and using differentiated human neuroblastoma cells. The individual roles of ALA, ARA, and EPA were investigated to determine the impact on neuronal membrane composition, membrane n-6:n-3 ratio, and morphology during neuronal differentiation. The human neuroblastoma cell line, SH-SY5Y, is utilized to model the process of development as the cells are induced to undergo the process of differentiation from neural progenitor cells into mature neuronal-like cells. The purpose of this study was to examine the impact of individual PUFAs on the n-6:n-3 ratio of membrane phospholipids and neuritogenesis in SH-SY5Y human neuroblastoma cells during neuronal development.

2.2. Methods

2.2.a. Materials

SH-SY5Y cells purchased from ATCC (Manassas, VA). Gibco 45% glucose, TrypLE – Express (trypsin), Dulbecco's Modified Medium (DMEM), Ham's F12 Medium, penicillin-streptomycin, and fetal bovine serum (FBS), Pierce BCA protein assay (Rockford, IL), and cell culture plates and flasks were purchased for Fisher Scientific (Waltham, MA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Cayman chemical. Fatty acid-free bovine serum albumin (BSA), and retinoic acid were purchased from Sigma-Aldrich (St. Louis, MO). The ALA, ARA, and EPA fatty acid sodium salts (>99.999%) were purchased from Nu-Chek Prep (Elysian, MN).

2.2.b. Cell culture

SH-SY5Y cells were cultured in growth media (DMEM, 1% penicillin-streptomycin, 8 mM glucose, and 10% FBS) at 37°C with 5% CO_2 for 24 hours to allow for cell adherence. The cells were plated in 75cm^2 flasks, 12-well plates, and 24-well plates at seeding densities of 2 x 10^6, 0.16 x 10^6, and 0.08 x 10^6, respectively. The total volume of media in each type of culture container was 20 mL, 1 mL/well, and 500 µL/well, respectively. After allowing for adherence, the growth media was aspirated and replaced with differentiating media (1:1 DMEM to Ham's F12, 8mM glucose, 1% FBS, and 10µM retinoic acid) supplemented with 70µM of fatty acid conjugated to BSA. The cells were incubated for 5 days for the cells to adequately differentiate into the neuronal phenotype. The differentiating media was not replaced during the 5-day incubation period.

To harvest the cells from the 75cm^2 flasks , the differentiating media was removed, and 10 mL of trypsin was added for cell detachment from the flask. An equal volume of differentiating media (without the 10µM retinoic acid) was added to neutralize the trypsin and the flask contents were transferred to a 50mL conical tube for centrifugation at 1400rpm at 5°C for 10 minutes to form a cell pellet. The liquid was aspirated from the tube, leaving the cell pellet. The cell pellet was broken up and resuspended in PBS warmed to 37°C, serving as a rinse. The PBS cell suspension was transferred into a 1.5mL microcentrifuge tube and was centrifuged for 10 minutes at 1400rpm. The PBS was removed, and the cell pellet was resuspended in warmed PBS to rinse

the cells. A sample of 100µM was taken from each microcentrifuge tube to be used for the BCA protein assay (Pierce, Rockford, IL) and stored at -20°C. The cells were centrifuged again for 10 minutes at 1400rpm. The PBS was removed, and the cells were resuspended in 200µL of ddH_2O. The cell suspensions were stored short-term at -20°C.

2.2.b.i. PUFA Stock Solution (20mM)

To dissolve the FA sodium salts, 1mL of methanol was added to the ampule. The solution was transferred to a pre-weighed glass, screw cap culture tube and dried with nitrogen. Once dried, the culture tube was weighed to determine the appropriate amount of ddH_2O to make a 20uM fatty acid solution, and warmed to 70°C. To prepare the FA stock solution for addition to the cell culture medium, the solution was sterile filtered, aliquoted into 1mL sterilized, plastic microcentrifuge tubes. The tubes were sealed with parafilm and stored at -20°C.

The 20mM FA stock solutions were analyzed using Gas Chromatography (GC) to confirm the presence of and purity of the corresponding FA. A modified version of the fatty acid analysis procedure was performed with FA stock solutions to form FA methyl esters for GC analysis. To start, 20 µL of the FA stock solution was combined with 10 µL of concentrated HCl and 175 µL ddH_2O in a glass, screw cap culture tube. The solution was vortex to thoroughly mix. Then, 3 mL of ethyl acetate was added to the culture tube and vortexed to combine. The solution was centrifuged for 5 minutes at 1400 rpm. The top ethyl acetate layer was transferred to a new culture tube and dried using nitrogen. Once dried, the fatty acid analysis procedure was followed beginning at the addition of Boron Trifluoride (BF_3).

2.2.b.ii. Fatty acid supplemented culture medium

To prepare the FA supplemented differentiating media, an aliquoted microcentrifuge tube of the 20mM FA stock solutions and 20% fatty acid-free BSA in PBS solution were warmed in a 37°C water bath for approximately 15 minutes. In a sterile, 1.5mL microcentrifuge tube, 250 µL of the warmed 20 mM FA stock solution and 750 µL of the 20% BSA in PBS solution were added and mixed using a micropipette to yield a 5mM FA-BSA conjugate solution. The 5mM FA-BSA conjugate solution was added to differentiating media to achieve a concentration of 70 µM of the FA.

2.2.c. Fatty acid analysis

The harvested SH-SY5Y cell pellets were prepared for GC analysis using a modified version of the previously reported methods[89–91] to prepare fatty acid methyl esters. In brief, the defrosted cell pellet suspended in 200µl of ddH_2O was combined with a 2:1 chloroform to methanol with 0.001% BHT solution. The solution was centrifuged until there was clear separation of the organic and inorganic layers. The lower organic phase was transferred to a culture tube. This process was three times to maximize the lipid extraction from the cells. The samples were dried using nitrogen. Then, 2N KOH in MeOH was added and the samples were heated at 100°C for 5 minutes. Once cooled, BF_3 was added and heated at 100°C for 5 minutes. The samples were allowed to cool and then were combined with hexane and saturated NaCl. The samples were centrifuged until there was clear separation of layers. The top hexane layer was transferred to a 2ml glass screw cap GC vial. The resulting fatty acid methyl ester samples were analyzed using a Varian 3900 GC with a fused silica capillary column and flame ionization detector.

The standards PUFA3 and Supelco37, with known PUFA composition, were analyzed with GC to identify the retention time that corresponds to each FA. The analyzed samples were compared to the standards to identify the FAs present in each sample. The fatty acid composition was normalized to the percentage of the total fatty acids (mol) in the sample and expressed as the percentage of the total mols (mol%).

2.2.d. Cell viability

2.2.d.i. MTT analysis

The relative cell viability was determined using an MTT assay that is previously described by Mosmann (1983).[92] SH-SY5Y cells were cultured in a 24-well plate per treatment group and incubated with the FA treatment for 5 days. On the last day of incubation, 5mg/mL MTT in PBS solution was added to each well to achieve a concentration of 0.5mg/mL. The cells were incubated with the MTT for 1.5 hours at 37C and 5% CO_2. The media was removed using a micropipette to avoid the removal of the resulting dark purple formazan crystals. Then, 300µL of DMSO was added to each well and placed on a microplate shaker to dissolve the crystals. The plates were incubated for approximately 12 hours until the crystals dissolved completely. Half of the well

volume was transferred to a 96-well plate and the absorbance was measured with a spectrophotometer (Bio-Tek Powerwave X200; Winooski, VT) at a wavelength of 570nm.

2.2.d.ii. BCA protein assay

Using the BCA Assay Kit (Pierce, Rockford, IL), cell viability was also estimated using a colorimetric-based assay. In short, the 100µL sample of cells suspended in PBS taken during the harvesting process were prepared in accordance with the instructions provided in the assay kit. In a 96-well plate, 25µL of the cell pellet was combined with the assay reagents and incubated at 37°C for 30 minutes. The absorbance was measured using the Bio-Tek Powerwave X200 (Winooski, VT) spectrophotometer at a wavelength of 590nm. The protein concentration was determined by comparing the absorbance of the samples to the BSA standard concentrations. Protein concentration was used as an indicator of cell viability in conjunction with the MTT assay.

2.2.e. SH-SY5Y morphological analysis

To document cell morphology during the differentiation process the cells were photographed throughout the FA treatment period. Once the differentiating media was added, photographs were taken of the cells daily for 5 days until the cells were harvested. The photographs were taken at the same location of the flasks over the course of the 5-day FA treatment period. Using phase-contrast microscopy, an inverted microscope, Zeiss Axio Vert. A1 (Oberkochem, Germany), with the Zeiss AxioCam ICm1 camera and Zeiss Zen2 imaging software was used to take the images. The cells were photographed at a 10x magnification with a 32.02 ms exposure time. The images were 1388 x 1038 pixels and analyzed with the NIH ImageJ software with the ImageScience NeuronJ plugin. After identifying the areas of the imaged field of view consistent across all 5 days for each flask, 10 neurons were selected for analysis. Neurons were selected based on defined criteria: neurite length must be a minimum the width of the cell body; the neurites originate from a single cell body with a clear end point; and neurites were not in a clustering of cells. The neurites were traced to determine the number of neurites per cell body.

2.2.f. Statistical analysis

To determine the treatment effect on fatty acid composition and cell morphology, one-way analysis of variance (ANOVA) was used. The results from the ANOVA test were further assessed using the Tukey HSD multiple comparisons of means test. The results with a $p>0.05$ are considered statistically significant. All statistical analysis was performed using SAS Statistics Software (Cary, NC).

2.3. Results

2.3.a. Membrane PUFA composition

To determine the impact of supplementing individual fatty acid treatments on the membrane PUFA composition, GC was used to analyze the fatty acids present in the SH-SY5Y phospholipids. The results for select fatty acids, expressed as (mol%), are shown in Figure 4-5. For all treatments and the BSA control there was no significant difference in LA. As expected, ALA treatment led to a significantly higher membrane proportion of ALA versus the other three treatments. Membrane arachidonic acid proportions were significantly higher with ARA treatment in comparison to ALA treatment, but not so in comparison to the EPA treatment or BSA control. The membrane EPA proportion was significantly higher in the EPA treatment, as anticipated, compared to BSA control, ALA, and ARA. Additionally, the EPA proportion was high with the ALA treatment in comparison with the BSA control and ARA treatment. Both the ALA and EPA treatments had significantly higher mean proportions of n-3 DPA (docosapentaenoic acid) compared to the BSA control; however, the ALA treatment was not significantly different from the ARA treatment. Lastly, the BSA control had a significantly higher mean proportion of DHA compared to the ARA treatment.

2.3.b. The n-6:n-3 ratio

To assess the effects of PUFA supplementation on the n-6:n-3 ratio, the mean proportions of PUFAs were totaled based on their n-6 and n-3 classification. The total mean n-6 PUFA proportion for each treatment was determined by summing the following: 18:2n6, 18:3n6, 20:3n6, 20:4n6; and 22:5n6. To determine the total mean n-3 PUFA proportion for each treatment, the

following PUFAs were summed: 18:3n3, 20:5n3, 22:5n3, and 22:6n3. To determine the total PUFA proportion, the total n-6 PUFA and total n-3 PUFA were summed to get the total mean PUFA proportion per treatment. The n-6:n-3 ratio was determined using the totals for the mean n-6 and n-3 PUFAs proportions.

The total PUFA, total n-6 PUFA, total n-3 PUFA, and the n-6:n-3 ratio across treatment groups are shown in Figure 6. There was no significant difference for total PUFA among treatment groups. As expected, ALA and EPA had significantly higher n-3 mean PUFA proportions than BSA and ARA treatments; EPA had a significantly higher mean proportion of n-3 PUFA than the ALA treatment. As for the mean n-6 PUFA proportion, the ARA treatment had a significantly higher proportion compared to the ALA treatment, but there was no difference with BSA and EPA treatments. For the n-6:n-3 ratio, the ARA treatment had a significantly higher n-6:n-3 ratio compared to all treatments. Both ALA and EPA had significantly lower n-6:n-3 ratios than the BSA control, but there was no significant difference between ALA and EPA treatments.

