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The Impact of an Omega-3 Enriched Diet on Hyperactivity and Biochemistry in an Animal Model for Attention-Deficit/Hyperactivity Disorder

Nadine M. Hammoud
Purdue University

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Is approved by the final examining committee:

John Burgess

Richard Mattes

Kimberly Kinzig

Megan McCrory

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THE IMPACT OF AN OMEGA-3 ENRICHED DIET ON HYPERACTIVITY AND BIOCHEMISTRY IN AN ANIMAL MODEL OF ATTENTION-DEFICIT/HYPERACTIVITY DISORDER

A Thesis

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Nadine M. Hammoud

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I dedicate this thesis to my parents, who always support my goals.

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LIST OF ABBREVIATIONS

Abbreviation	**Term**
5-HT	Serotonin
5-HTAA	5-Hydroxyindoleacetic acid
AA	Arachidonic acid
ADHD	Attention-deficit/hyperactivity disorder
ALA	Alpha-linolenic acid
CI	Confidence interval
D1-D5	Dopamine receptors 1-5
DA	Dopamine
DAT	Dopamine active transporter
DAT1	Dopamine active transport gene
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
DRD4	Dopamine D4 Receptor
DSM-V	Diagnostic & Statistical Manual of Mental Disorders
EFA	Essential fatty Acid
EPA	Eicosapentaenoic acid
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HVA	Homovanillic acid
LA	Linoleic acid
LC	Long chain
OR	Odds ratio
PUFA	Polyunsaturated fatty acid

ROS	Reactive oxygen species
SEM	Standard Error of Mean
SHR	Spontaneously Hypertensive Rat
TH	Tyrosine Hydroxylase
VNTR	Variable number tandem repeats
VTA	Ventral Tegmental Area
WKY	Wistar Kyota Rat

ABSTRACT

Hammoud, Nadine M. M.S., Purdue University, December 2014. The Impact of an Omega-3 Enriched Diet on Hyperactivity and Biochemistry in an Animal Model for Attention-Deficit/Hyperactivity Disorder. Major Professor: John Burgess.

Attention-deficit/hyperactivity disorder (ADHD) is the most diagnosed behavioral disorder in children. It affects around 5% of children worldwide and 11% of children in the United States, with rates increasing. Pharmaceutical treatments, such as amphetamines and methylphenidates, are not effective for everyone and are known to have unwanted side effects. While the etiology of the disorder is not yet fully understood, there are clear genetic and environmental components. Nutritional insufficiencies have recently become a popular environmental risk factor under investigation. Essential fatty acids (EFA), omega-3 polyunsaturated fatty acids (PUFA) in particular, are needed for proper brain development and function. Our lab has found lower proportions of omega-3 PUFA in the phospholipids and red blood cell membranes of about 40% of the children and adults with ADHD. Other research groups have subsequently confirmed similar findings. It is not yet known why a subgroup of the ADHD population seem to display EFA insufficiency, or if supplementation can reliably prevent or alleviate symptoms of the disorder. However, multiple human and animal studies have reported a reduction in ADHD-symptoms with omega-3 PUFA supplementation. Thus, we hypothesized that an omega-3 PUFA enriched diet would reduce the ADHD symptom of hyperactivity,

modulate dopamine and serotonin turnover, and increase omega-3 PUFA proportion in plasma and brain phospholipids in the Spontaneously Hypertensive Rat (SHR) animal model for ADHD. Additionally, we explored the relationship between oxidative stress, EFA status, and ADHD behavior with the prediction that SHR will display greater oxidative stress than the control strain, Wistar Kyota Rat (WKY). In order to develop a protocol that elucidates the behavioral differences between the two rat strains, we conducted a pilot study on various behavioral tests on the WKY and SHR while on standard rat chow. Results from our preliminary data led us to use the open field test as a measure of hyperactivity. In our intervention study, the omega-3 enriched diet (omega-3 diet) had no impact on measures of hyperactivity. However, our intervention successfully increased omega-3 PUFA proportions in plasma and brain phospholipid membranes. WKY had a higher proportion of eicosapentaenoic acid (EPA) in both plasma and brain than SHR, and SHR had a higher proportion of docosahexaenoic acid in plasma for both diets. Results of the liver total glutathione (GSH) analysis suggested that the omega-3 diet reduced oxidative stress, but that the SHR had lower oxidative stress than the WKY. SHR on the omega-3 diet had a lower concentration of dopamine in the neostriatum than SHR on the omega-6 dominant diet, and both rat strains on the omega-3 diet had lower serotonin concentration. Consistent with the lack of impact on behavior, dopamine and serotonin turnover were not modulated by diet. However, dopamine turnover in the SHR was lower than that in the WKY. In summary, our dietary intervention did not impact behavior, which was consistent with the lack of impact on neurotransmission, despite the alteration in phospholipid proportions. Future studies should focus on determining the most effective dose, EPA/DHA ratio, and time period for an omega-3 PUFA intervention.

CHAPTER 1. INTRODUCTION

1.1 Objectives and Organization

The overall goal was to examine the possible impact of an omega-3 enriched diet on Attention-deficit/hyperactivity disorder (ADHD) behavior, particularly as it relates to neurotransmission, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) proportion in plasma and brain and oxidative stress. The specific hypothesis that was tested is that a higher plasma and brain phospholipid proportion of EPA and DHA, obtained from diet, can reduce hyperactivity, improve dopamine and serotonin utilization, and reduce oxidative stress.

The specific objectives were to:

1. Evaluate the behavioral effects of an omega-3 enriched diet on hyperactivity in comparison to an omega-6 dominant diet in an animal model.

2. Examine biochemical markers in the animal brain, plasma, and liver to determine the relationship between behavior, neurotransmission, oxidative stress, and brain and plasma phospholipid composition, along with any impact of diet or strain.

This thesis covers topics regarding the relationship between ADHD, essential fatty acids (EFAs), and oxidative stress in 4 chapters. Chapter 1 is a literature review that covers general background ADHD information, the relationship between essential fatty acids and brain function and behavior, and studies reporting the impact of omega-3

supplementation on ADHD behavior. It will also include a review of omega-3 polyunsaturated essential fatty acids, oxidative stress, and the history of the validated animal model for ADHD. Chapter 2 is a supplemental chapter detailing a pilot study used to validate behavioral differences between the SHR and WKY, and behavioral tests. Chapter 3 describes the design and results of our animal study on the impact of an omega-3 enriched diet on behavior, neurotransmission, essential fatty acid proportion, and oxidative stress. Chapter 4 will conclude with a summary of the work, conclusions, and future directions.

1.2 Attention-Deficit/Hyperactivity Disorder

Attention-deficit/hyperactivity disorder is the most commonly diagnosed behavioral disorder in children. Diagnosis is based off of criteria set by the Diagnostic and Statistical Manual of Mental Disorders (5^{th} edition) (DSM-V), which is a classification and diagnostic tool used by psychologists and researchers. According to the DSM-V, ADHD is characterized by symptoms of inattention, hyperactivity, and impulsiveness. Manifestations of these symptoms include: frequent daydreaming, interrupting others, inability to remain seated, and/or not listening when spoken to. Children under the age of 17 must exhibit at least 6 of the 9 inattentive and/or hyperactive and impulsive symptoms, while adults need only to display 5 or more symptoms[1]. This is a change from the previous criteria reported in the DSM-IV, in order to reflect the reduction of symptoms that commonly occurs with age[2]. Furthermore, there must be clear evidence that the symptoms interfere with, or reduce, the quality of social,

academic, or occupational functioning. Finally, more accountable causes of the symptoms must be ruled out before reaching a diagnosis.

ADHD is divided into three subtypes: Combined presentation, Predominantly Inattentive presentation, and Predominantly Hyperactive-Impulsive presentation. In Combined presentation, the individual displays symptoms of inattention, hyperactivity, and impulsiveness. In the Inattentive or Hyperactive-Impulsive presentation, the individual primarily displays symptoms of inattention or both hyperactivity and impulsiveness. The clinician must also specify whether the client's ADHD is mild, moderate, or severe, which is determined by the number of symptoms presented[1].

ADHD is commonly co-morbid with other psychological disorders. In reviews of ADHD diagnosis, symptoms, and treatment, 25-75% of teens with ADHD are reported to meet the criteria for oppositional defiant or conduct disorder. Depression and anxiety are two other common co-morbid disorders with 48% and 36% receiving the additional diagnosis, respectively[3,4]. ADHD is also commonly co-morbid with other disorders such as tic disorders, Obsessive Compulsive Disorder, and sleep difficulties[4].

1.2.1 Demographics

As previously stated, ADHD is the most common behavioral disorder in children. It affects around 5% of children (ages 4-17) worldwide, and up to 11% of children in the United States (US)[5]. The percentage of children with an ADHD diagnosis has been increasing yearly, with rates at 7.8% in 2003, 9.5% in 2007 and rising to 11% in 2011[6]. This is an average increase of 5%/year from 2003-2011[7]. Males are three times more

likely to have an ADHD diagnosis than females. ADHD diagnosis also varies by state, with the highest rates in the Midwest and some states on the East Coast[6].

1.2.2 Neurobiology

There are multiple theories regarding the physiology of ADHD, and dopamine (DA) neurotransmission abnormalities are heavily implicated in most of them. Dopamine is a catecholamine neurotransmitter, a chemical messenger in the brain, which plays an essential role in attention, motivation, learning, and memory. Major dopamine pathways run through the striatum and the frontal cortex regions of the brain – areas implicated in ADHD. Dopamine is primarily produced in the substantia nigra or the ventral tegmental area (VTA), both of which are located in the midbrain (Figure 1). Consequently, these areas are often analyzed when researching dopamine. Projections from these neurons go to the striatum, the nucleus accumbens, and the prefrontal cortex in addition to other areas of the brain. Dopamine exerts an effect by binding to cell-surface receptors.

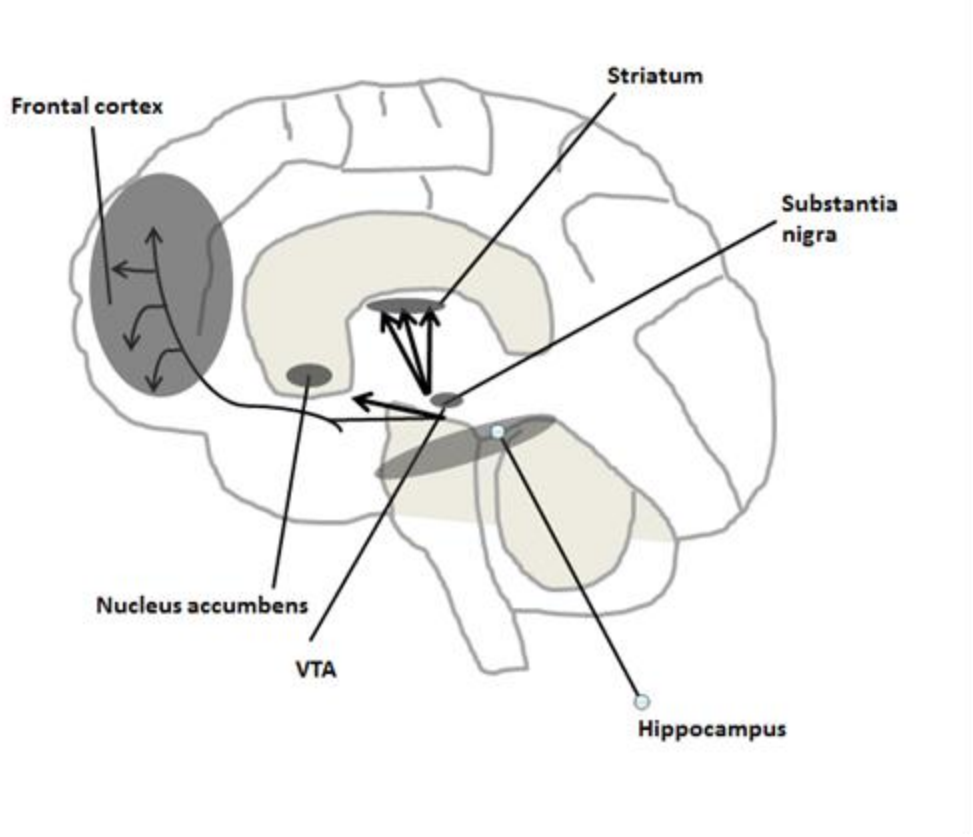

Figure 1: Dopaminergic Pathways

There are 5 types of dopamine receptors (D1-D5), which all function as G-protein receptors. However, D2 and D4 are currently the main dopamine receptors of interest in ADHD research[8,9]. A simplified model of how dopamine is controlled at the synaptic level could be described by: (1) pre-synaptic cell firing releases dopamine into the synaptic cleft; (2) dopamine then reversibly binds to dopamine receptors on the post synaptic cell; (3) dopamine is either reabsorbed by dopamine transporters and degraded into homovanillic acid (HVA) or is recycled for further use (see Figure 2)[9]. Of note, dopamine is a precursor to norepinephrine, which is also crucial for short-term memory and attention.

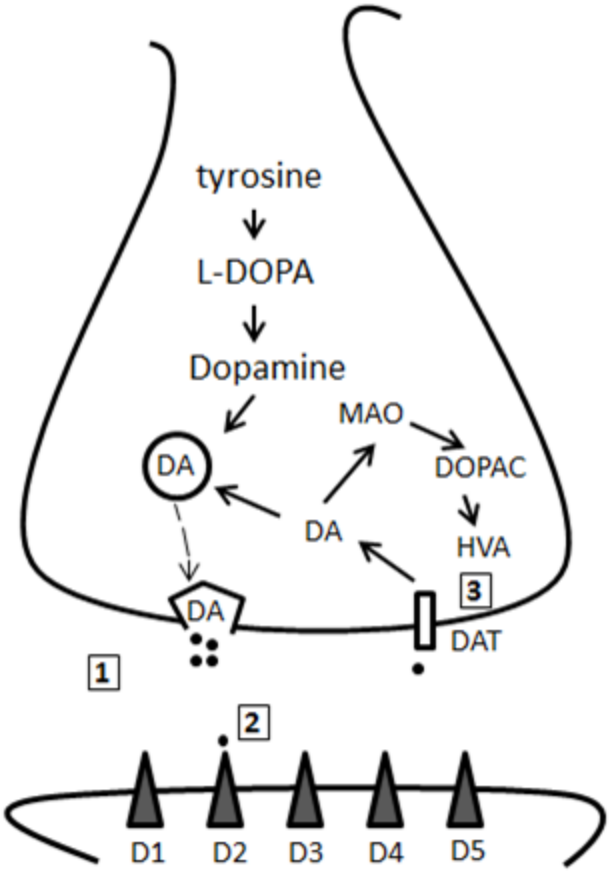

Figure 2: Dopamine Metabolism

Among the multiple theories that have been proposed for the cause of ADHD behavior, it is generally agreed upon that the cause involves disturbances in the dopaminergic pathway. Some points of contingency stem from whether these disturbances result in a hyperdopaminergic state, hypodopaminergic state, or if there are alternative consequences. Currently, one of the most supported theories is that those with ADHD have a dopamine deficit, meaning they have low dopamine production or

response for reasons not fully understood[9]. Multiple neurobiological studies have been conducted to decipher the cause of the proposed dopamine deficit. Castellanos et al.[9] investigated whether there are size abnormalities in dopamine-modulated areas of the brain. The researchers measured brain volumes of 152 children and adolescents with ADHD and compared the sizes to healthy controls using magnetic resonance images. The researchers found that brain regions with high dopamine receptors were significantly smaller in the ADHD group than the control. Their longitudinal growth curves suggested that this decrease in volume occurs early in development, as fundamental developmental processes appeared to be healthy[10]. Another area to investigate is dopamine function. Volkow et al.[11] measured dopamine receptor availability and dopamine release in ADHD adults using positron emission tomography. The researchers found that dopamine receptor availability in the left caudate was significantly lower in subjects with ADHD ($p < 0.04$), and showed a trend in the right caudate ($p < 0.07$). In addition to lower dopamine receptor availability, the ADHD subjects were reported to have lower dopamine release as well[11].

In congruence with the hypodopaminergic theory, there is also evidence that patients with ADHD have low dopamine response. Wigal et al.[12] tested ten untreated children with ADHD versus eight age-matched controls by having them undergo two separate exercise sessions. Exercise is known to elicit a dopamine and norepinephrine response and peripheral nervous system activity is correlated with brain activity. They found that the children with ADHD had significantly lower plasma norepinephrine at all-time points and significantly lower dopamine at the peak of the exercise compared to controls. However, it is yet to be determined whether the lack of dopamine response is

due to a systematic dopamine deficit or to less stimulation of the adrenals in response to exercise[12]. Nonetheless, the results provide further support that ADHD symptoms may be a result of a hypodopaminergic state.

1.2.3 Treatment

ADHD is normally treated with the use of amphetamines (ex. Adderall) or methylphenidates (ex. Ritalin). Both treatments either indirectly or directly increase the amount of dopamine in the synapse. Amphetamines cause an increase in dopamine release. Methylphenidates, on the other hand, block dopamine reabsorption by blocking the dopamine active transporter (DAT). Blocking reabsorption increases the amount of dopamine in the synapse, which allows dopamine to repeatedly bind to dopamine receptors[13]. However, stimulants may act in other additional ways that help reduce hyperactivity. While stimulant medication can alleviate symptoms, it is only effective for around 70% of adolescents[4]. Common side effects include upset stomach, headache, decreased sleep, and decreased appetite. Recently, non-stimulant atomoxetine has been approved for ADHD treatment. Atomoxetine is a selective inhibitor of the presynaptic noradrenaline transporter with low affinity for serotonin (5-HT) and dopamine transporter receptors[14]. Preliminary evidence suggests that atomoxetine may be beneficial for ADHD that is co-morbid with anxiety and depression, two common co-morbid disorders[15].

1.2.4 Etiology

The etiology of ADHD is not yet known, but there are clear genetic and environmental components. Based on many twin and adoption studies, the estimated

heritability of ADHD is almost 80% and this rate has not changed since 1973[16]. Parents of adopted children with ADHD are less likely to have the disorder than biologically related relatives[17]. Thus, genetics seems to play an important role in initiating ADHD.

There are a few genetic studies that have identified genes with polymorphisms that are associated with ADHD[18]. The most frequently implicated genetic factors are the dopamine D4 receptor (DRD4) and the dopamine transporter gene (DAT1). Both these genes play important roles in the dopaminergic system. DRD4 has been frequently analyzed due to its prevalence in the frontal-subcortical networks. Dopamine, along with norepinephrine, are potent agonists for this receptor[19]. Many researchers have assayed a tandem repeat polymorphism in exon III of DRD4 because it has been shown that the 7-repeat allele variant results in a low response to dopamine. In fact, a significant association between the 7-repeat allele and ADHD in both case-control and family studies has been reported[20,21]. However, other studies found no overall association of any allele with ADHD[22]. Tyrosine hydroxylase, which plays a role in DA synthesis by catalyzing the conversion of tyrosine to dihydroxy-phenylalanine, was also investigated for its role in ADHD. However, studies have shown no association between polymorphisms in the TH gene and ADHD[23,24,25,26]. There are many studies investigating abnormalities in the dopamine transporter gene as a potential instigator of ADHD. DAT is responsible for the reuptake DA, a preliminary step to its degradation. A polymorphism consisting of an allele containing a 10-repeat allele of a 40-base pair variable number of tandem repeats (VNTR) located at the 3' untranslated, non-coding end has been primarily investigated. The association of ADHD with this polymorphism was first proposed by Cook et al.[27], who conducted a study including 119 children with ADHD and found an

association between this polymorphism and response to treatment. This has later been confirmed by multiple other studies[27,28]. Furthermore, Dougherty et al.[29] measured striatal DA transporter activity and found that activity was elevated by 70% in ADHD adults. Higher DA transporter activity could result in higher dopamine degradation. On the other hand, the results of studies assessing DAT density in ADHD individuals have not been consistent[30]. While there is some promising evidence supporting genetic factors, no single gene polymorphism has enough support to be considered the sole cause of ADHD. Thus environmental factors are also researched.

1.3 Environmental Risk Factors and ADHD

Environmental factors have become an increasingly popular focus in ADHD research. When assessing ADHD risk, environment factors are commonly classified as prenatal, perinatal, and postnatal[31]. Multiple meta-analyses and reviews have assessed common environmental factors for positive correlation with ADHD risk. The risk factors that will be discussed in this review include: cigarette and alcohol exposure, lead exposure, and nutritional insufficiencies.

1.3.1 Maternal Smoking

Maternal smoking during pregnancy is significantly associated with increased ADHD risk in the offspring[32]. Braun et al.[33] obtained data from the National Health and Nutrition Examination Survey from 1999-2002. Among 4,704 children aged 4-15 years old, 4.2% had ADHD and took stimulant medication. Using multivariable analysis, they found that prenatal tobacco exposure [odds ratio (OR) = 2.5;95% confidence interval (CI),

1.2-5.2] was significantly associated with ADHD, however, postnatal maternal smoking was not. The researchers suggest that prenatal exposure to tobacco could account for up to 270,000 excess cases of ADHD [33].

1.3.2 Prenatal Alcohol Exposure

The risk of prenatal exposure to alcohol seems to be dependent on severity and duration of alcohol consumption, thus the research has not been uniform[31]. However, in a retrospective, case-control study conducted by Mick et al.[34] it was reported that those exposed to alcohol in utero were 2.5 times more likely to have ADHD, independent of nicotine exposure. The researchers concluded that alcohol is a risk factor of ADHD. In addition, Knopik et al.[35] reported that offspring of twins with a history of alcohol abuse were significantly more likely to develop ADHD than offspring of nonalcoholic controls.

1.3.3 Lead Exposure

In addition to evaluating maternal smoking as a risk factor, Braun et al.[33] investigated the risk of lead exposure. Using the same data obtained from the National Health and Nutrition Examination Survey, the researchers found higher blood lead concentrations in mothers that have children with ADHD, which could account for 290,000 excess cases of ADHD in the US. This number is equal to the excess number resulting from tobacco exposure. Studies involving lead screening of children with ADHD report positive correlations between mean blood lead level and ADHD[36]. Other studies report variable results, but enough evidence supports that prenatal exposure to lead is a risk factor[31].

1.3.4 Nutritional Insufficiencies

Low nutrient status has been associated with a number of disorders. For example, lack of vitamin C can cause the rare disease scurvy, while vitamin D deficiency can lead to a disorder of the bone, known as rickets. Nutritional insufficiencies may also impact behavioral disorders, and have been investigated within ADHD research.