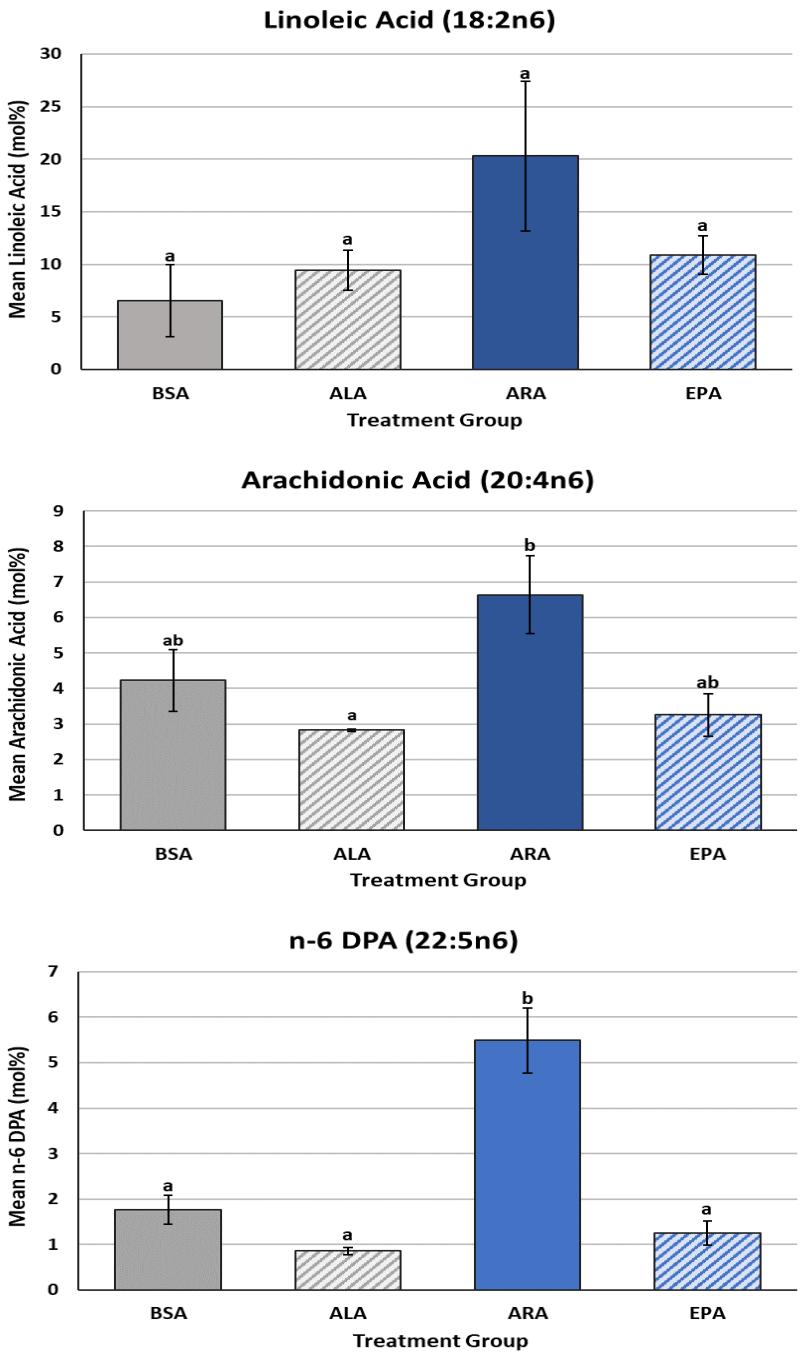

Figure 4. Effect of PUFA supplementation on mean n-6 PUFA proportions in phospholipids of SH-SY5Y cells. SH-SY5Y cells were supplemented with 70µM of ALA, ARA, or EPA during the differentiation process to the neuronal phenotype. Phospholipid fatty acid proportions were determined using GC analysis of fatty acid methyl esters. Proportions are expressed as mol%. The data were analyzed using one-way ANOVA with Tukey's HSD post-hoc analysis. The letters indicate a significant different ($p<0.05$) between treatments. Bars that share the same letter are not significantly different.

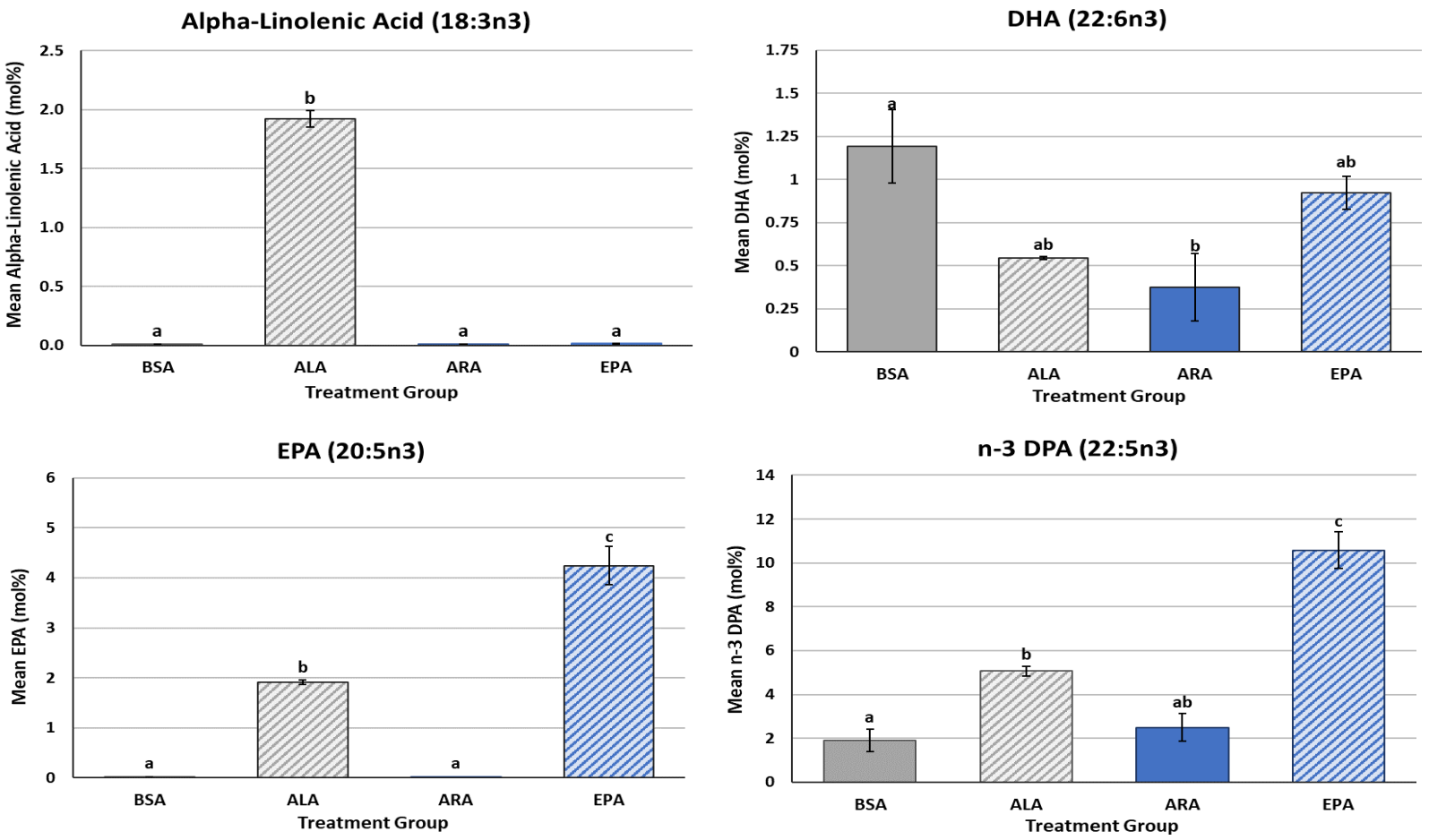

Figure 5. Effect of PUFA supplementation on mean n-3 PUFA proportions in phospholipids of SH-SY5Y cells. SH-SY5Y cells were supplemented with 70µM of ALA, ARA, or EPA during the differentiation process to the neuronal phenotype. Fatty acid proportions were determined using GC analysis of fatty acid methyl esters. Proportions are expressed as mol%. The data were analyzed using one-way ANOVA with Tukey's HSD post-hoc analysis. The letters indicate a significant different ($p<0.05$) between treatments. Bars that share the same letter are not significantly different.

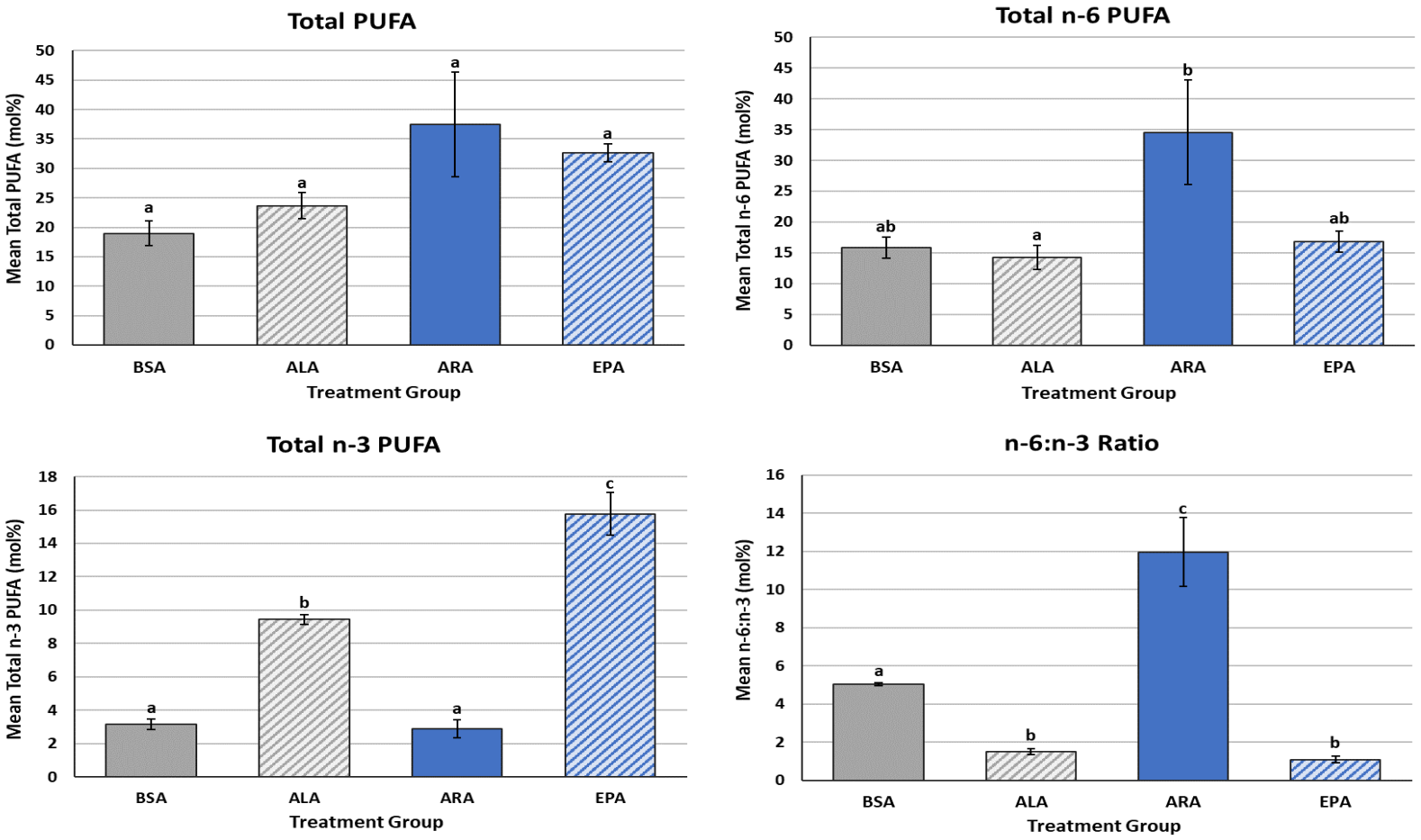

Figure 6. Omega-3 PUFA (70µM) supplementation increases total n-3 PUFA proportion and decreases the n-6:n-3 ratio in phospholipid composition. Differentiated SH-SY5Y cells were treated with 70µM of ALA, ARA, or EPA upon initiation of the differentiation process for 5 days. The mean FA proportions (mol%) were summed to determine the total PUFA, total n-6 PUFA, total n-3 PUFA, and the n-6:n-3 ratio. The data were analyzed using one-way ANOVA with Tukey's HSD Test post-hoc analysis. The letters indicate a significant different ($p<0.05$) between treatments. Bars that share the same letter are not significantly different.