A relatively new risk factor associated with ADHD is vitamin D insufficiency. The US Endocrine Society characterizes vitamin D deficiency as vitamin D levels less than 20ng/ml, and insufficiency as vitamin D between 21-29ng/mol. Low vitamin D is a major health concern that occurs in both low sunshine and abundant sunshine areas[37]. Not only can insufficiency result from low sun exposure, but also from poor vitamin D status during pregnancy[37,38]. Vitamin D is implicated as an essential component of normal brain development as it enhances neuroprotection and modulates anti-inflammatory mechanisms[39,40]. Recently, vitamin D insufficiency has been investigated for its impact on ADHD risk. Bener et al.[41] measured serum levels of vitamin D in 1331 children displaying symptoms of ADHD or healthy controls. The researchers found that vitamin D insufficiency was significantly greater in the children with ADHD and that these children were more likely to have severe vitamin D deficiency[41]. This is one of the first investigations into the correlation between vitamin D insufficiency and ADHD and it is a risk factor that should be investigated further[42].

Essential fatty acid insufficiency is another nutritional factor that is currently being researched. Essential fatty acids are so named because they cannot be synthesized in the body, and thus need to be consumed in the diet. There are two polyunsaturated fatty acid types of interest: omega-3 polyunsaturated fatty acids (PUFAs) and omega-6

polyunsaturated fatty acids. Inadequate intake, or excessive breakdown and excretion, could result in a number of abnormal symptoms manifesting in the hair and skin, or excessive thirst and urination[43]. Omega-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been a popular topic in ADHD research due to their known role in brain development and behavior. A study conducted in our lab on children with ADHD and age-matched controls, identified a subgroup of children with ADHD displaying symptoms of EFA deficiency and presenting with low proportions of omega-3 and omega-6 fatty acids in the blood[44]. In fact, about 40% of subjects had significantly lower plasma omega-3 and omega-6 PUFAs than both healthy controls and those with ADHD not displaying EFA symptoms. Chen et al.[45] investigated dietary intake and blood phospholipid levels in children with ADHD and also found that those with ADHD had significantly lower levels of arachidonic acid (AA), an omega-6 fatty acid, and DHA, despite similar diets to controls. Another study reported a similar finding[46]. Interestingly, a recent meta-analysis on the correlation between common environmental risk factors and ADHD reported a significant correlation between babies that were formula fed and those that developed ADHD[47]. According to the authors, this finding was consistent with previous studies and the results may be due to the greater amounts of essential fatty acids found in breast milk. Up until 2001, commercial formula did not contain omega-3 fatty acids[48]. The next section will discuss essential fatty acid biology, intake, and their relationship with brain development and behavior in detail.

1.4 Essential Fatty Acids and Brain

1.4.1 Essential Fatty Acid Background and Synthesis

Essential fatty acids are fatty acids that are required for biological processes but need to be ingested from the diet. Alpha-linolenic acid (ALA) and linoleic acid (LA) are two fatty acids that are known to be essential for humans. They are also the precursors to omega-3 and omega-6 long-chain polyunsaturated fatty acids. The two main families of EFAs are the omega-6 family and the omega-3 family and they are named based on the location of the first double bond closest to the terminal (omega) end of the fatty acid (Figure 3).

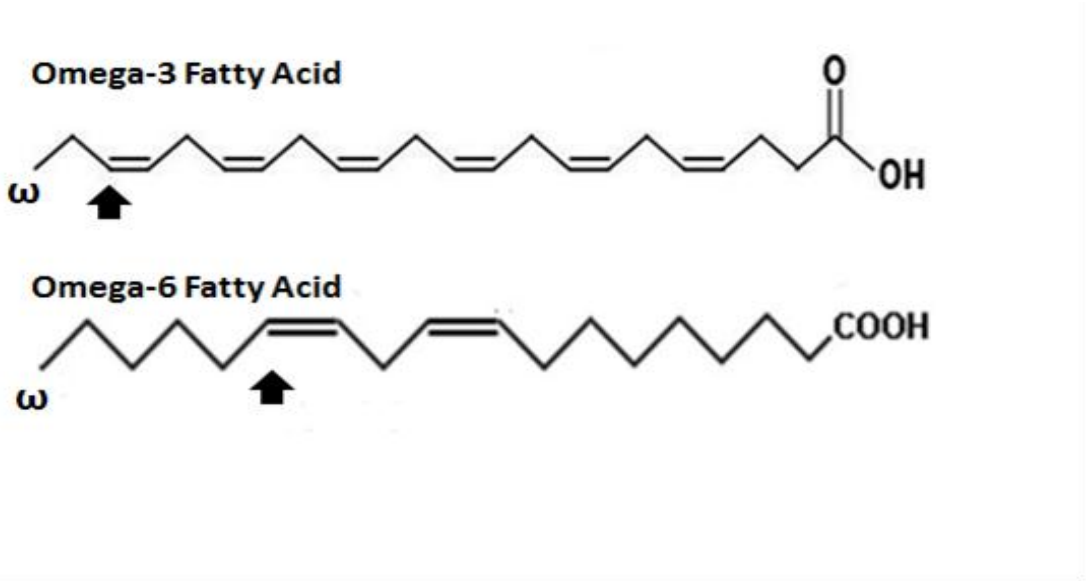

Figure 3: Omega-6 and Omega-3 Fatty Acids

Both ALA and LA contain 18-carbon long fatty acid tails. Through a series of desaturation and elongation enzymatic steps, arachidonic acid (AA) or EPA and DHA are synthesized, respectively. The two families share the same enzymes as illustrated in their synthesis pathway diagram (Figure 4).

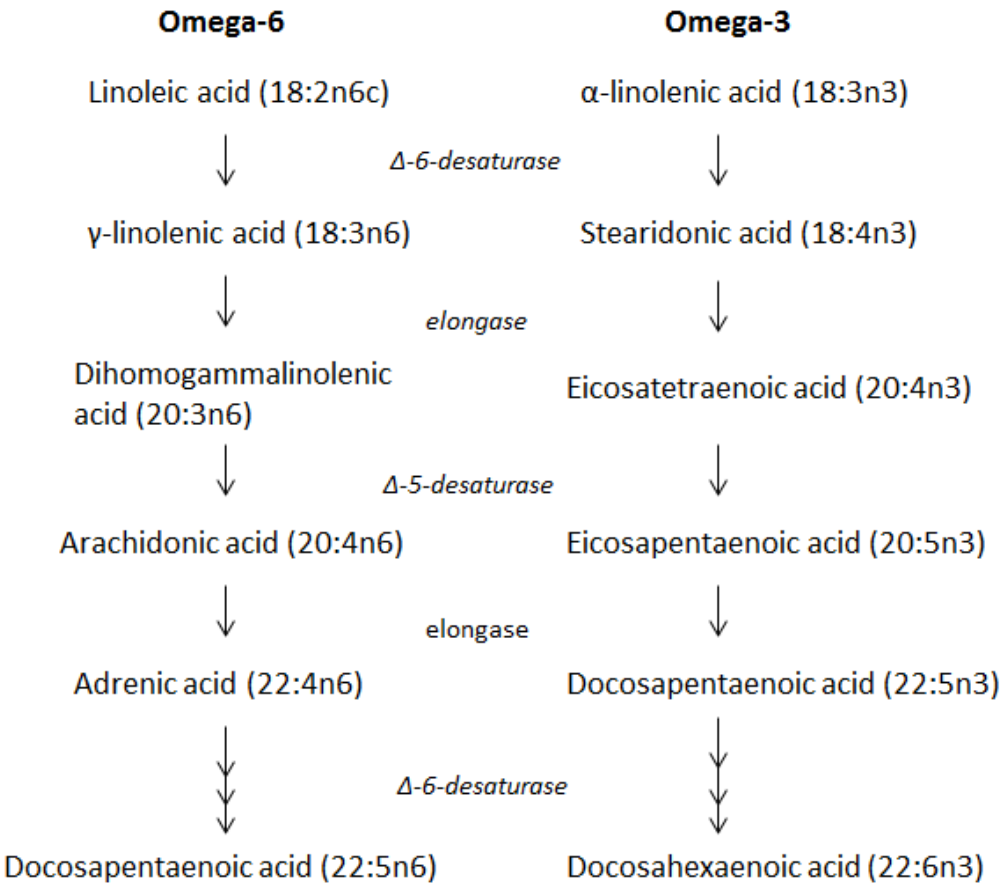

Figure 4: Essential Fatty Acid Metabolism

1.4.2 Essential Fatty Acids in the Western Diet

Omega-3 PUFAs are very low in the typical Western Diet. On average, the ratio of omega-6 EFAs to omega-3 EFAs is 15:1[49]. Most omega-6 EFAs come from meat such as red meat and poultry, which are two common foods in the western diet. They are also found in corn and soy. On the other hand, omega-3 PUFAs are primarily found in fatty fish such as salmon and sardines. ALA is found in flax seed and chia seeds, but the

conversion to EPA and DHA is small[49,50]. An international board of lipid experts recommended a ratio of 2:1 as most ideal[51], but 4:1 is also recommended and more attainable[52,53]. The acceptable intake of omega-3 EFAs is 1.6g/day for men and 1.1g/day for women. The FDA advises that 3g/day of EPA + DHA can be ingested safely, with up to all 2g from supplements[54].

1.4.3 Essential Fatty Acid Status

Essential fatty acid status is determined by the quantity of EFAs and their products in cells and tissues, and is usually measured via plasma analysis. Status is impacted by dietary intake, metabolism, absorption, and degradation. Deficiency can be caused by one of two ways: primary deficiency or secondary deficiency. Primary deficiency results from inadequate dietary intake while secondary deficiency is the result of something internal, such as competition for the metabolic enzymes, or an increased degradation rate. One such factor than can increase PUFA degradation rate is oxidation from exposure to free radicals. An accumulation of free radicals can cause macromolecule damage. When the damage is sufficient to destroy cells and tissue, the occurrence is referred to as oxidative stress. Oxidative stress will be discussed in more detail in a later section. Insufficiency of one type of fatty acid can also occur when the intake of one family of fatty acid dominates the other, as the same enzymes are used for both the omega-6 and omega-3 PUFA synthesis pathway (i.e. too much omega-6 intake could result in lower omega-3 PUFA synthesis). Furthermore, long chain PUFA synthesis is not 100%. For the omega-3 family, conversion from ALA to EPA and DHA is as low as 5-15%[55,56].

1.4.4 Brain Fatty Acid Composition

The brain is one of the fattiest organs in the body, second only to adipose tissue. Thus, lipids are crucial for brain development and function[57]. Almost 66% of the brain's weight is due to phospholipids, 35% of which are omega-3 PUFAs. Of all the fat in the brain, DHA makes up 10-20% and is the highest omega-3 found in the brain[57]. The most dramatic brain development occurs prenatally and during the first few years of life. Consequently, this is when adequate availability of EPA and DHA is most important. DHA delivery to the central nervous system is most efficient during times of synaptogenesis, which occurs most rapidly during early brain development[58]. Many studies confirm that DHA is essential for optimal brain development and function[59,60]. Studies conducted by Jumpsen et al.[52,53] demonstrated that a ratio of 4:1 was optimal for frontal cortex, hippocampus, and cerebellum development in growing rats. The researchers reported that even a small change in the ratio impaired the rate of development[52,53]. Furthermore, a reduced amount of DHA is compensated by an increased amount of docosapentaenoic acid (DPA) (22:5n-6), which reduces membrane fluidity[61].

1.4.5 Oxidative Stress and Essential Fatty Acids

Fatty acids, especially those with multiple double bonds, are prone oxidation in elevated states of oxidative stress. Oxidative stress is the imbalance of the biological system's antioxidant defense and the manifestation of reactive oxygen species. A disturbance in the normal redox state in cells can result in the production of peroxides or free radicals, which can damage the DNA, carbohydrates, lipids, and proteins[62]. Free

radicals are species that contain one or more unpaired electrons. Normal oxidative metabolism results in a constant stream of oxygen derived free radicals, which are more reactive than ground state oxygen and are thus named reactive oxygen species (ROS). ROS can include radicals (i.e. hydroxyl radical) and non-radicals that easily degrade to radicals (i.e. hydrogen peroxide), while the antioxidant defense system includes both dietary and endogenously produced antioxidants such as carotenoids, tocopherols, and thiols[62].

The upregulation of antioxidant genes is one of the major mechanisms by which cells protect themselves against oxidative stress[63]. Intracellular thiol groups act as antioxidants by scavenging free radicals through enzymatic reactions[62]. Reduced glutathione (GSH) is one of the most abundant and important intracellular thiols in cells[63]. Therefore cells tightly regulate the synthesis, export, and utilization of GSH. GSH elicits its antioxidant effect by removing potentially toxic electrophiles and metals, which protects cells from toxic oxygen products[64]. GSH also exhibits control in membrane transport[65]. In fact, it is able to protect biological membranes against lipid peroxidation as it prevents damage in a lipid environment[62]. However, its ability to protect membranes is enzymatically mediated, and therefore dependent on, vitamin E.

Vitamin E, which encompasses a small group of tocopherols, is a major lipid-soluble antioxidant. It is primarily responsible for protecting biological membranes by protecting membrane PUFAs from lipid peroxidation. The amount of vitamin E in membranes often indicates the susceptibility of low-density lipoproteins, whole organs, or microsomal membranes to oxidative damage[62,66,67]. Tocopherols scavenge peroxyl radicals without reacting in chain-propagating steps, thereby protecting lipids.

1.4.6 Essential Fatty Acids and Neural Function

As discussed previously, ADHD symptoms are thought to result from disturbances in the dopaminergic system. Therefore, the areas of neural function that will be discussed in this review are either directly or non-directly related to neurotransmission: membrane fluidity, neurotransmitter activity, and neuron health.

1.4.6.1 Membrane Fluidity

Omega-3 PUFAs are thought to influence neural function in a variety of ways. One of the most known ways is by increasing membrane fluidity. The membrane fluidity index is primarily influenced by the percentage and composition of membrane PUFA and membrane cholesterol content. Neuronal membranes are made up of a phospholipid bilayer, which incorporates phospholipids from fatty acids in the diet. Saturated fatty acids have a hydrocarbon tail with the maximum amount of hydrogen atoms possible. An unsaturated fatty acid has a hydrocarbon tail with one or more carbon-carbon double bonds instead of hydrogen. These double bonds cause kinks in the tail, which contribute to membrane fluidity. Therefore, higher PUFA content results in a more fluid membrane. On the other hand, cholesterol is needed to maintain rigidity despite high temperatures. Interestingly, omega-3 fatty acids can actually displace cholesterol in the membrane and thereby reduce its amount, while omega-6 fatty acids only redistribute membrane cholesterol[68]. A number of research studies have shown that changing the level of EFAs in the diet will affect the fatty acid profile in the neuronal membrane, therefore these changes can be induced via dietary intervention[69].

Maintaining proper membrane fluidity is important for healthy neural processes. A rigid membrane, or low membrane fluidity index, will affect receptor function, ion channel activity, and neurotransmitter release such as dopamine[68]. When nerve cell membranes are fluid, neurotransmitter receptors are better able to recognize and bind the appropriate neurotransmitters. Since dopamine is a neurotransmitter that is heavily implicated in ADHD behavior, improving membrane fluidity could alleviate symptoms.

Oxidative stress can also increase cholesterol levels in the brain, thereby decreasing membrane fluidity and affecting neurotransmission[70–72]. In young rats, oxidative stress was found to raise levels of brain cholesterol to that of aged rats[70]. The increase of free radicals in states of heightened oxidative stress can decrease membrane fluidity as well[73,74]. Some researchers have successfully corrected the harmful impact of oxidative stress on membranes using PUFA supplementation[75–78]. Treatment with omega-3 PUFA can also restore a long-lasting increase in synaptic efficacy following stimulation of afferent fibers in aged rats.

1.4.6.2 Neurotransmission

Na^+, K^+-ATPase couples ATP hydrolysis for the active transport of 3 Na^+ ions out of the cell in exchange for 2 K^+ ions into the cell. This process is integral in neurotransmission and signaling[79]. Na^+, K^+-ATPase activity allows for rapid repolarization of the neuron, an action needed for repetitive firing. Na^+ and K^+ exchange, mediated by Na^+, K^+-ATPase, plays an active role in normal action potential conduction[80]. Bourre et al.[81] investigated the impact of an alpha-linolenic acid deficient diet on Na^+,

K^+-ATPase activity in nerve endings. The researchers fed Wistar rats an ALA-deficient diet (1.8% sunflower oil) or an ALA-adequate diet (1.9% soybean oil) for two generations. The third generation males were used for behavioral experiments and physiological analysis. The deficient diet successfully resulted in reduced amounts of DHA, and increased amounts of DPA, in brain cells and organelles compared to control. The researchers found that the nerve terminal Na^+, K^+-ATPase activity in rats fed the ALA-deficient diet was reduced to 60% of the control group. In addition, those rats on the deficient diet performed significantly worse on the shuttle box test for learning capacity.

Dopamine and serotonin (5-HT) are implicated in many neurological disorders[82]. In a study conducted by Delion et al.[83] rats were fed an ALA-deficient diet over several generations and the dopaminergic and serotoninergic systems were analyzed in the frontal cortex, striatum, and cerebellum. The rats on the deficient diet had a 40-75% lower level of frontal cortex endogenous dopamine. There was also an 18-46% increase in serotonin 5-HT^2 receptor density but without a change in endogenous serotonin levels. The increase in 5-HT^2 receptor density could be due to the decrease in D2 dopaminergic receptor density which also occurred, as the two are thought to be interlinked[83]. Many other studies have shown this effect of PUFA deficiency on dopamine and serotonin[84]. In a study where piglets were fed an AA and DHA-deficient diet, dopamine and serotonin concentration decreased in the frontal cortex[85]. Similar studies feeding rats an n-3 PUFA-deficient diet also reported a reduction in the dopaminergic vesicle pool and inadequate storage of newly synthesized dopamine[86,87]. On the other hand, a study on rats given fish

oil reported a 40% increase in dopamine concentrations in the frontal cortex and greater binding to D2 receptors[84].

1.4.6.3 Neurons

It is widely accepted that DHA plays a crucial role in brain development, especially during periods of dramatic growth. Cao et al.[88] demonstrates this fact by testing the effect of DHA on neurite outgrowth and viability in growing cortical neurons[88]. The researchers prepared cortical neurons from fetal Sprague-Dawley rat pups. Cells were divided into DHA-treated or control groups. Different concentrations of DHA were added to the neurobasal medium after plating. The researchers found that the DHA-treated group had significantly more neurite outgrowth and accelerated neurite elongation than controls[88]. Interestingly, only a narrow concentration range of DHA had a desired effect on neurite growth while high concentrations were actually neurotoxic to the cortical cells. This is in agreement with other studies that report that high-dose DHA supplementation can lead to higher peroxidation and oxidative stress, but these side effects can be reduced with antioxidant vitamin E intake[89–91]. These findings support that there may be an optimal intake of DHA to receive the best results.

1.4.7 Impact of an Essential Fatty Acid Deficit on Learning and Behavior

Several studies have shown that omega-3 PUFA deficiency can lead to functional and learning deficits, similar to ADHD-associated problems. Rats on an ALA-deficient diet exhibited 187% higher locomotor activity in a novel environment than control rats

over the course of 2 hours[92]. Of note, this increase in activity was seen after 15 minutes until the end of the testing period. The diet was successful in decreasing brain DHA by 80%, which was accompanied by a 575% increase in brain DPA. The alterations in locomotor activity observed are also consistent for the potential role that omega-3 PUFA, such as low DHA, can play in the development of ADHD. The researchers also had a remedial diet group where rats that were previously deficient were supplemented with DHA at weaning, a growth period that is developmentally similar to a full-term infant[93]. The supplementation, however, did not have an effect on locomotor activity[92].

Omega-3 PUFA deficiency has been shown to have a detrimental impact on learning ability in multiple studies. Mice fed an ALA-deficient or ALA-adequate diet for multiple generations demonstrated significantly decreased spatial learning and memory in comparison to controls[94,95]. In one such study, previously deficient 3rd generation mice were supplemented with omega-3 PUFA from egg yolk or pig brain for two months prior to testing in an elevated plus maze. The supplemented mice performed just as well as the control mice, and all groups performed better than the ALA-deficient group[95], further demonstrating that omega-3 PUFA supplementation can, in fact, reverse the negative effects of omega-3 PUFA deficiency.

1.5 Essential Fatty Acid Intervention Studies

1.5.1 Human Intervention Studies

Several human omega-3 intervention studies have investigated the potential for omega-3 supplements to alleviate ADHD symptoms. A meta-analysis reviewing 10 trials involving 669 children total concluded that omega-3 supplementation was modestly

effective in alleviating ADHD symptoms[96]. Bloch and Qawasmi[96] also note that supplementation with high EPA was most effective, and that while the impact is only moderate in comparison to standard pharmaceutical treatments, it is often preferred due to the benign nature of omega-3 supplements[96]. Milte et al.[97] conducted a recent study with the aim to evaluate whether a supplement with high DHA or high EPA is more effective in treating ADHD. The 12-month randomized controlled three-way crossover trial was conducted in children aged 6 to 13. Children were given one of three treatment conditions: EPA-DHA-LA, DHA-LA-EPA, or LA-EPA-DHA. Each supplement was taken for 4 months, with the omega-6 linoleic acid (LA) used for comparison. No washout period was included because erythrocyte PUFA levels are thought to return to baseline after 16 weeks[98]. Mean changes in erythrocyte fatty acids reflected what was expected with each treatment, with EPA and DHA increasing the most after the EPA or DHA treatment, respectively. However, mean EPA and DHA did not return completely to baseline after LA, hinting that the washout period was not fully effective. While conclusion statements could not be made in regards to which treatment was best (as the washout failed), behavioral changes were compared to change in erythrocyte PUFAs. An improvement in literacy, attention, and behavior was associated with within-subject changes in erythrocyte PUFA. Increases in EPA, DHA, and total omega-3 PUFA and decreases in the omega-6:omega-3 ratio had the most consistent correlations. Subscales of parent-rated behavior and hyperactivity was also improved with high erythrocyte omega-3 PUFA[99]. In a separate study, Vaisman et al.[100] investigated whether omega-3 fatty acids esterified to phospholipids or triglycerides are most effective for EPA and DHA delivery and ADHD treatment. Children were given a daily aliquot of a relatively

low amount of EPA and DHA (153mg EPA, 95mg DHA) compared to most intervention trials. The omega-3 PUFAs were either esterified to phospholipids or delivered via fish oil (naturally esterified to triglyercides). Of note, the authors emulsified the fatty acids into a chocolate spread, which may improve child adherence to the supplement regimen. Interestingly, the EPA and DHA esterified to phospholipids resulted in the greatest increase in plasma EPA and DHA levels. Furthermore, this increase was correlated with the greatest improvement in behavior scores. The fish oil also increased plasma EPA and DHA levels but the impact on behavior, though not significantly different from the impact in the phospholipid group, was not significantly improved from placebo[100]. These studies raise the question of not simply what kind of omega-3 fatty acids to supplement with, but what kind of delivery method to use.

When reviewing all the studies investigating the impact of an omega-3 supplement on ADHD behavior, the results are not conclusive. Just as there are studies reporting an effect, there are also studies reporting none[101–103]. This could be because the intervention may best impact a subgroup of those with ADHD, most likely the same subgroup that demonstrate omega-3 insufficiency. This is a conclusion that Belanger et al. reported[104]. In their study comparing the effect of an omega-3 supplement versus an omega-6 supplement (control) on children with ADHD, only a subgroup of the children (30.7%) saw significant improvement in their attention. More studies are needed to specifically evaluate treatment intervention on those displaying EFA deficiency symptoms.