2.3.d. SH-SY5Y morphology during differentiation

To assess the impact of PUFA supplementation on the morphology of SH-SY5Y cells during the differentiation from progenitor to neuronal phenotype (Figure 7), the neurite number was measured each day of the PUFA treatment. The neurite number during the 5-day differentiation for each treatment is shown in Figure 8. The number of neurites for 10 selected cells per sample were counted each day of the PUFA treatment incubation from the captured images. The mean number of neurites per treatment was used for comparison. There was no significant difference between groups within each day of the treatment. However, there was a trend for the number of neurites to increase each day for all treatments ($p<0.05$).

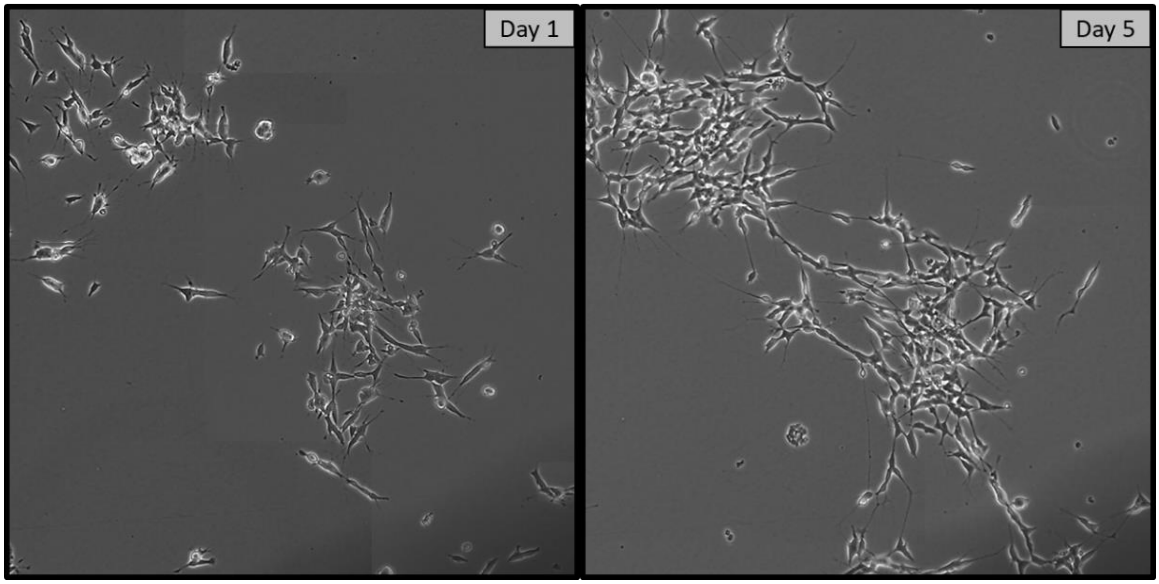

Figure 7. Morphological changes in differentiated SH-SY5Y cells. BSA control treated SH-SY5Y cells were incubated for 5 days with 10μM of retinoic acid to induce differentiation from progenitor cells into a neuronal phenotype.

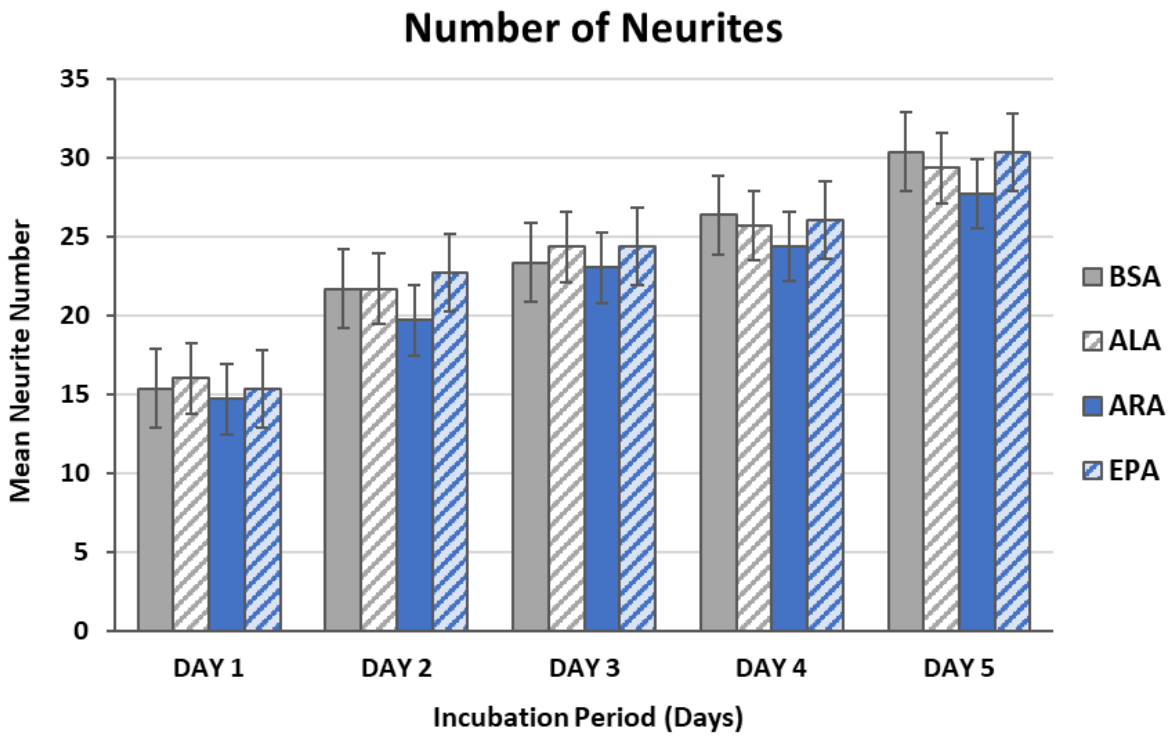

Figure 8. Number of neurites during each day of PUFA treatment. SH-SY5Y cells were supplemented with 70µM PUFA during a differentiation period of 5 days. The number of neurites per 10 selected cells was determined for each day of the treatment. The data were analyzed using one-way ANOVA with Tukey's HSD Test post-hoc analysis. The letters indicate a significant difference ($p<0.05$) between treatments. Bars that share the same letter are not significantly different.

2.3.e. The effect of PUFA supplementation on cell viability

To determine whether the PUFA supplementation impacted cell viability an MTT assay and BCA assay were performed. The results of the MTT and BCA assay analysis are shown in Figure 9. When the cells were treated with ALA and EPA there was no significant difference in the cell viability compared to the BSA control. When the cells were treated with ARA, there was a significant ($p<0.05$) reduction in cell viability compared to ALA treatment and BSA control groups. There was no significant difference between EPA and ARA treatment on cell viability. Even though there wasn't significant difference between EPA treatment and the ALA and BSA treatments, the cell viability with the EPA treatment appears to be slightly lower in comparison.

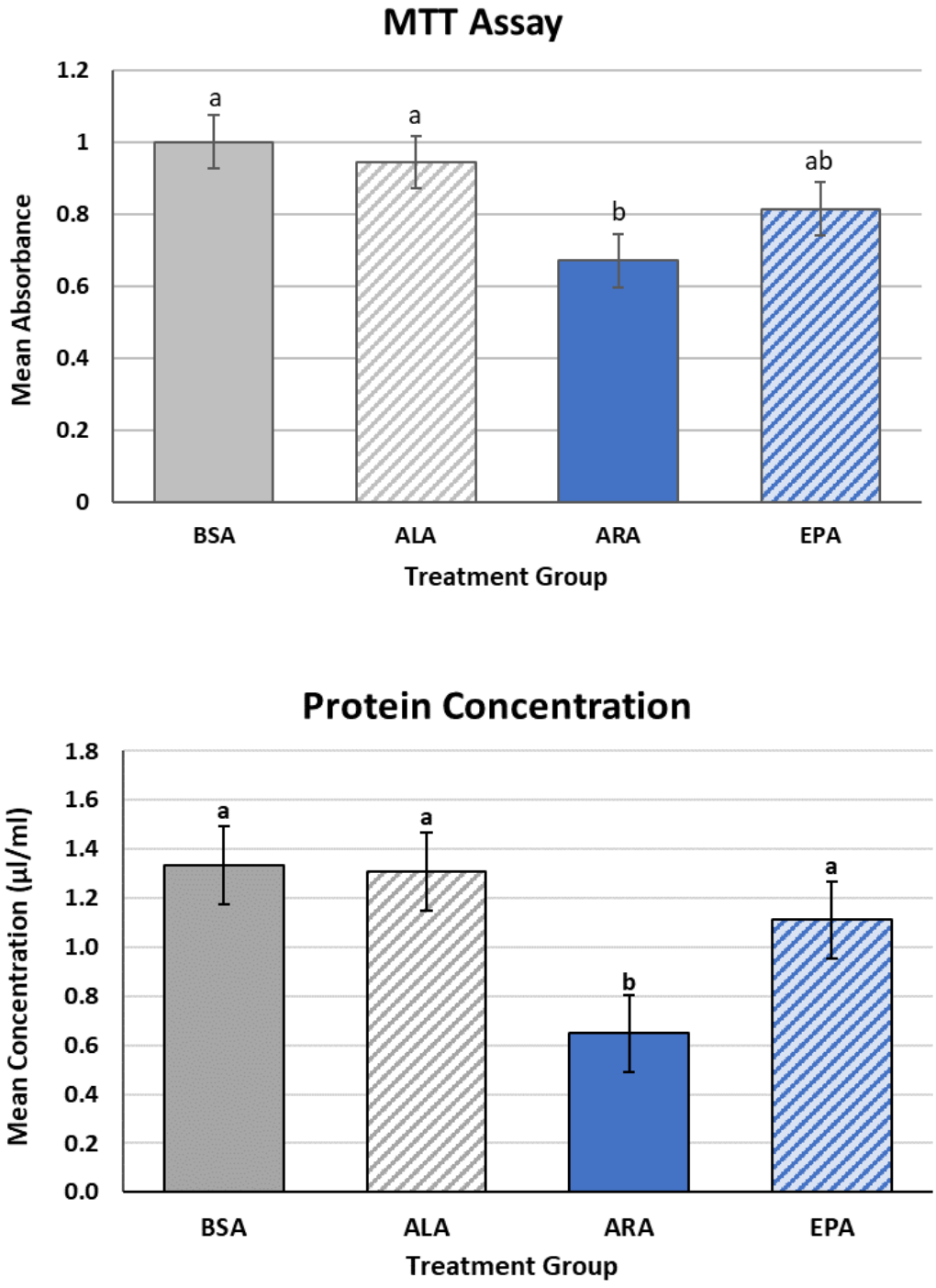

Figure 9. Decreased cell viability with ARA supplementation. SH-SY5Y cells were treated with 70µM of ALA, ARA, or EPA as the cells underwent differentiation for 5 days. The data were analyzed using one-way ANOVA with Tukey's HSD Test post-hoc analysis. The letters indicate a significant difference ($p<0.05$) between treatments. Bars that share the same letter are not significantly different.