Due to the inconsistent results from human studies, and the recent validation of a rat model for ADHD, animal studies are a relatively recent and valuable addition to

ADHD research. By utilizing an animal model, researchers can control for the placebo effect, perform more invasive analysis, and maintain a controlled environment. Research from animal studies may help determine how effective an omega-3 intervention is and pinpoint which physiological processes are being affected.

1.5.2 Animal Models and Animal Behavioral Tests for ADHD

An ideal animal model for behavioral disorders should have similar biochemistry, etiology, symptoms, and effective treatments as the disorder in humans[105]. Animal models are often advantageous over human models in that they allow for easier controlled environments, in-depth physiological analysis, and low risk of "placebo effect". As ADHD diagnosis is currently only behaviorally based, the validation of an ADHD animal model is based primarily on behavior[105]. Therefore, a validated animal model for ADHD should conform to construct validity, predictive validity, and face validity. Construct validity confirms that the animal model conforms to a theoretical rational. Predictive validity confirms that the animal model correlates to ADHD in humans in regards to behavior and neurological functions, while face validity means the animal model mimics the fundamental behavioral symptoms of ADHD. Currently the Spontaneously Hypertensive Rat (SHR/NCrl) from Charles River, Germany (Rat Genome Database 2008) is the most validated animal model for ADHD research[106]. While the best control strain is the Wistar Kyoto Rat (WKY/NHsd) from Harlan, UK.

Many studies have established face validity in the SHR. Both children with ADHD vs control and SHR vs control have been tested on a multiple fixed-interval/extinction schedule of reinforcement[107–111]. A multiple schedule is defined as two

or more schedule components alternating in the presence of different stimulus. A fixed-interval component is used to measure reactivity to reinforcers and impulsiveness, while the extinction component measures sustained attention and sensitivity to change[105]. These tests can be used to measure attention and impulsiveness. The ADHD symptom of impulsiveness can be seen as brief, short sequences of activity and rapid changes. It is also marked by the inability to wait, such as blurting out answers or choosing short-term rewards over bigger, long-term ones. These bursts of activity and rapid changes are typical in the SHR. Motor impulsiveness can be tested using the multiple fixed-interval/extinction schedule discussed above with impulsiveness measured as bursts of responses with short inter-response times. In behavioral tests with children with ADHD, this type of behavior is seen towards the end of testing.

Sagvolden et al.[105] have conducted numerous studies in validating the SHR as a model for ADHD. One of the earlier studies compared SHR/NCrl and WKY/NHsd among other rat strains on their behavior when trained to respond to cue lights in a dual lever operant chamber. The rats were trained to press a lever once the light turned on over it and were rewarded with water. The rats were trained for weeks until ready for their final schedule, which was used for behavioral analysis. Sustained attention was measured as percent correct lever responses. Impulsiveness was determined by number of short inter-response times (<0.67s) and general activity was expressed as total number of lever presses.

The researchers reported that the male SHR/NCrl rats had significantly poorer sustained attention compared to the WKY/NHsd and also greater general activity based on lever presses. The SHR/NCrl was moderately more impulsive than the other groups,

and more often responded to a previous lever press with a following lever press within 0.67s despite no reinforcement for this extra press. However, while there was a trend, there was no significant difference between groups in terms of impulsiveness. On the other hand, in another study by Sagvolden et al.[112] impulsiveness was found to be significantly higher for SHR than WKY.

An open field maze can also be used to measure hyperactivity by measuring general locomotion. In this behavioral test, an animal is placed on one spot in a square open field with four walls. The floor is normally divided into squares and locomotion is measured as number of boxes crossed or via video recording and computer analysis. In addition, number of center box entries and number of rearings can also be measured. Normal rats are expected to stick to the perimeter and have relatively few rearings. The idea is that if an animal is hyperactive, they will not only travel farther and more frequently, but will also have more rearings and center box entries. The SHR is frequently found to have high measures of locomotion in this test[113].

In terms of construct validity, there is support that SHR genetics, behavior, and neurobiology conform to a theoretical rationale for ADHD. Recently, genetic similarities have been found between the SHR and those with ADHD. A 160bp insertion was found in the non-coding region of the DAT1 gene, just as genetic differences in the DAT1 gene of humans with ADHD have also been reported[27,29,114–116]. In addition, DAT1 expression is altered as it has been found to be reduced in SHR midbrain during the first postnatal month compared to controls, but then is increased after one month[117,118]. Adult SHR also have decreased extracellular dopamine levels in the caudate nucleus[120,121]. In addition, SHR also exhibit smaller brain volume, such as in the prefrontal cortex and hippocampus,

than controls, which has also been observed in humans with ADHD[122]. Furthermore, DA uptake, storage and metabolism are disturbed in SHR, consistent with one of the primary propositions for ADHD physiology[111,123–125]. In animal studies, DA turnover is used as a reflection of dopamine utilization, with the lower ratio indicating low dopamine utilization, and possibly hypodopaminergic signaling[120,126]. Homovanillic acid (dopamine metabolite) / dopamine ratio has been found to be lower in several brain areas in SHR versus WKY.

Given the strength of the behavioral similarities of the SHR to ADHD-like behavior, it is argued that it passes predictive validity[105]. Many researchers have argued for altered reinforcement processes as a rationale for ADHD symptoms, and these processes were discovered via the use of an animal model[107,110,127–129]. Future studies utilizing the SHR may help localize neurobiology, genetic, or physiological abnormalities that underlie the disorder.

One potential confounder with the SHR is the development of hypertension. Hypertension develops naturally as the SHR reach adulthood, therefore it is advised to study juvenile, pre-hypertensive rats for ADHD research (before 10-12 weeks of age)[112,130]. However, it has been shown that the hyperactive behavior is independent of hypertensive status[131,132].

1.5.3 Animal Intervention Studies

The use of SHR in ADHD studies is becoming increasingly popular. Dervola et al.[133] tested the impact of an omega-3 enriched diet on male and female SHR sustained attention, impulsiveness, and hyperactivity. The researchers used a dual lever operant

chamber and a behavioral test as described previously. The male rats on the omega-3 diet displayed improved attention, decreased hyperactivity and decreased impulsiveness. The female rats, on the other hand, displayed no change or the opposite effect. One thought is that the female SHR is not validated as a proper representation of ADHD and thus may not respond to treatment as expected. Furthermore, the omega-3 diet was much higher in fat than the control diet. Thus, the higher energy the omega-3 diet provided may have affected the rats' behavior, explaining the opposite response of the females. In addition to behavioral outputs, the authors measured dopamine and serotonin and their respective degradation products (HVA and 5-HTAA) in order to measure dopamine and serotonin turnover. Dopamine turnover was improved with the omega-3 diet in the male SHR, which correlated with the improved changes in behavior. A recent study by Hauser et al. compared the effects of an omega-3 enriched diet to an omega-3 deficient diet on SHR hyperactivity using an open field maze. The researchers found that the SHR on the omega-3 enriched diet were less hyperactive than rats on the deficient diet, further supporting the importance of omega-3 fatty acids in healthy motor control and behavior[134].

1.6 Oxidative Stress and Attention-Deficit/Hyperactivity Disorder

Given the subgroup of those with ADHD exhibiting secondary omega-3 PUFA insufficiency, our lab suspected systematic oxidative stress as a contributing factor. There are several ways to measure oxidative stress. For example, total GSH intracellular concentration is an indicator of oxidative stress[135]. GSH is found in two forms, the reduced GSH form and the oxidized glutathione disulfide form (GSSG). Oxidative stress

can impact the GSH/GSSG ratio as well as total GSH. GSH can be measured by a wide variety of high-pressure liquid chromatography (HPLC) methods, which can detect GSH at picomolar concentrations. However, this process can have poor recovery of GSH[135]. Another method is to conduct a recycling kinetic assay, which allows for rapid and accurate measurements of GSH, GSSG, and GSH + GSSG.

It has been established that a decrease in GSH concentration may be associated with the pathogenesis of many diseases, such as: AIDS, alcoholic liver disease, respiratory distress syndrome, and rheumatoid arthritis[136]. A decrease is also known to be associated with aging and has been observed in the substantia nigra of Parkinson Disease patients[137,138]. A depletion of total GSH (GSH + 2GSSG + protein-bound glutathione) and a decreased GSH/GSSG ratio are known indicators of oxidative stress in ischemic brain disease[139], cancer[140], and cardiovascular disease[141]. However, GSH concentrations are increased in the epithelial lining fluid of chronic smokers[142].

In 2003 our lab found significant correlations between an increase in red blood cell (RBC) α-tocopherol concentrations and a decrease in ADHD symptoms. Furthermore, Ross et al. reported increased exhalation of ethane in subjects with ADHD, a marker of omega-3 PUFA per oxidation[143]. Thus, we have suspected systematic increased oxidative stress as a potential factor in ADHD[144]. In 2006 we measured glucose-6-phosphate dehydrogenase activity in red blood cells, a marker of oxidative stress, in young adults with ADHD from Purdue University. Surprisingly, there was not a significant difference in activity in the ADHD group versus healthy controls. Furthermore, F^2-isoprostane concentrations measured in urine were not elevated in those with ADHD[145]. Nonetheless,

given the current evidence, it is still worth examining this potential factor in animal models for ADHD.

1.7 Summary and Research Questions

ADHD is a prominent behavioral disorder affecting 5% of children and adults worldwide[6]. While medical treatments are available, they only work for up to 70% of the population and have unwanted side effects[146]. Thus, alternative treatment options are frequently investigated. Omega-3 fatty acids continue to be a common topic in ADHD research. While many research studies support the efficacy of omega-3 supplementation, continued research is needed to determine the best dosage, delivery method, and identify whether a subgroup of the ADHD population would benefit more from omega-3 supplementation than others. Furthermore, the impact of omega-3 deficiency or supplementation on ADHD-symptoms may bring light as to the etiology of the disorder. Animal models can be used to elucidate the mechanism by which omega-3 fatty acids elicit their impact on behavior, pending that this effect is consistent.

At present there are only a few studies investigating dietary interventions on ADHD-like behavior in the SHR. In Chapter 3 we describe our study in which we aim to address the question of whether an omega-3 dietary intervention will reduce hyperactivity and modulate neurobiology and biochemistry in the SHR. Our first aim was to evaluate the impact of an omega-3 enriched diet on ADHD-like behavior. We hypothesized that an omega-3 enriched diet will reduce hyperactivity in the SHR, as seen in previous studies. We designed a 2x2 study in which we also tested the validated control strain, WKY, in order to control for a potential differential impact of the diet, and to record any

physiological or behavioral differences between strains. In regards to this, our second aim was to evaluate the impact of diet or strain on neurotransmission, oxidative stress, and fatty acid proportions in the brain and plasma. We hypothesized that the omega-3 enriched diet would modulate neurotransmission, reduce oxidative stress, and increase omega-3 polyunsaturated fatty acid proportions in the brain and plasma phospholipids. We also hypothesized that the SHR will be more hyperactive, have lower dopamine and serotonin turnover, higher oxidative stress, and lower omega-3 PUFA proportions. In addition, we conducted a preliminary study on SHR and WKY on standard rat chow to establish protocols for testing ADHD-like behavior (Chapter 2).