2.4. Discussion

PUFAs are instrumental in brain development and consequentially deficiency has been shown to impair cognitive functions, such as learning, memory, and planning. It has been suggested that the availability of PUFAs alters the neuronal membrane composition, elongation, neuritogenesis, synaptogenesis, and neurotransmission. However, the current literature is riddled with inconsistencies in methodology, such as the use of undifferentiated cells, and a high variability in FA diet compositions across studies. Additionally, very few studies have accounted for the competitive relationship of n-6 and n-3 PUFAs when assessing neuronal development; this has further contributed to the lack of consensus on the role of PUFAs seen in the literature. Overall, the mechanism of the role of PUFAs in neuronal and brain development needs further elucidation. The goal of this study was to investigate how PUFA supplementation affects membrane PUFA composition, the n-6:n-3 ratio of membrane PUFAs, and morphology during neuronal development. Human neuroblastoma cells, SH-SY5Y, were utilized for their ability to differentiate from a progenitor to a neuronal-type cells to simulate the process of neuronal development.

Many previous studies have consistently shown that supplementing individual PUFAs resulted in alterations to the PUFAs composition of membrane phospholipids. These findings suggest that the availability of PUFAs is reflected in the membrane composition, thus higher availability infers increased membrane incorporation. Despite the agreement, the utilization of undifferentiated cell models in most of these studies needs to be considered; the relevance of these results to the differentiating process is questionable since undifferentiated cells are highly proliferative while the rate of proliferation significantly decreases upon differentiation. More recent studies that have used differentiated primary embryonic rat cell cultures and PC12 cell have supported the previous finding of altered membrane composition following PUFA supplementation. However, the use of rodent-derived cells has limited translational application to humans. Additionally, many of the primary cell cultures were taken from rodent embryos subjected to n-3 deficient maternal diets in gestation that lack consistent, controlled the amounts of PUFAs. These limitations make it difficult to interpret the current evidence to determine if the availability of PUFAs alters membrane composition to the extent as proliferative, undifferentiated cells. In the present study, we have demonstrated that PUFA supplementation alters the PUFA membrane composition and the changes reflect the supplemented PUFA and its n-6 or n-3 classification

(Figure 4-5). These results are in accordance with previous studies and support that the availability of PUFAs imparts alterations to membrane PUFA composition in differentiated neuronal cells.

Previous studies have focused on varying the availability of n-3 PUFAs, whether individually or in combinations, on membrane composition. The n-3 PUFAs used in the literature are highly variable as the definition of what constitutes 'n-3 PUFAs' in these studies is poorly outlined. Also, the concentration of each n-3 PUFA is inconsistent and is often not controlled across treatment groups within studies. In this study, ALA, ARA, and EPA were individually supplemented during the differentiation of SH-SY5Y cells. The results of this study suggest that the supplementation of the single PUFA does affect the phospholipid fatty acid proportion of that PUFA as well as other PUFAs present in the cell membrane. The cells supplemented with ALA had a significantly higher ALA mean proportion compared to all treatment (Figure 5), but also had significantly higher EPA and n-3 DPA mean proportions compared to BSA (Figure 5). The mean proportion of ARA was significantly higher in the ARA treatment compared to ALA, but there wasn't a significant difference among BSA control, and treatments with EPA and ALA (Figure 4). This result suggests that increasing the availability of the n-3 PUFAs, ALA and EPA, may decrease the ARA membrane proportion compared to the control. Conversely, ARA supplementation significantly decreased the mean proportion of DHA in comparison to the BSA control (Figure 5), suggesting that increased availability of ARA displaces the membrane DHA proportion to compensate. The results from assessing the FA composition of the membrane suggest that increased availability of individual PUFAs alter membrane composition not only increasing the proportion of that PUFA but also impacts the other PUFAs.

There is significantly more attention placed on n-3 PUFAs than n-6 PUFAs, and even less on the relationship between the two. It is important to consider the competitive relationship between n-6 and n-3 PUFAs as they use the same enzymes for conversion to longer chain PUFAs and incorporation in cellular membranes, as well as use the same transport mechanism across the BBB[96]. The availability of n-6 and n-3 PUFAs are an important determinant in their uptake and utilization by cells. Currently, very few studies have assessed the impact of n-3 PUFAs in conjunction with n-6 PUFAs[96]. Additionally, the n-6:n-3 ratio is ill-defined across these studies as well as the n-6 and n-3 PUFA ratio used vary widely. This study addresses this discrepancy in the literature by clearly defining the PUFAs that compose the n-6:n-3 ratio, how the n-6:n-3 ratio was determined, and analyzes the effect that individual PUFA supplementation has on the

membrane n-6:n-3 ratio. As shown in Figure 6, supplementation of individual PUFAs significantly changes the membrane n-6:n-3 ratio. Both ALA and EPA treatments resulted in significant reductions in the n-6:n-3 ratio in comparison the BSA, but there was no significant difference between ALA and EPA treatments. Conversely, ARA treatment resulted in a significant increase in the membrane n-6:n-3 ratio compared to BSA.

It has been proposed that PUFAs affect the neuritogenesis, neurite elongation, and synaptogenesis. Many studies have demonstrated that ARA plays a role in neuritogenesis and the initial neurite elongation process.[65,67–69] In contrast, few studies have demonstrated that ARA does not affect neurite number and length of neuronal cells[70]. In this study, we assessed the number of neurites per cell body of 10 selected cells, based on the criteria discussed in the methods section, for each day of treatment. There was no significant difference between the treatment groups each day, however, all treatment groups followed a trend of increasing number of neurites from the first to the last day of treatment (Figure 8). The results from this study support prior research that has suggests that ARA has no impact on the neurite number.

Lastly, in this study we assessed whether the concentration of the PUFA supplementation impacted cell viability. It was observed that the only treatment that affected cell viability was the ARA treatment, which significantly decreased cell viability compared to the other groups (Figure 9). There were no significant differences among ALA, EPA, and BSA treatment groups on cell viability. The detrimental effects of a high media concentration of ARA were expected as prolonged exposure to high levels of ARA can impair cell survival through the induction of apoptosis, which has been associated with increased oxidative stress[76,93]. Additionally, studies have shown that ARA can be cytotoxic to many cell lines at concentrations of 50 – 100 μM [76,93]. Studies conducted with hippocampal and spinal cord neurons have shown that ARA can induce oxidative stress and decrease cell viability[94]. These results contradict some of the previous studies that have found that ARA supplementation did not impact cell viability[65,73]. The results may vary due to the difference in concentrations of ARA supplemented between our study and the previous studies.

2.5. Conclusion

The current literature evidence about the role of PUFAs in neuronal development is inadequate to provide enough support and insight into a clear mechanism. This uncertainty can be

attributed to the vast inconsistencies across studies regarding methods such as diet composition and the use of undifferentiated cell models. Additionally, the definition of what constitutes ‘n-6 PUFAs’ and ‘n-3 PUFAs’ in these studies ranges in the number of PUFAs included. With some studies focusing on single fatty acids while others focusing on different combinations of PUFAs, these discrepancies provide more uncertainty about the current evidence. To better understand the mechanism of action, it is imperative to first understand the roles of individual PUFAs. Also, it is necessary to account for the complex physiological interactions of n-6 and n-3 PUFAs in the determination of the mechanism of each PUFA in neuronal development. The lack of consideration for the relationship dynamics between n-6 and n-3 limits the translational capacity of the current research. In this study, we have attempted to address some of these issues.

The results of this study have shown that supplementation of individual PUFAs alter neuronal membrane PUFA composition and the membrane n-6:n-3 ratio in SH-SY5Y cells. Additionally, ARA supplementation at 70µM does have adverse effects on cell viability, while supplementation with ALA and EPA had no impact. Overall, PUFA supplementation impacted neuronal membrane composition and the n-6:n-3 ratio, but this study does not provide enough evidence to demonstrate that ALA, ARA, or EPA individually influence neuritogenesis during differentiation. Further experiments are needed to assess functional outcomes and other morphological measures of developing neurons.

2.6. References

1. Georgieff, M. K., Ramel, S. E., & Cusick, S. E. (2018). Nutritional Influences on Brain Development. *Acta Paediatr*, *107*(8), 1310–1321. https://doi.org/10.1111/apa.14287

2. National Research Council (US) and Institute of Medicine (US) Committee on Integrating the Science of Early Childhood Development. (2000). From Neurons to Neighborhoods: The Science of Early Childhood Development. In J. . Shonkoff & D. A. Philips (Eds.), *National Academy of Sciences* (pp. 182–217). National Academies Press. https://www.ncbi.nlm.nih.gov/books/NBK225562/

3. Mousa, A., Naqash, A., & Lim, S. (2019). Macronutrient and Micronutrient Intake during Pregnancy: An Overview of Recent Evidence. *Nutrients*, *11*(2), 443. https://doi.org/10.3390/nu11020443

4. Healy-Stoffel, M., & Levant, B. (2018). N-3 (Omega-3) Fatty Acids: Effects on Brain Dopamine Systems and Potential Role in the Etiology and Treatment of Neuropsychiatric Disorders. *CNS & Neurological Disorders - Drug Targets*, *17*(3), 216–232. https://doi.org/10.2174/1871527317666180412153612

5. Bourre, J. M. (2009). Diet, Brain Lipids, and Brain Functions: Polyunsaturated Fatty Acids, Mainly Omega-3 Fatty Acids. In G. G. Lajtha A., Tettamanti G. (Ed.), *Handbook of Neurochemistry and Molecular Neurobiology* (3rd ed., pp. 409–441). Springer. https://doi.org/10.1007/978-0-387-30378-9_17

6. van Elst, K., Bruining, H., Birtoli, B., Terreaux, C., Buitelaar, J. K., & Kas, M. J. (2014). Food for thought: Dietary changes in essential fatty acid ratios and the increase in autism spectrum disorders. *Neuroscience and Biobehavioral Reviews*, *45*, 369–378. https://doi.org/10.1016/j.neubiorev.2014.07.004

7. Purandare, C. N. (2012). Maternal nutritional deficiencies and interventions. *Journal of Obstetrics and Gynecology of India*, *62*(6), 621–623. https://doi.org/10.1007/s13224-013-0347-9

8. Bhatia, H. S., Agrawal, R., Sharma, S., Huo, Y. X., Ying, Z., & Gomez-Pinilla, F. (2011). Omega-3 fatty acid deficiency during brain maturation reduces neuronal and behavioral plasticity in adulthood. *PLoS ONE*, *6*(12). https://doi.org/10.1371/journal.pone.0028451

9. Haag, M. (2003). Essential fatty acids and the brain. *Can J Psychiatry*, *48*(3), 195–203. https://doi.org/10.1177/070674370304800308

10. Makrides, M., Smithers, L. G., & Gibson, R. A. (2010). Role of long-chain polyunsaturated fatty acids in neurodevelopment and growth. *Nestle Nutrition Workshop Series: Pediatric Program*, *65*, 123–136. https://doi.org/10.1159/000281154

11. Fleith, M., & Clandinin, M. T. (2005). Dietary PUFA for preterm and term infants: Review of clinical studies. *Critical Reviews in Food Science and Nutrition*, *45*(3), 205–229. https://doi.org/10.1080/10408690590956378

12. Belkind-Gerson, J., Carreón-Rodríguez, A., Contreras-Ochoa, C., Estrada-Mondaca, S., Parra-Cabrera, M., Scanlon, M. J., Owada, Y., Yamamoto, Y., Porter, C. J. H. H., Nicolazzo, J. A., Rapoport, S. I., Larqué, E., Hanebutt, F. L., Koletzko, B., Demmelmair, H., Schiessl, B., Chen, C. T., Green, J. T., Orr, S. K., … Gillberg, C. (2008). Fatty Acids and Neurodevelopment. *Journal of Pediatric Gastroenterology and Nutrition*, *47*, S7–S9.

13. Herrera, E., & Amusquivar, E. (2000). Lipid metabolism in the fetus and the newborn. *Diabetes/Metabolism Research and Reviews*, *16*(3), 202–210. https://doi.org/10.1002/1520-7560

14. Pfisterer, U., & Khodosevich, K. (2017). Neuronal survival in the brain: Neuron type-specific mechanisms. *Cell Death and Disease*, *8*(3), e2643. https://doi.org/10.1038/cddis.2017.64

15. Akerele, O. A., & Cheema, S. K. (2016). A balance of omega-3 and omega-6 polyunsaturated fatty acids is important in pregnancy. *Journal of Nutrition and Intermediary Metabolism*, *5*, 23–33. https://doi.org/10.1016/j.jnim.2016.04.008

16. Richter, C. K., Bowen, K. J., Mozaffarian, D., Kris-Etherton, P. M., & Skulas-Ray, A. C. (2017). Total Long-Chain n-3 Fatty Acid Intake and Food Sources in the United States Compared to Recommended Intakes: NHANES 2003–2008. *Lipids*, *52*(11), 917–927. https://doi.org/10.1007/s11745-017-4297-3

17. Zhang, Z., Fulgoni, V. L., Kris-Etherton, P. M., & Mitmesser, S. H. (2018). Dietary intakes of EPA and DHA omega-3 fatty acids among US childbearing-age and pregnant women: An analysis of NHANES 2001–2014. *Nutrients*, *10*(4). https://doi.org/10.3390/nu10040416

18. Thompson, M., Hein, N., Hanson, C., Smith, L. M., Anderson-Berry, A., Richter, C. K., Bisselou, K. S., Appiah, A. K., Kris-Etherton, P., Skulas-Ray, A. C., & Nordgren, T. M. (2019). Omega-3 fatty acid intake by age, gender, and pregnancy status in the United States: National health and nutrition examination survey 2003–2014. *Nutrients*, *11*(1), 177–189. https://doi.org/10.3390/nu11010177

19. Nordgren, T. M., Lyden, E., Anderson-Berry, A., & Hanson, C. (2017). Omega-3 fatty acid intake of pregnant women and women of childbearing age in the united states: Potential for deficiency? *Nutrients*, *9*(3). https://doi.org/10.3390/nu9030197

20. Braarud, H. C., Markhus, M. W., Skotheim, S., Stormark, K. M., Frøyland, L., Graff, I. E., & Kjellevold, M. (2018). Maternal DHA status during pregnancy has a positive impact on infant problem solving: A Norwegian prospective observation study. *Nutrients*, *10*(5). https://doi.org/10.3390/nu10050529

21. Makrides, M., Collins, C. T., & Gibson, R. A. (2011). Impact of fatty acid status on growth and neurobehavioural development in humans. *Maternal and Child Nutrition*, *7*(SUPPL. 2), 80–88. https://doi.org/10.1111/j.1740-8709.2011.00304.x

22. Koletzko, B., Agostoni, C., Carlson, S., Clandinin, T., Hornstra, G., Neuringer, M., Uauy, R., Yamashiro, Y., & Willatts, P. (2007). Long chain polyunsaturated fatty acids (LC-PUFA) and perinatal development. *Acta Paediatrica*, *90*(4), 460–464. https://doi.org/10.1111/j.1651-2227.2001.tb00452.x

23. Larque, E., Demmelmair, H., & Koletzko, B. (2002). Perinatal Supply and Metabolism of Long-Chain Polyunsaturated Fatty Acids Importance for the Early Development of the Nervous System. *Annals New York Academy of Science*, *967*, 299–310.

24. Saini, R. K., & Keum, Y. S. (2018). Omega-3 and omega-6 polyunsaturated fatty acids: Dietary sources, metabolism, and significance - A review. *Life Sciences*, *203*, 255–267. https://doi.org/10.1016/j.lfs.2018.04.049 R

25. Janssen, C. I. F., & Kiliaan, A. J. (2014). Long-chain polyunsaturated fatty acids (LCPUFA) from genesis to senescence: The influence of LCPUFA on neural development, aging, and neurodegeneration. *Progress in Lipid Research*, *53*(1), 1–17. https://doi.org/10.1016/j.plipres.2013.10.002

26. Khaire, A. A., Kale, A. A., & Joshi, S. R. (2015). Maternal omega-3 fatty acids and micronutrients modulate fetal lipid metabolism: A review. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *98*, 49–55. https://doi.org/10.1016/j.plefa.2015.04.007

27. Lauritzen, L., Brambilla, P., Mazzocchi, A., Harsløf, L. B. S., Ciappolino, V., & Agostoni, C. (2016). DHA effects in brain development and function. *Nutrients*, *8*(1), 1–17. https://doi.org/10.3390/nu8010006

28. Coletta, J. M., Bell, S. J., & Roman, A. S. (2010). Omega-3 Fatty Acids and Pregnancy. *Reviews in Obstetrics & Gynecology*, *3*(4), 163–171. https://doi.org/10.3909/riog0137

29. Hibbeln, J. R., & Gow, R. V. (2014). Omega-3 fatty acid and nutrient deficits in adverse neurodevelopment and childhood behaviors. *Child and Adolescent Psychiatric Clinics of North America*, *23*(3), 555–590. https://doi.org/10.1016/j.chc.2014.02.002.

30. Greiner, R. S., Moriguchi, T., Slotnick, B. M., Hutton, A., & Salem, N. (2001). Olfactory discrimination deficits in n - 3 fatty acid-deficient rats. *Physiology and Behavior*, *72*(3), 379–385. https://doi.org/10.1016/S0031-9384(00)00437-6

31. Catalan, J., Moriguchi, T., Slotnick, B., Murthy, M., Greiner, R. S., & Salem, N. (2002). Cognitive deficits in docosahexaenoic acid-deficient rats. *Behavioral Neuroscience*, *116*(6), 1022–1031. https://doi.org/10.1037/0735-7044.116.6.1022

32. García-Calatayud, S., Redondo, C., Martín, E., Ruiz, J. I., García-Fuentes, M., & Sanjurjo, P. (2005). Brain docosahexaenoic acid status and learning in young rats submitted to dietary long-chain polyunsaturated fatty acid deficiency and supplementation limited to lactation. *Pediatric Research*, *57*(5 I), 719–723. https://doi.org/10.1203/01.PDR.0000156506.03057.AD

33. van Elst, K., Brouwers, J. F., Merkens, J. E., Broekhoven, M. H., Birtoli, B., Helms, J. B., & Kas, M. J. H. (2019). Chronic dietary changes in n-6/n-3 polyunsaturated fatty acid ratios cause developmental delay and reduce social interest in mice. *European Neuropsychopharmacology*, *29*(1), 16–31. https://doi.org/10.1016/j.euroneuro.2018.11.1106

34. Fedorova, I., & Salem, N. (2006). Omega-3 fatty acids and rodent behavior. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *75*(4–5), 271–289. https://doi.org/10.1016/j.plefa.2006.07.006

35. Vancassel, S., Blondeau, C., Lallemand, S., Cador, M., Linard, A., Lavialle, M., & Dellu-Hagedorn, F. (2007). Hyperactivity in the rat is associated with spontaneous low level of n-3 polyunsaturated fatty acids in the frontal cortex. *Behavioural Brain Research*, *180*(2), 119–126. https://doi.org/10.1016/j.bbr.2007.02.032

36. Lozada, L. E., Desai, A., Kevala, K., Lee, J.-W., & Kim, H.-Y. (2017). Perinatal Brain Docosahexaenoic Acid Concentration Has a Lasting Impact on Cognition in Mice. *The Journal of Nutrition*, *12*, jn254607. https://doi.org/10.3945/jn.117.254607

37. DeMar, J. C., Ma, K., Bell, J. M., Igarashi, M., Greenstein, D., & Rapoport, S. I. (2006). One generation of n-3 polyunsaturated fatty acid deprivation increases depression and aggression test scores in rats. *Journal of Lipid Research*, *47*(1), 172–180. https://doi.org/10.1194/jlr.M500362-JLR200

38. Levant, B., Radel, J. D., & Carlson, S. E. (2004). Decreased brain docosahexaenoic acid during development alters dopamine-related behaviors in adult rats that are differentially affected by dietary remediation. *Behavioural Brain Research*, *152*(1), 49–57. https://doi.org/10.1016/j.bbr.2003.09.029

39. Zamberletti, E., Piscitelli, F., De Castro, V., Murru, E., Gabaglio, M., Colucci, P., Fanali, C., Prini, P., Bisogno, T., Maccarrone, M., Campolongo, P., Banni, S., Rubino, T., & Parolaro, D. (2017). Lifelong imbalanced LA/ALA intake impairs emotional and cognitive behavior via changes in brain endocannabinoid system. *Journal of Lipid Research*, *58*(2), 301–316. https://doi.org/10.1194/jlr.M068387

40. Sakayori, N., Tokuda, H., Yoshizaki, K., Kawashima, H., Innis, S. M., Shibata, H., & Osumi, N. (2016). Maternal Nutritional Imbalance between Linoleic Acid and Alpha-Linolenic Acid Increases Offspring's Anxious Behavior with Sex-Dependent Manner in Mice. *Tohoku J Exp Med*, *240*(1), 31–37. https://doi.org/10.1620/tjem.240.31.

41. Moreira, J. D., Knorr, L., Ganzella, M., Thomazi, A. P., de Souza, C. G., de Souza, D. G., Pitta, C. F., e Souza, T. M., Wofchuk, S., Elisabetsky, E., Vinadé, L., Perry, M. L. S., & Souza, D. O. (2010). Omega-3 fatty acids deprivation affects ontogeny of glutamatergic synapses in rats: Relevance for behavior alterations. *Neurochemistry International*, *56*(6–7), 753–759. https://doi.org/10.1016/j.neuint.2010.02.010

42. Yamamoto, N., Saitoh, M., Moriuchi, A., Nomura, M., & Okuyama, H. (1987). Effect of dietary alpha-linolenate/linoleate balance on brain lipid compositions and learning ability of rats. *Journal of Lipid Research*, *28*, 144–145.

43. Chalon, S., Delion-Vancassel, S., Belzung, C., Guilloteau, D., Leguisquet, A. M., Besnard, J. C., & Durand, G. (1998). Dietary fish oil affects monoaminergic neurotransmission and behavior in rats. *Journal of Nutrition*, *128*(12), 2512–2519. https://doi.org/10.1093/jn/128.12.2512

44. Lacombe, R. J. S., Chouinard-Watkins, R., & Bazinet, R. P. (2018). Brain docosahexaenoic acid uptake and metabolism. *Molecular Aspects of Medicine*. https://doi.org/10.1016/j.mam.2017.12.004

45. Innes, J. K., & Calder, P. C. (2018). Omega-6 fatty acids and inflammation. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *132*(March), 41–48. https://doi.org/10.1016/j.plefa.2018.03.004

46. Tracey, T. J., Steyn, F. J., Wolvetang, E. J., & Ngo, S. T. (2018). Neuronal Lipid Metabolism: Multiple Pathways Driving Functional Outcomes in Health and Disease. *Frontiers in Molecular Neuroscience*, *11*(January), 1–25. https://doi.org/10.3389/fnmol.2018.00010

47. Uauy, R., Mena, P., & Rojas, C. (2000). Essential fatty acids in early life: Structural and functional role. *Proceedings of the Nutrition Society*, *59*, 3–15. https://doi.org/10.1017/S0029665100000021

48. Uauy, R., & Dangour, A. D. (2006). Nutrition in brain development and aging: Role of essential fatty acids. *Nutrition Reviews*, *64*(5), S24–S33. https://doi.org/10.1301/nr.2006.may.S24

49. Coti Bertrand, P., O'Kusky, J. R., & Innis, S. M. (2006). Maternal dietary (n-3) fatty acid deficiency alters neurogenesis in the embryonic rat brain. *Journal of Nutrition*, *136*, 1570–1575. https://doi.org/136/6/1570 [pii]

50. Ahmad, A., Moriguchi, T., & Salem, N. (2002). Decrease in neuron size in docosahexaenoic acid-deficient brain. *Pediatric Neurology*, *26*(3), 210–218. https://doi.org/10.1016/S0887-8994(01)00383-6

51. Innis, S. M. (2007). Dietary (n-3) Fatty Acids and Brain Development. *The Journal of Nutrition*, *137*(4), 855–859. https://doi.org/10.1093/jn/137.4.855

52. Davis-Bruno, K., & Tassinari, M. S. (2011). Essential fatty acid supplementation of DHA and ARA and effects on neurodevelopment across animal species: A review of the literature. *Birth Defects Research Part B - Developmental and Reproductive Toxicology*, *92*(3), 240–250. https://doi.org/10.1002/bdrb.20311