CHAPTER 2. PILOT STUDY

2.1 Introduction

A pilot study was conducted to confirm a behavioral difference between SHR and WKY under our testing conditions. SHR and WKY were fed standard rat chow under food restriction so that they remained around 90th percentile for weight. Sucrose pellets were used as a reward for lever pressing in a dual lever operant chamber. An open field maze was utilized to record locomotion as a measure of hyperactivity.

2.2 Methods

2.2.1 Animal Models

The study was approved by Purdue Animal Care and Use Committee (PACUC), and conducted in concordance with the laws regulating experiments on live animals in the United States. 8 male, 3-week old Spontaneously Hypertensive Rats (SHR/NCrl) and 8 Wistar Kyoto Rats (WKY/NCrl) were purchased from Charles River International, Inc. Rats were initially housed 2/cage for 1 week with access to rat chow and water ad libitum, after which they were housed in individual cages. Rats were handled daily for the first two weeks and weighed continuously throughout the study. At 4 weeks of age food was restricted so that rats would be at their 90th percentile for weight. Rats were exposed to a few sucrose pellets a few days before testing to reduce their novelty. Growth rate and

body weight were monitored to ensure proper growth, and rats maintained 80% of their average expected weight. The animals were caged under standard conditions (humidity about 55%, temperature about 22ºC, reverse 12h light/dark cycle).

2.2.2 Diet

Rats were fed standard rat chow (Nestle Purina Pet Care Company, St. Louis, MO). Dustless Precision 45mg Sucrose Pellets (Bio-Serv, Flemington, NJ) were used as a reward during testing. Sucrose pellets were composed of: sucrose, dextrose, cellulose, tablet binder, magnesium stearate, natural and artificial flavors, calcium silicate, and food dye (protein 0%, fat 0%, carb 97.5%).

2.2.3 Behavioral Testing

Table 1: Behavioral Training and Testing Schedule

Behavioral Procedure	Number of Sessions	Reinforcement Schedule
Magazine Training	1	
Flap Training	2-3	FT 10
Shaping of lever-pressing	4-6	CRF
30 minute training	7-9	FT 10
60 minute training	10-14	VI 15
60 minute Testing	15-34	VI 60

Reinforcement was given either as a continuous reinforcement schedule (CRF), fixed time of reinforcement (FT), or variable interval schedule of reinforcement (VI)

At 5 weeks of age the 16 animal started the training schedule detailed above (Table 1). Testing ran daily between 10:00 and 14:00. Rats were randomized to one of the four chambers, and were tested at the same time each day. The 34 sessions included

training, lever shaping, and testing with reinforcers. Sessions used in the behavioral analysis lasted 60 minutes or until 45 pellets were rewarded.

After animals were magazine trained, lever-pressing training began on one lever. The animals ran on continuous reinforcement and then fixed interval 10s sessions to strengthen the behavior. For their final schedule, rats were put on a variable interval 60s (VI60) schedule of reinforcement. Pressing the lever, signaled by a lit cue light above the lever, produced a reward on a VI60 schedule of reinforcement. While this occurs, the cue light above the lever turns off and the cue light in the reinforce-delivery hopper turns on. Following the reinforcer delivery, a VI60 timer is initiated to determine the time until the cue light turns on again.

2.2.4 Apparatus

The rats were tested in 4 operant chambers (Model 80004NS, Lafayette Instrument, Lafayette, IN). The chambers were equip with stainless steel floors, fixed lever press bars requiring a weight of 28mg to activate, and a white, opaque cue light above each lever. The animals had a working space of 30.5cm L x 26cm D x 20.0cm H. 45mg sucrose reward pellets were dispensed in a small, recessed cubicle with a cue light that lit when rewards were dispensed. Abet II 2.16 (Lafayette Instruments Abet II, Lafayette, IN, USA) was used to record behavior and schedule reinforcements and lights.

2.2.5 Measurement of Behavior

The number of presses on the reinforcer-producing lever, time of events, and number of reinforcers produced and collected were recorded. Attention was calculated as

a measure of accurate responses on the reinforcer-producing lever as the animal had to pay attention to the cue light above the lever before pressing. Hyperactivity was measured as total number of lever-presses in the operant chamber, and distance travelled and rearings in the open field test[133].

2.2.6 Open Field

The open field test was used to measure locomotor activity. The open field apparatus was a wooden 107cm x 107cm open-field box, with a Plexiglas floor. A 25-box grid was created with masking tape placed underneath the Plexiglas. Each square was 20cm. Testing was conducted at 10 weeks of age. Rats started at the same corner of the box each time and allowed to roam uninterrupted for 15 minutes. Two researchers tallied total box crossings, rearings, and center box entries. Lights were off during testing with the exception of a red lamp. The maze was cleaned with 70% ethanol and allowed to dry between sessions. Rats were tested between 11:00-12:00 after their last day in the operant chamber.

2.2.7 Statistics

SPSS 22 (IBM SPSS Statistics, Armonk, NY) was used to conduct one-way ANOVA with repeated measures on the operant chamber behavioral outcomes, after data passed for normality and equal-variance. Kruskal Wallis test was used to determine normality for all data. Values were considered outliers if they were more than two standard deviations away from the mean. Student's t-test was used to evaluate the open field test outcome measures. Significance was defined as $p < 0.05$. Values in figures

reported as mean + standard error of mean (SEM), values in text reported as mean ± SEM.

2.3 Results

2.3.1 Operant Chambers

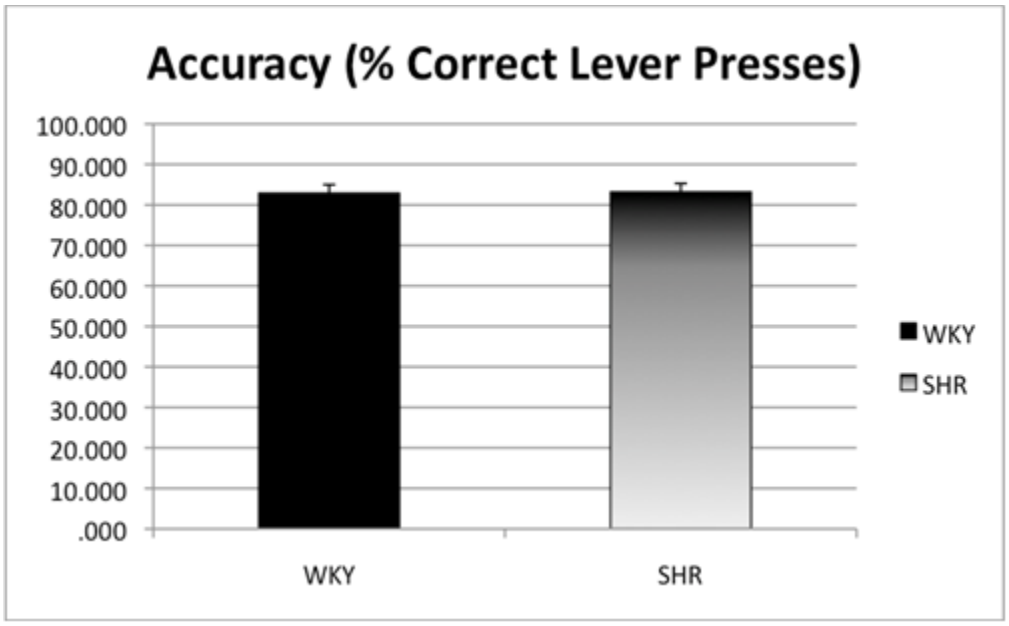

Figure 5: Mean percent accuracy (correct presses/total presses) for WKY and SHR (mean + SEM), n = 8

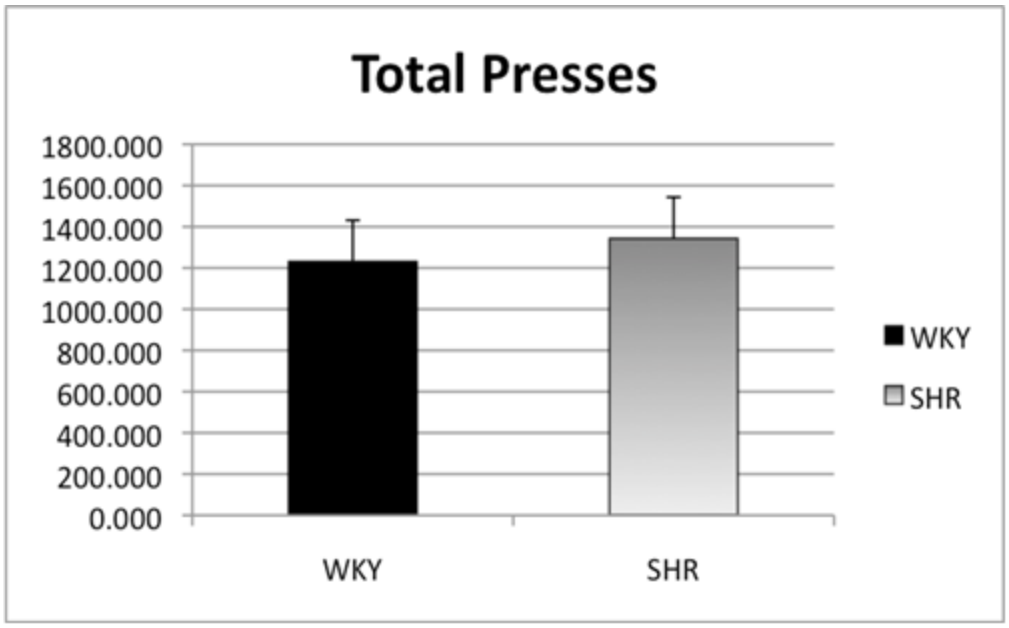

Figure 6: Mean total lever presses for WKY and SHR (mean + SEM), n = 8

No statistically significant differences were observed between strains. Mean percent accuracy was 82.9±2.1 for WKY and 83.2±2.1 for SHR. Mean total lever presses were 1231.24±200.13 and 1343.26±200.13 for WKY and SHR, respectively. There was, however, a significant impact of day as both percent accuracy ($p = 0.002$) and total lever presses ($p = 0.028$) increased throughout the study.

2.3.2 Open Field Maze

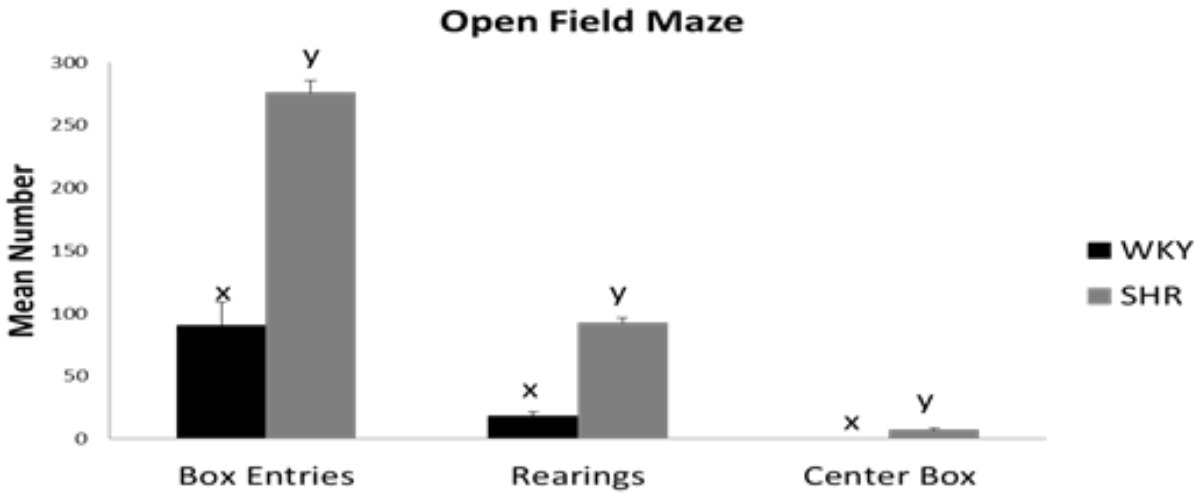

Figure 7: Mean box entries, rearings, and center box entries for the open field maze test for WKY and SHR. ^{x,y}Signifies significant difference between strains ($p < 0.05$). Mean + SEM, n = 8

SHR had significantly more box crossings (276.38±9.34 vs 90.38±18.52), rearings (92.25±4.51 vs 18.25±3.02) and center box entries (7.38±0.86 vs 0) than WKY ($p < 0.001$).