53. Yavin, E., Himovichi, E., & Eilam, R. (2009). Delayed cell migration in the developing rat brain following maternal omega 3 alpha linolenic acid dietary deficiency. *Neuroscience*, *162*(4), 1011–1022. https://doi.org/10.1016/j.neuroscience.2009.05.012

54. Wainwright, P. E., Bulman-Fleming, M. B., Lévesque, S., Mutsaers, L., & McCutcheon, D. (1998). A Saturated-Fat Diet during Development Alters Dendritic Growth in Mouse Brain. *Nutritional Neuroscience*, *1*(1), 49–58. https://doi.org/10.1080/1028415X.1998.11747212

55. Ahmad, A., Murthy, M., Greiner, R. S., Moriguchi, T., & Salem, N. (2002). A decrease in cell size accompanies a loss of docosahexaenoate in the rat hippocampus. *Nutritional Neuroscience*, *5*(2), 103–113. https://doi.org/10.1080/10284150290018973

56. Cetin, I., & Koletzko, B. (2008). Long-chain v-3 fatty acid supply in pregnancy and lactation. *Current Opinion in Clinical Nutrition and Metabolic Care*, *11*, 297–302. https://doi.org/10.1111/j.1477-8947.1982.tb01029.x

57. Grayson, D. S., Kroenke, C. D., Neuringer, M., & Fair, D. A. (2014). Dietary omega-3 fatty acids modulate large-scale systems organization in the rhesus macaque brain. *The Journal of Neuroscience*, *34*(6), 2065–2074. https://doi.org/10.1523/JNEUROSCI.3038-13.2014

58. Madore, C., Nadjar, A., Delpech, J. C., Sere, A., Aubert, A., Portal, C., Joffre, C., & Layé, S. (2014). Nutritional n-3 PUFAs deficiency during perinatal periods alters brain innate immune system and neuronal plasticity-associated genes. *Brain, Behavior, and Immunity*, *41*(1), 22–31. https://doi.org/10.1016/j.bbi.2014.03.021

59. Yoshida, S., Yasuda, a, Kawazato, H., Sakai, K., Shimada, T., Takeshita, M., Yuasa, S., Kobayashi, T., Watanabe, S., & Okuyama, H. (1997). Synaptic vesicle ultrastructural changes in the rat hippocampus induced by a combination of alpha-linolenate deficiency and a learning task. *Journal of Neurochemistry*, *68*(3), 1261–1268.

60. Pongrac, J. L., Slack, P. J., & Innis, S. M. (2007). Dietary polyunsaturated fat that is low in (n-3) and high in (n-6) fatty acids alters the SNARE protein complex and nitrosylation in rat hippocampus. *The Journal of Nutrition*, *137*(8), 1852–1856. https://doi.org/10.1093/jn/137.8.1852

61. Chalon, S. (2006). Omega-3 fatty acids and monoamine neurotransmission. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *75*(4–5), 259–269. https://doi.org/10.1016/j.plefa.2006.07.005

62. Zimmer, L., Hembert, S., Durand, G., Breton, P., Guilloteau, D., Besnard, J. C., & Chalon, S. (1998). Chronic n-3 polyunsaturated fatty acid diet-deficiency acts on dopamine metabolism in the rat frontal cortex: A microdialysis study. *Neuroscience Letters*, *240*(3), 177–181. https://doi.org/10.1016/S0304-3940(97)00938-5

63. Zimmer, L., Delion-Vancassel, S., Durand, G., Guilloteau, D., Bodard, S., Besnard, J. C., & Chalon, S. (2000). Modification of dopamine neurotransmission in the nucleus accumbens of rats deficient in n-3 polyunsaturated fatty acids. *The Journal of Lipid Research*, *41*(1), 32–40.

64. Zimmer, L., Delion-Vancassel, S., Cantagrel, S., Breton, P., Delamanche, S., Guilloteau, D., Durand, G., & Chalon, S. (2002). The dopamine mesocorticolimbic pathway is affected by deficiency in n-3 polyunsaturated fatty acids. *AM J Clin Nutr*, *75*, 662–667.

65. Petroni, A., Blasevich, M., Papini, N., La Spada, P., & Galli, C. (1996). Changes in Arachidonic Acid Levels and Formation and in Lipid Synthesis in the Human Neuroblastoma SK-N-BE During Retinoic Acid-Induced Differentiation. *Journal of Neurochemistry*, *67*, 549–556.

66. Martin, R. E., & Bazan, N. G. (1992). Changing Fatty Acid Content of Growth Cone Lipids Prior to Synaptogenesis. *Journal of Neurochemistry*, *59*(1), 318–325. https://doi.org/10.1111/j.1471-4159.1992.tb08906.x

67. Dehaut, F., Bertrand, I., Miltaud, T., Pouplard-Barthelaix, A., & Maingault, M. (1993). N-6 polyunsaturated fatty acids increase the neurite length of PC12 cells and embryonic chick motoneurons. *Neuroscience Letters*, *161*(2), 133–136. https://doi.org/10.1016/0304-3940(93)90277-R

68. Williams, E. J., Walsh, F. S., & Doherty, P. (1994). The Production of Arachidonic Acid Can Account for Calcium Channel Activation in the Second Messenger Pathway Underlying Neurite Outgrowth Stimulated by NCAM, N-Cadherin, and L1. *Journal of Neurochemistry*, *62*(3), 1231–1234. https://doi.org/10.1046/j.1471-4159.1994.62031231.x

69. Robson, L. G., Dyall, S., Sidloff, D., & Michael-Titus, A. T. (2010). Omega-3 polyunsaturated fatty acids increase the neurite outgrowth of rat sensory neurones throughout development and in aged animals. *Neurobiology of Aging*, *31*(4), 678–687. https://doi.org/10.1016/j.neurobiolaging.2008.05.027

70. Ikemoto, A., Kobayashi, T., Watanabe, S., & Okuyama, H. (1997). Membrane fatty acid modifications of PC12 cells by arachidonate or docosahexaenoate affect neurite outgrowth but not norepinephrine release. *Neurochemical Research*, *22*(6), 671–678. https://doi.org/10.1023/A:1027393724676

71. Calderon, F., & Kim, H. Y. (2004). Docosahexaenoic acid promotes neurite growth in hippocampal neurons. *Journal of Neurochemistry*, *90*(4), 979–988. https://doi.org/10.1111/j.1471-4159.2004.02520.x

72. Novak, E. M., Dyer, R. A., & Innis, S. M. (2008). High dietary ω-6 fatty acids contribute to reduced docosahexaenoic acid in the developing brain and inhibit secondary neurite growth. *Brain Research, 1237*, 136–145. https://doi.org/10.1016/j.brainres.2008.07.107

73. Kan, I., Melamed, E., Offen, D., & Green, P. (2007). Docosahexaenoic acid and arachidonic acid are fundamental supplements for the induction of neuronal differentiation. *Journal of Lipid Research*, *48*(3), 513–517. https://doi.org/10.1194/jlr.C600022-JLR200

74. Maekawa, M., Takashima, N., Matsumata, M., Ikegami, S., Kontani, M., Hara, Y., Kawashima, H., Owada, Y., Kiso, Y., Yoshikawa, T., Inokuchi, K., & Osumi, N. (2009). Arachidonic acid drives postnatal neurogenesis and elicits a beneficial effect on prepulse inhibition, a biological trait of psychiatric illnesses. *PLoS ONE*, *4*(4), 1–9. https://doi.org/10.1371/journal.pone.0005085

75. Grootendorst-van Mil, N. H., Tiemeier, H., Steenweg-de Graaff, J., Koletzko, B., Demmelmair, H., Jaddoe, V. W. V., Steegers, E. A. P., & Steegers-Theunissen, R. P. M. (2018). Maternal plasma n-3 and n-6 polyunsaturated fatty acids during pregnancy and features of fetal health: Fetal growth velocity, birth weight and duration of pregnancy. *Clinical Nutrition*, *37*, 1367–1374. https://doi.org/10.1016/j.clnu.2017.06.010

76. Tallima, H., & Ridi, R. El. (2018). Arachidonic acid: Physiological roles and potential health benefits – A review. *Journal of Advanced Research*, *11*, 33–41. https://doi.org/10.1016/j.jare.2017.11.004

77. Tanaka, K., Farooqui, A. A., Siddiqi, N. J., Alhomida, A. S., & Ong, W. Y. (2012). Effects of docosahexaenoic acid on neurotransmission. *Biomolecules and Therapeutics*, *20*(2), 152–157. https://doi.org/10.4062/biomolther.2012.20.2.152

78. Cao, D., Kevala, K., Kim, J., Moon, H.-S., Jun, S. B., Lovinger, D., & Kim, H.-Y. (2009). Docosahexaenoic acid promotes hippocampal neuronal development and synaptic function. *Journal of Neurochemistry*, *111*(2), 510–521. https://doi.org/10.1111/j.1471-4159.2009.06335.x

79. Géraldine, M., Stéphanie, D., Bénédicte, L., Isabelle, D., Monique, L., & Sylvie, V. (2010). DHA enhances the noradrenaline release by SH-SY5Y cells. *Neurochemistry International*, *56*(1), 94–100. https://doi.org/10.1016/j.neuint.2009.09.006

80. Blanpied, T. A., & Ehlers, M. D. (2004). Microanatomy of dendritic spines: Emerging principles of synaptic pathology in psychiatric and neurological disease. *Biological Psychiatry*, *55*(12), 1121–1127. https://doi.org/10.1016/j.biopsych.2003.10.006

81. Khoury, S., Soubeyre, V., Cabaret, S., Merle, L., Grégoire, S., Deprêtre, N., Jarriault, D., Grosmaitre, X., Bretillon, L., Berdeaux, O., Acar, N., & Le Bon, A. M. (2020). Perinatal exposure to diets with different n-6:n-3 fatty acid ratios affects olfactory tissue fatty acid composition. *Scientific Reports*, *10*(1), 1–15. https://doi.org/10.1038/s41598-020-67725-9

82. Fan, C., Sun, W., Fu, H., Dong, H., Xia, L., Lu, Y., Deckelbaum, R. J., & Qi, K. (2015). Dietary Ratios of N-6/N-3 Polyunsaturated Fatty Acids During Maternal Pregnancy Affect Hippocampal Neurogenesis and Apoptosis in Mouse Offspring. *Nutricion Hospitalaria*, *32*(3), 1170–1179. https://doi.org/10.3305/nh.2015.32.3.9259

83. Tian, C., Fan, C., Liu, X., Xu, F., & Qi, K. (2011). Brain histological changes in young mice submitted to diets with different ratios of n-6/n-3 polyunsaturated fatty acids during maternal pregnancy and lactation. *Clinical Nutrition*, *30*(5), 659–667. https://doi.org/10.1016/j.clnu.2011.03.002

84. Hajjar, T., Goh, Y. M., Rajion, M. A., Vidyadaran, S., Li, T. A., & Ebrahimi, M. (2013). Alterations in neuronal morphology and synaptophysin expression in the rat brain as a result of changes in dietary n-6: n-3 fatty acid ratios. *Lipids in Health and Disease*, *12*(1), 1–9. https://doi.org/10.1186/1476-511X-12-113

85. Hejr, H., Ghareghani, M., Zibara, K., Ghafari, M., Sadri, F., Salehpour, Z., Hamedi, A., Negintaji, K., Azari, H., & Ghanbari, A. (2017). The ratio of 1/3 linoleic acid to alpha linolenic acid is optimal for oligodendrogenesis of embryonic neural stem cells. *Neuroscience Letters*, *651*, 216–225. https://doi.org/10.1016/j.neulet.2017.05.020

86. Guo, B. Q., Ding, S. Bin, & Li, H. Bin. (2019). Maternal n-3 PUFAs deficiency during pregnancy inhibits neural progenitor cell proliferation in fetal rat cerebral cortex. *International Journal of Developmental Neuroscience*, *76*(June), 72–79. https://doi.org/10.1016/j.ijdevneu.2019.07.001

87. Tang, M., Zhang, M., Cai, H., Li, H., Jiang, P., Dang, R., Liu, Y., He, X., Xue, Y., Cao, L., & Wu, Y. (2016). Maternal diet of polyunsaturated fatty acid altered the cell proliferation in the dentate gyrus of hippocampus and influenced glutamatergic and serotoninergic systems of neonatal female rats. *Lipids in Health and Disease*, *15*(1), 1–10. https://doi.org/10.1186/s12944-016-0236-1

88. Katakura, M., Hashimoto, M., Okui, T., Shahdat, H. M., Matsuzaki, K., & Shido, O. (2013). Omega-3 polyunsaturated fatty acids enhance neuronal differentiation in cultured rat neural stem cells. *Stem Cells International*, 9. https://doi.org/10.1155/2013/490476

89. Metcalfe, L. D., Schmitz, A. A., & Pelka, J. R. (1966). Rapid Preparation of Fatty Acid Esters from Lipids for Gas Chromatography Analysis. *Analytical Chemistry*, *38*(3), 514–515. https://doi.org/10.1007/BF02641197

90. Liu, K.-S. (1994). Preparation of Fatty Acid Methyl Esters for Gas-Chromatographic Analysis of Marine Lipids : Insight Studies AND. *JAOCS*, *71*(11), 1179–1187.

91. Folch, J., Lees, M., & Stanley, G. H. S. (1957). A Simple Method For The Isolation And Purification Of Total Lipides From Animal Tissues*. *J Biol Chem*, *226*, 497–509. https://doi.org/10.1016/j.ultrasmedbio.2011.03.005

92. Mosmann, T. (1983). Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *Journal of Immunological Methods*, *65*(1–2), 55–63. https://doi.org/10.1016/0022-1759(83)90303-4

93. Pompeia, C., Lima, T., & Curi, R. (2003). Arachidonic acid cytotoxicity: Can arachidonic acid be a physiological mediator of cell death? *Cell Biochemistry and Function*, *21*(2), 97–104. https://doi.org/10.1002/cbf.1012

94. Toborek, M., Malecki, A., Garrido, R., Mattson, M. P., Hennig, B., & Young, B. (1999). Arachidonic acid-induced oxidative injury to cultured spinal cord neurons. *Journal of Neurochemistry*, *73*(2), 684–692. https://doi.org/10.1046/j.1471-4159.1999.0730684.x

95. Schmitz, G., & Ecker, J. (2008). The opposing effects of n-3 and n-6 fatty acids. *Progress in Lipid Research*, *47*(2), 147–155. https://doi.org/10.1016/j.plipres.2007.12.004

96. Harris, W. S. (2018). The Omega-6:Omega-3 ratio: A critical appraisal and possible successor. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *132*(March), 34–40. https://doi.org/10.1016/j.plefa.2018.03.003

APPENDIX A. FATTY ACID DATA

Table A.1.Fatty Acid Phospholipid Composition of PUFA Supplemented SH-SY5Y Cells.

Peak Name	BSA (mol%)	ALA (mol%)	ARA (mol%)	EPA (mol%)
14:0	1.28	1.23	1.08	1.21
16:0	33.58	32.59	25.98	24.72
16:1	1.08	1.19	0.56	1.14
17:0	0.79	0.50	0.52	0.57
17:1	0.01	0.43	0.96	0.55
18:0	18.26	20.71	17.90	18.80
18:1n9c	19.86	16.88	11.35	15.51
18:1n9t	5.23	2.68	2.15	3.14
18:2n6c	6.51	9.44	20.29	10.92
18:3n3	0.01	1.92	0.01	0.01
20:3n6	0.01	0.43	0.48	0.56
20:4n6	4.23	2.82	6.64	3.25
20:5n3	0.01	1.91	0.01	4.25
22:2	1.71	0.65	1.53	1.31
22:5n6	1.76	0.86	5.48	1.26
22:5n3	1.92	5.07	2.49	10.59
22:6n3	1.19	0.55	0.38	0.92
Total %	100.00	100.00	100.00	100.00

Table A.2. Fatty Acid Analysis Data from Gas Chromatography.

Peak Name	Treatment Groups											
	BSA 1	BSA 2	BSA 3	ALA 1	ALA 2	ALA 3	ARA 1	ARA 2	ARA 3	EPA 1	EPA 2	EPA 3
	mol%											
14:0	0.90	1.00	1.23	0.94	1.12	1.14	0.90	1.00	0.72	0.66	0.97	1.00
14:1	0.76	- - -	- - -	- - -	- - -	- - -	- - -	0.55	2.61	1.36	- - -	- - -
15:0	- - -	- - -	- - -	- - -	- - -	- - -	0.46	0.29	- - -	0.35	0.30	- - -
15:1	- - -	- - -	- - -	0.38	- - -	- - -	0.68	0.32	- - -	0.41	0.38	- - -
16:0	20.09	33.83	29.35	25.63	30.14	29.26	22.46	28.3	12.14	9.37	22.59	24.76
16:1	0.95	0.61	1.04	0.92	1.06	1.13	0.43	0.42	0.50	0.64	0.80	1.02
17:0	0.87	0.61	0.38	0.43	0.50	0.38	0.41	0.47	0.38	0.32	0.43	0.48
17:1	0.01	0.01	0.01	0.37	0.38	0.36	0.58	0.27	1.48	0.29	0.43	0.48
18:0	12.59	13.59	18.74	15.76	19.32	18.99	15.75	17.20	10.46	9.04	15.96	16.65
18:1n9c	16.11	13.76	18.31	13.15	15.48	15.41	9.58	10.52	7.42	7.80	12.04	14.29
18:1n9t	4.81	3.01	4.72	2.15	2.40	2.43	1.62	1.73	1.86	1.75	2.32	2.71
18:2n6c	1.18	11.44	4.19	11.03	6.34	7.00	17.61	5.99	25.9	4.38	12.39	8.02
18:3n6	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	0.39	- - -	- - -	- - -
18:3n3	0.01	0.01	0.01	1.71	1.65	1.64	0.01	0.01	0.01	0.01	0.01	0.01
20:1n9	0.47	0.54	0.42	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
20:2	1.37	1.00	1.48	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
20:3n6	0.01	0.01	0.01	0.37	0.47	0.29	0.38	0.3	0.48	0.44	0.35	0.32
20:4n6	4.00	2.40	3.72	2.35	2.45	2.56	4.62	4.37	7.12	2.17	2.26	2.31
20:5n3	0.01	0.01	0.01	1.55	1.65	1.77	0.01	0.01	0.01	2.41	3.08	3.64
22:2	1.54	0.92	1.64	0.42	0.69	0.60	0.92	1.81	0.96	- - -	1.27	0.99
22:5n6	1.62	1.07	1.54	0.84	0.74	0.65	3.85	3.89	5.56	0.87	0.85	0.86
22:5n3	0.91	2.56	1.35	4.53	4.19	4.46	2.96	1.07	2.07	5.9	7.86	9.08
22:6n3	1.05	0.69	1.13	0.46	0.47	0.49	0.01	0.34	0.55	0.53	0.79	0.66
Total %	69.26	87.07	89.28	82.99	89.05	88.56	83.24	78.86	80.62	48.70	85.08	87.28

Table A.3. Fatty Acid Category Totals.

Fatty Acid Category	Treatment Groups											
	BSA 1	BSA 2	BSA 3	ALA 1	ALA 2	ALA 3	ARA 1	ARA 2	ARA 3	EPA 1	EPA 2	EPA 3
	mol %											
Saturated Fatty Acids (SFA)	49.74	56.31	55.67	51.52	57.36	56.20	48.03	59.93	29.40	40.53	47.31	49.14
Monounsaturated Fatty Acids (MUFAs)	33.37	20.59	27.44	20.45	21.70	21.83	15.49	17.51	17.20	25.15	18.77	21.20
Polyunsaturated Fatty Acids (PUFAs)	16.89	23.10	16.89	28.03	20.94	21.97	36.48	22.56	53.40	34.31	33.92	29.66
Omega-6 PUFAs	14.03	19.34	14.09	18.09	12.00	12.53	32.89	20.75	50.12	16.14	20.12	14.32
Omega-3 PUFAs	2.86	3.76	2.80	9.94	8.94	9.44	3.59	1.81	3.27	18.17	13.80	15.34
n-6:n-3	4.91	5.15	5.03	1.82	1.34	1.33	9.16	11.44	15.31	0.89	1.46	0.934

APPENDIX B. SH-SY5Y CELL IMAGES

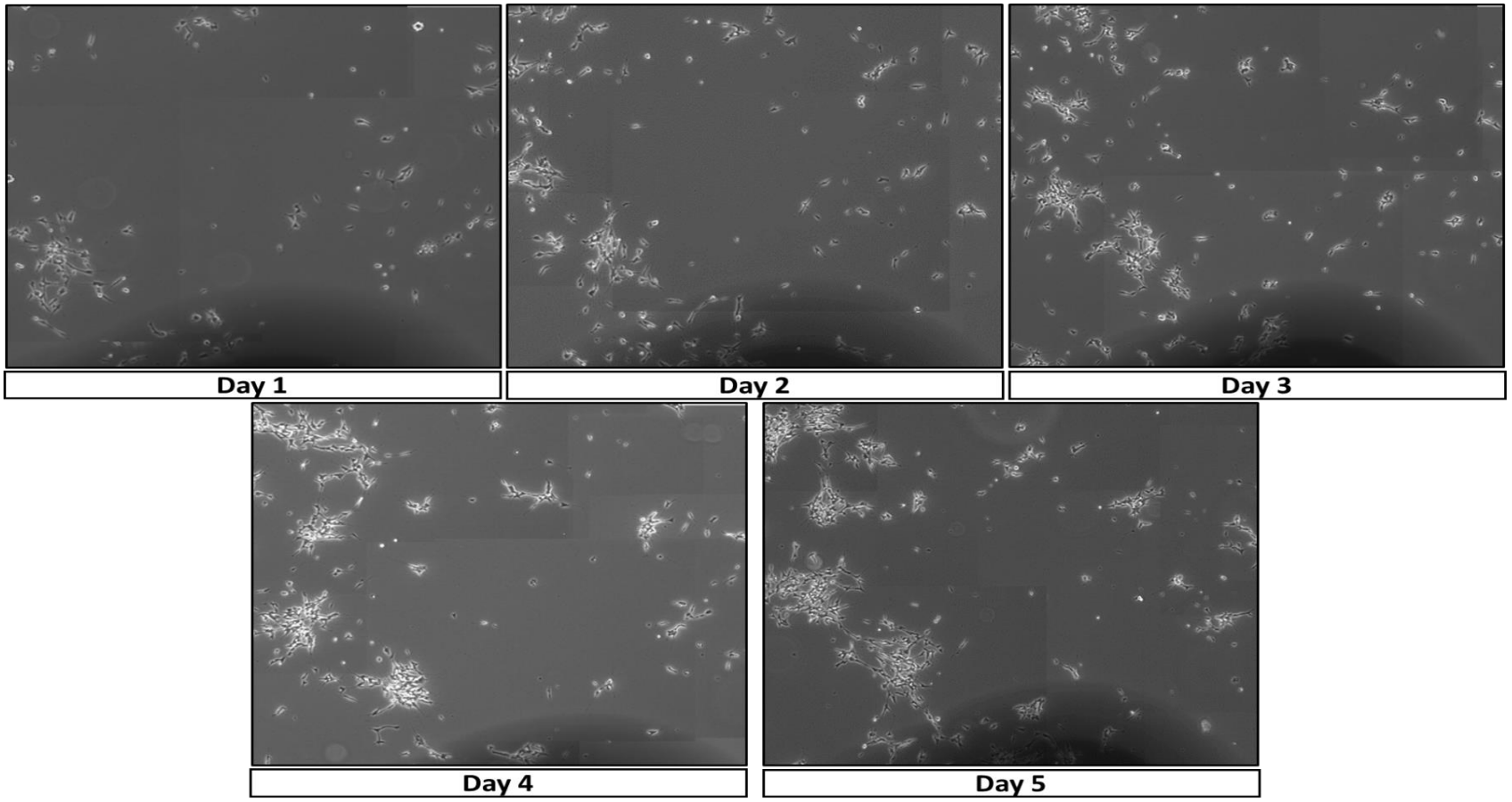

Figure B.1. BSA Replicate #1 Microscopy Images. Pictures were taken each day during the 5-day treatment incubation period.