2.4 Discussion and Conclusion

Rats were only at 80th percentile of their expected growth, which is lower than the target 85-90th percentile desired. While an increase in diet was enlisted to promote further weight gain, the intervention was not strong enough. Consequently, the animals could have been more motivated than usual because of the increased need for food. While a behavioral strain difference has been observed in multiple studies[112], our single-lever operant chamber tests did not reveal a difference in behavior between the two strains. However, the open field maze did show a significant strain effect. Thus, we chose to

focus on hyperactivity and proceed with the open field maze test for our subsequent study. Our data suggests that a dual-lever discrimination test may be needed to reveal the behavioral differences observed in other studies.

CHAPTER 3. INTERVENTION STUDY

3.1 Introduction

Attention-deficit/hyperactivity disorder is the most commonly diagnosed behavioral disorder in children worldwide. Diagnosis rates have been increasing steadily in the United States, with up to 11% of children diagnosed with the disorder as of 2011[6]. ADHD is diagnosed based on symptoms detailed in the DSM-V, which include: inattention, hyperactivity, and impulsivity[1]. Dopamine abnormalities have been heavily implicated in the physiology of ADHD[30]. Multiple studies have confirmed a low dopamine response, lower dopamine receptor availability, and smaller brain region volumes in areas known to be rich in dopamine receptors[10,29,147,148]. Stimulant medications that prevent dopamine reuptake or increase dopamine release are effective for treating symptoms, but these medications only work for around 70% of the population[146].

The complete etiology of ADHD remains unknown, but clear environmental and genetic factors have been identified[31,149]. Nutritional insufficiencies, particularly essential fatty acids, have been a topic of interest in ADHD research. Essential polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are crucial for healthy brain development. EPA and DHA, or their precursor alpha-linolenic acid (ALA), must be obtained from the diet as the body cannot synthesize them otherwise.

Most brain development occurs prenatally and during the first few years of life, therefore having adequate omega-3 PUFA is particularly important during this time[150,151]. The typical Western Diet consists of a very high omega-6 fatty acid: omega-3 fatty acid ratio (15:1) as opposed to the recommended ratio of 4:1[52,53,152]. Our lab has investigated essential fatty acid (EFA) status in children with ADHD, as many displayed EFA deficiency symptoms (i.e. increased thirst, dandruff, frequent urination). We found that a subgroup of children with ADHD, about 40%, had insufficient plasma EFA levels despite reporting a similar diet to the other children tested[44,153]. This finding has been subsequently reported by other labs as well, both within and outside the United States[45,46]. Furthermore, feeding infants baby formula, which lacked added essential omega-3 PUFA until 2002, is significantly correlated with increased ADHD risk[47]. Consequently, omega-3 PUFAs have been implicated as a possible environmental factor in ADHD risk and development.

The importance of adequate omega-3 PUFAs for healthy brain physiology has been demonstrated in several neurobiology and behavior studies on omega-3 PUFA deficient animals. Omega-3 PUFA deficiency, especially during times of heightened brain development, can have detrimental effects on learning, brain structure and function[151]. EPA and DHA are crucial for membrane fluidity, proper neurotransmission, and brain development[154]. Animal studies evaluating the impact of animals on an omega-3 deficient diet for multiple generations report decreased performance on learning and memory tasks, lower dopamine and serotonin concentration, and lower dopamine storage[69,81,83,94,155,156]. Interestingly, both learning and neurotransmitter deficits have been successfully reversed with omega-3 PUFA supplementation[92,157]. Omega-3 supplementation has been evaluated

for its impact on ADHD and several human and animal studies have reported a reduction in ADHD-symptoms. The results of these intervention studies, however, have not been unanimous[158].

In order to eliminate the risk of "placebo effect" and evaluate more invasive physiological factors, we utilized the Spontaneously Hypertensive Rat (SHR). The SHR is a well-validated animal model for ADHD, and displays all three major behavioral characteristics of the disorder[105,112,159]. In our study, we examine the impact of an omega-3 enriched diet (2:1 omega-6:omega-3 ratio) versus a typical Western Diet (13.3:1) on hyperactivity, a common ADHD behavior. Juvenile SHR and their validated control strain, Wistar Kyota Rat (WKY), were fed either an omega-3 fatty acid dominant diet (omega-3 diet) or an omega-6 fatty acid dominant diet (omega-6 diet) followed by locomotion and rearing measurement in an open field maze. Dopamine abnormalities are heavily implicated in ADHD behavior, so we measured DA and its downstream neurotransmitter, serotonin (5-HT), turnover in the neostriatum. Additionally, both plasma and brain phospholipid compositions were assessed. Lastly, as oxidative stress could be a causative factor in secondary omega-3 PUFA deficiency, we analyzed liver glutathione (GSH) concentrations as a measure of oxidative stress. We hypothesize that the omega-3 diet will increase omega-3 PUFA proportions in plasma and brain phospholipids, increase DA and 5-HT turnover, and reduce oxidative stress, which will be correlated with a decrease in hyperactivity. We also hypothesize that the SHR will be more hyperactive, have lower neurotransmitter turnover, and a higher state of oxidative stress than the WKY.

3.2 Materials and Methods

3.2.1 Animal Models

The study was approved by Purdue Animal Care and Use Committee (PACUC), and conducted in concordance with the laws regulating experiments on live animals in the United States. 16 male, 3-week old Spontaneously Hypertensive Rats (SHR/NCrl) and 17 Wistar Kyoto Rats (WKY/NCrl) were purchased from Charles River International, Inc. Rats were initially housed four/cage for four days with access to standard rat chow and water ad libitum, after which they were randomized into two diet groups: omega-3 enriched diet or omega-6 dominant diet. The extra WKY was placed in the omega-3 diet group. After one week rats were switched to individual cages. Rats were handled daily for the first two weeks and weighed continuously (every 3 days) throughout the study. Both groups had ad libitum access to their specified food and water. Food intake was calculated daily to ensure similar intake between groups. Growth rate and body weight increased as expected, and there was no difference in weight gain between dietary groups. The animals were caged under standard conditions (humidity about 55%, temperature about 22ºC, reverse 12h light/dark cycle).

3.2.2 Diet

Table 2: Dietary Composition of the Omega-3 and Omega-6 Diets

Modifications	Omega-3 Diet	Omega-6 Diet
Soybean Oil	35g/kg	35g/kg
Menhaden Oil	36g/kg	-
Corn Oil	-	36g/kg
α-tocopherol	133.5 IU/kg	133.5 IU/kg

Table 3: Fatty Acid Composition of Both Diets (% Proportion)

Fatty Acids	Omega-3 Diet	Omega-6 Diet
16:0	16.96	12.60
18:0	6.27	0.13
16:1n7	4.19	3.12
18:1n9c	16.15	26.34
18:1n9t	2.58	0.94
18:2n6c (LA)	33.23	56.08
18:3n6	0.83	0.59
18:3n3	4.87	0.19
20:3n6 (DGLA)	0.03	-
20:4n6 (AA)	0.62	-
20:5n3(EPA)	7.36	-
22:6n3 (DHA)	6.90	-

Diets were modified from Bio-Serv AIN-93G standard rodent diet (http://www.bio-serv.com/Rodent_Standard_Diets/RDAIN93G.html). Solely the fat content was modified to ensure equivalence between diets with exception to fatty acid composition. The composition of both diets was as follows: 59.3% carbohydrate, 18.1%

protein, 7.1% fat, 4.8% fiber. The omega-3 enriched diet contained menhaden oil, while the omega-6 diet contained an equivalent amount of corn oil. Both diets had an equivalent amount of soybean oil. Consequently, the omega-3 enriched diet contained omega-3 long chain polyunsaturated fatty acid (LC-PUFA) EPA and DHA whereas the omega-6 diet had high amounts of omega-6 LC-PUFA precursor linoleic acid (LA) but no omega-3 LC-PUFAs. Furthermore, the omega-3 enriched diet had an omega-6:omega-3 ratio of 2:1, whereas the omega-6 diet had a ratio of 13.3:1. α-tocopherol was added to both diets to protect against lipid peroxidation. See Table 2 and 3 for detailed composition information.

3.2.3 Behavioral Experiment

The open field test was used to measure locomotor activity. The open field apparatus was a wooden 107cm x 107cm open-field box, with a Plexiglas floor. A 25-box grid was created with masking tape placed underneath the Plexiglas. Each square was 20.3cm. Testing was conducted at 8 weeks of age. Rats started at the same corner of the box each time and allowed to roam uninterrupted for 15 minutes. Two researchers tallied total box crossings and rearings. Lights were off during testing with the exception of a red lamp. All testing sessions were recorded by a camcorder and reevaluated by researchers. The maze was cleaned with 70% ethanol and allowed to dry between sessions. Rats were tested between 10:00-13:00 six days a week. Each rat was tested at the same time every other day, for a total of three trials.

3.2.4 Euthanasia

Rats were euthanized at 10 weeks of age via CO_2 exposure and cardiac stick. Blood was collected via cardiac stick and stored in EDTA tubes (EDTA used as an antiocoagulant). Tubes of blood were mixed on a rocking mixer and then stored on ice until final storage in freezer. Kidney, liver, brain, and spleen were collected, wrapped in foil, and immediately frozen in liquid nitrogen. The neostriatum was separated from the rest of the brain and also frozen.

3.2.5 Analysis

Blood was centrifuged for 20 minutes at 3000g. Plasma was collected and aliquoted for storage in -80ºC. Liver, brain, spleen, and kidney were also washed and stored in -80ºC until analysis.

3.2.5.1 Phospholipid and Oxidative Stress Analysis

Chemicals and Materials: Methanol and chloroform were purchased from Macron Fine Chemicals (Central Valley, PA). Solid phase extraction (SPE) silica cartridges (500mg, 6ml) and holder were purchased from Burdick & Jackson (Muskegon, MI). Bovine serum albumin (BSA) protein assay kit and dye reagent concentrate were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). 96 Well Tissue Culture Plates (flat bottom) and 15ml polypropylene conical tubes were purchased from Falcon (Corning Science Mexico S.A. de C.V.). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

3.2.5.1.1 Phospholipid Composition Method

Plasma: 0.5ml of plasma was thawed and added to a medium test tube. 3.5ml of methanol was added to the plasma followed by 7ml of chloroform. The solution was filtered through Whatman #5 paper then washed with 6ml chloroform:methanol. 3ml 0.88% KCL was added and the solution was vortexed for 2 minutes. Samples were then refrigerated overnight. The bottom chloroform layer was removed and dried under nitrogen in a 37ºC water bath. Afterwards samples underwent column separation via solid phase extraction silica cartridges. Cartridges were washed with 10ml of chloroform then dried plasma lipids were dissolved in 0.5ml of chloroform and poured through the column. Once the lipid solution was absorbed, another 10ml of chloroform was added to the cartridge, followed by 10ml of methanol. The fraction was collected and then an additional 10ml of methanol was added. The columns were then washed with 20ml methanol and 20ml of chloroform. Methanol fractions were combined and dried under nitrogen. Then 1ml of methanol with butylated hydroxytoluene (BHT) and 1ml 14% boron trifluoride (BF_3) were added and the solution was vortexed. Afterwards the test tube was capped tightly and heated in a 100ºC heating block for 30 minutes. Once cooled, 1ml hexane with butylated hydroxytoluene and 3ml of double-dionized water (DDI) were added. After vortexing, the top layer was used for gas chromatography (GC) analysis. Protocol adapted from Juaneda and Rocquelin and Ohta et al.[160,161].