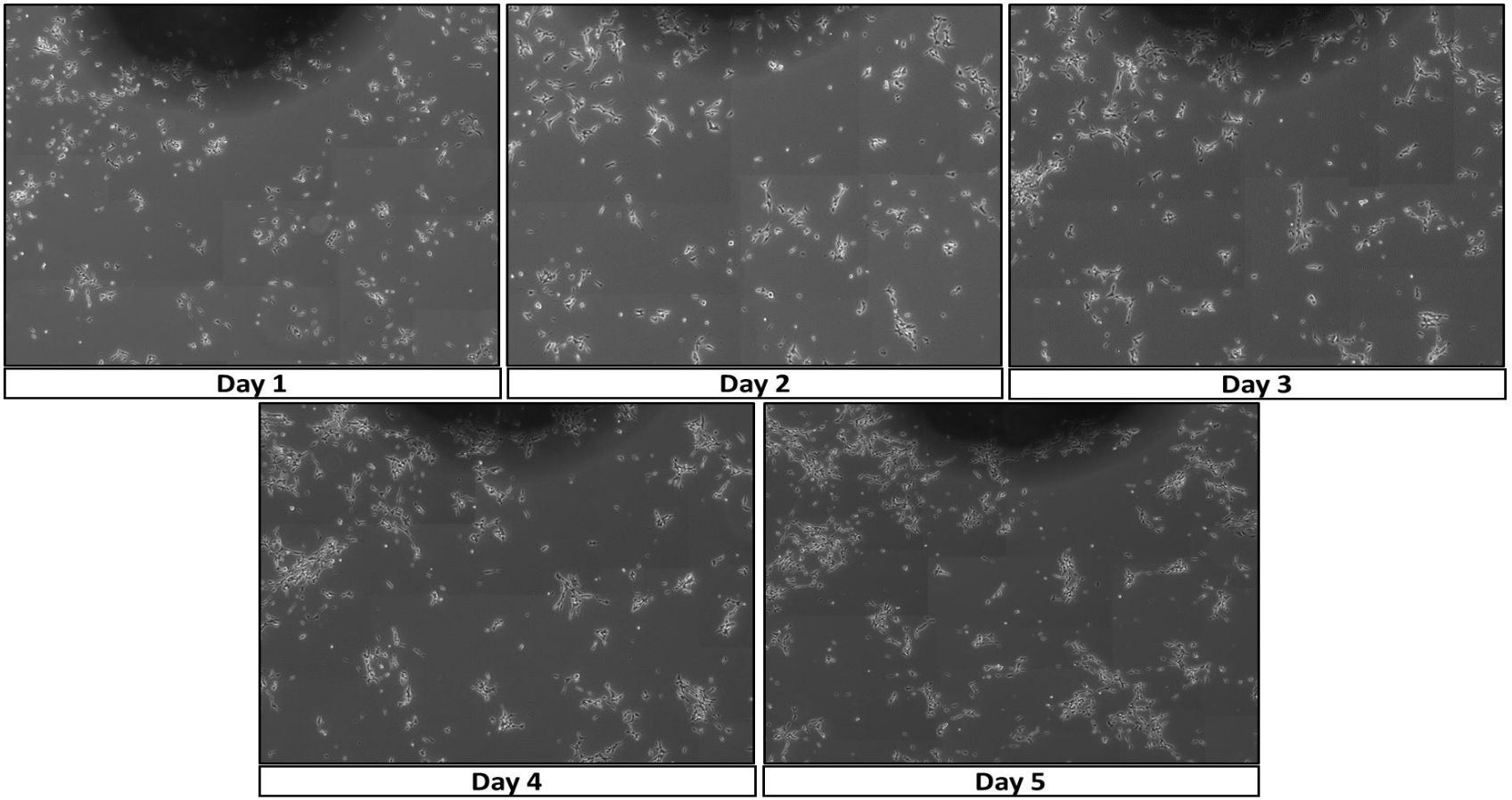

Figure B.2. ALA Replicate #1 Microscopy Images. Pictures were taken each day during the 5-day treatment incubation period.

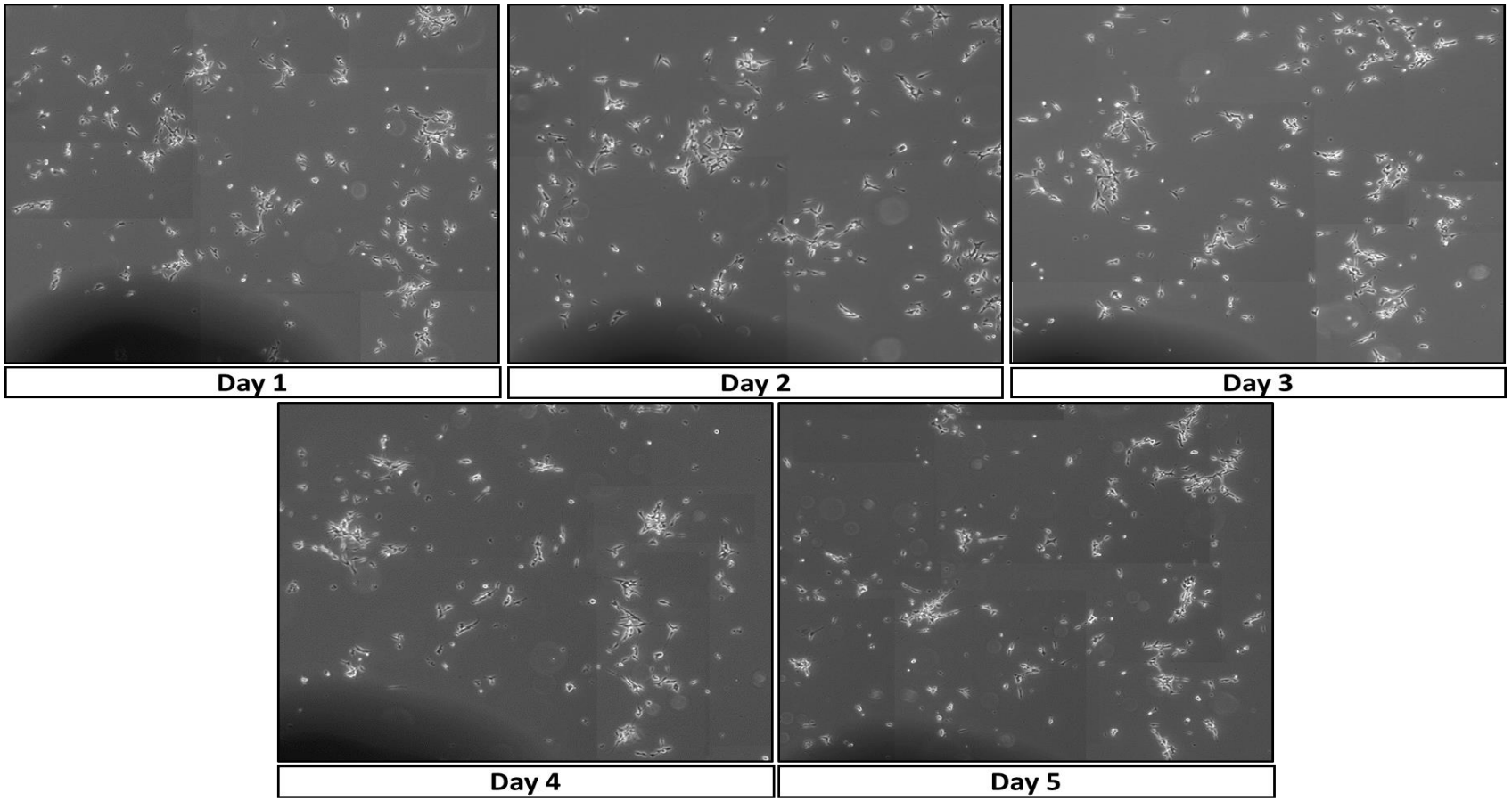

Figure B.3. ARA Replicate #1 Microscopy Images. Pictures were taken each day during the 5-day treatment incubation period.

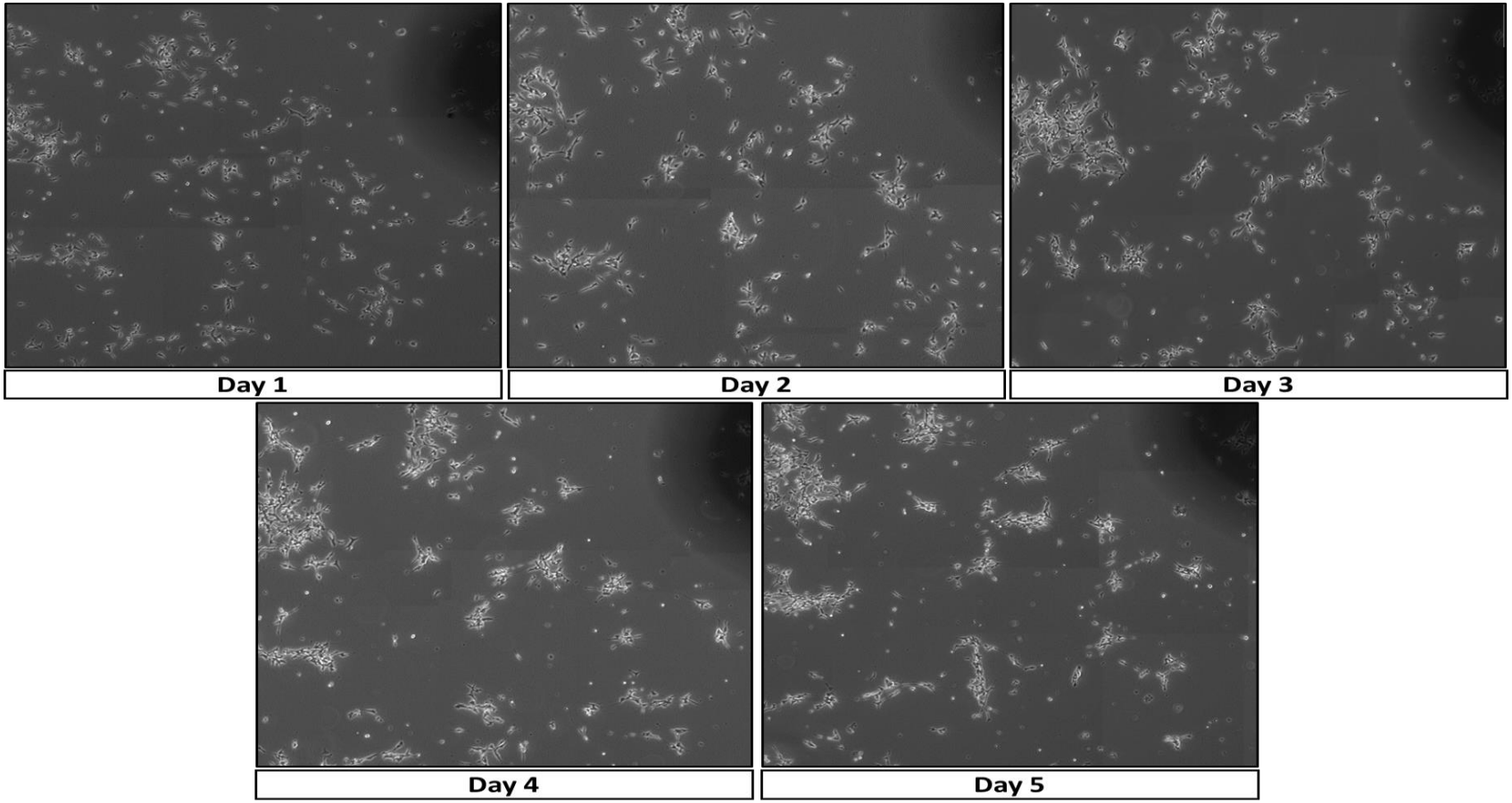

Figure B.4. EPA Replicate #1 Microscopy Images. Pictures were taken each day during the 5-day treatment incubation period.