Whole brain: Around 0.120g whole brain was cut, weighed and homogenized in homogenizing buffer (50mM tris-HCl, pH 7.4, 2mM EDTA) to make a 10% weight/volume (w/v) solution. Phospholipid extraction was conducted as described above with the exception of the column separation, which was not needed.

3.2.5.1.2 Oxidative Stress Method

Liver total glutathione (GSH) was analyzed as a marker of oxidative stress, using a protocol adapted from Rahman et al.[135]. A section of liver tissue was cut and weighed, ranging from 0.100g-0.250g in mass. The tissue was then homogenized using a mortar attached to a Sears Craften drill in enough ice-cold 5% metaphosphoric acid to make a 20% w/v solution. The samples were then centrifuged at 3000g for 20 minutes at 4ºC in 15ml conical vials. Reagent buffers were made fresh according to instructions with the exception of DTNB and β-NADPH solution. DTNB solution was made using 4mg of Elmands reagent, and β-NADPH was made using 6mg β-NADPH. GSH standards ranged from 3.13µg/ml – 50µg/ml. For the spectrometer analysis, 25µL of sample or standard was loaded into each well of the 96-well plate, followed by 115µL of the GR/DTNB mixture. After 90 seconds, 60µL of β-NADPH was added. The plate was immediately read via spectrometer (PowerWave Bio-Tek Instruments, Inc. Winooski, VT) and GSH concentration was calculated using a standard curve. GSH concentration was then normalized to protein for statistical analysis.

3.2.5.1.3 Protein Assay

A 1:10 dilution of Bio-Rad protein assay dye reagent concentrate and bovine serum albumin were used for the protein assay. BSA standard concentrations ranged from 0.0625mg to 0.50mg. 5µl of DDI water, standard, or sample was pipetted into a 96-well plate. 200µl of dye was added to each well. Plate was immediately analyzed at 595nm via spectrometer.

3.2.5.2 Neurotransmitter Analysis

3.2.5.2.1 Chemicals

A high performance liquid chromatogrpahy (HPLC) machine was utilized to conduct monoamine analysis. Dopamine, homovanillic acid, and serotonin standards were obtained from Arcos Organics, while 5-hydroxyindole-3-acetic acid (5-HIAA) and isoproterenol were obtained from Sigma.

3.2.5.2.2 Sample Preparation

Neostriatum samples were weighed and homogenized in 500uL of ice-cold 0.2M $HCLO_4$. The homogenizer was the same mortar and pestle mounted to a Sears Craften drill used for the previous tissue sample analyses. The suspension was mixed with ISO (a monoamine internal standard) in 0.12mM ascorbic acid, bringing the final concentration to 500µM ISO. The homogenates were centrifuged at 12,0000g at 2ºC for 30 minutes. Supernatents were stored at -20ºC until analysis. Samples were filtered through 0.22µm nylon syringe filters into glass vials for HPLC analysis.

3.2.5.2.3 Monoamine Analysis

Reverse-phased HPLC (ESA), equipped with a phenomenex Kinetex 2.6µM C18 column (inner diameter 4.6mm, length 100mm) was used for monoamine analysis. ESA CoulArray electrochemical detectors had working potentials 200, 225, 250, 275, 325, 350, and 375mV. The mobile phase consisted of 20mM sodium phosphate buffer, pH 2.5, and 1% methanol in a 1:1 solution of the 20mM sodium phosphate buffer, pH 2.5, and

100% methanol. Mobile phase was varied on a gradient so that the methanol percentage ranged from 1%-25%. Individual samples were eluted at 35 minutes with a flow rate of 0.7mL/min. Known concentrations of external standards were analyzed on the same day. Chromatograms were analyzed using ESA CoulArray and monoamine concentrations were expressed in pmol/mg protein.

3.2.6 Statistical Analysis

Behavioral outcomes were analyzed using two-way ANOVA with repeated measures on SPSS 22. GSH, neurotransmitter, and phospholipid comparisons were analyzed using two-way ANOVA, passing tests for normality and equal variance on SigmaStat (Jandel Scientific Software, San Jose, CA) or SPSS 22 (IBM SPSS Statistics, Armonk, NY). Kruskal Wallis test was used to determine normality for all data. Outliers were considered any value greater or less than two standard deviations. Variables for all ANOVA tests were strain and diet. Data that did not pass normality were transformed using either $1/x^2$ or $1/x$ transformation. Student t-test was used to analyze plasma EPA data as only the omega-3 diet group had non-zero values. For all tests $p < 0.05$ established significance. One-way ANOVA was run using SPSS 22 if there was a strain x diet interaction.

3.3 Results

3.3.1 Behavioral Analysis

The results of the open field maze test for hyperactivity are graphed below.

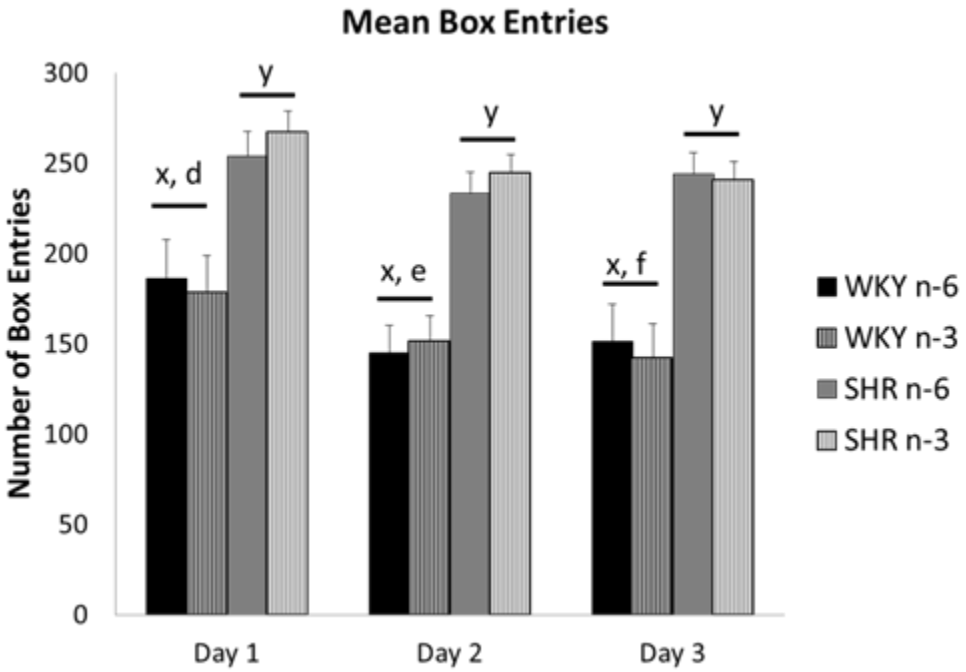

Figure 8: Mean total box entries spanning the three trial days of WKY and SHR for both diets, n = 7-9. ^{x,y}Signify significant difference between strains, ^{d,e,f}signify significant impact of day. Values are mean + SEM.

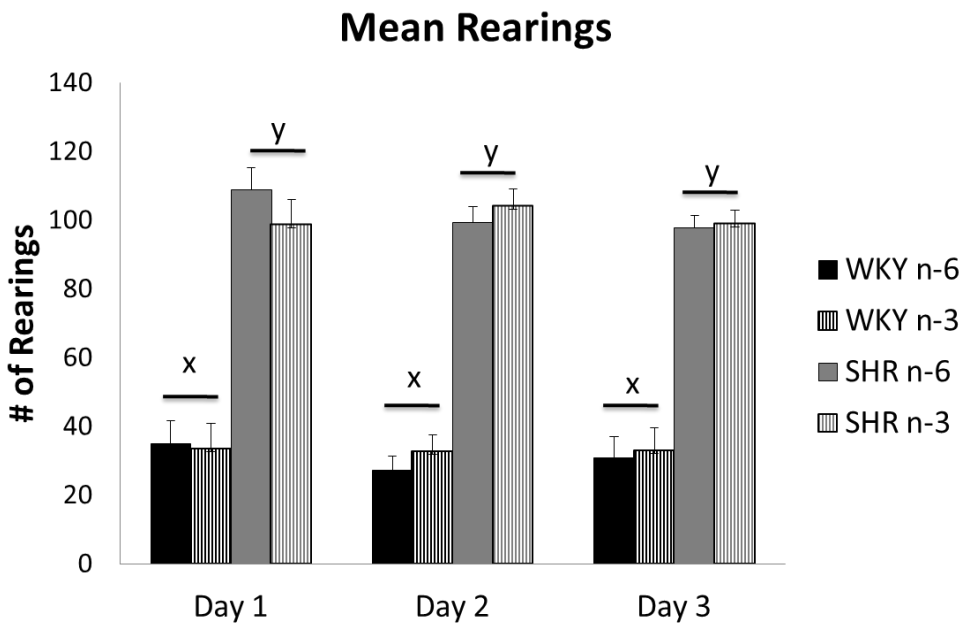

Figure 9: Mean total rearings spanning the three trial days of WKY and SHR for both diets, n = 7-9. [x,y]Signify significant differences between strains, [a,b]signify significant impact of diet. Values are mean + SEM.

Total box crossings and rearings were analyzed with two-way ANOVA with repeated measures (Figures 8 and 9). No effect of diet was found for total box crossings or rearings. However, there was a significant difference among strains for both measures ($p = 0.003$ box crosses, $p = 0.000$ rearings). SHR crossed a mean of 244.98 ± 11.96 boxes and averaged 101.52 ± 3.65 rearings over the three testing days. WKY scored significantly less than the SHR, with an average of 166.72 ± 11.62 box crossings and 32.11 ± 3.31 rearings. For the WKY, the average box crossings were impacted by day ($p = 0.024$), while average box crossings for SHR did not change significantly. WKY stuck to the perimeter of the maze or rested in one corner, while the SHR moved quickly and frequently across the entire maze.

3.3.2 Plasma and Brain Phospholipid Composition

Data for plasma and brain fatty acid proportions are presented in tables 4 and 5, while the three main omega-6 and omega-3 LC-PUFAs are graphed (Figures 10 and 11).

Table 4: Fatty acid composition of the plasma of WKY and SHR on both diets (% proportion)

	Plasma Phospholipids			
Fatty Acids	WKY		SHR	
	Omega-6	Omega-3	Omega-6	Omega-3
16:0	23.41 ± 0.60^{a}	26.88 ± 0.60^{b}	25.23 ± 0.63^{a}	26.76 ± 0.60^{b}
18:0	27.46 ± 1.60	24.45 ± 1.60	29.40 ± 1.67	27.98 ± 1.60
16:1n7	$0.59\pm0.10^{a,x}$	$0.95\pm0.10^{b,x}$	$0.27\pm0.10^{a,y}$	$0.83\pm0.10^{b,y}$
18:1n9c	6.93 ± 0.40^{a}	8.10 ± 0.40^{b}	6.83 ± 0.50^{a}	7.91 ± 0.40^{b}
18:1n9t	2.90 ± 0.20^{x}	3.01 ± 0.20^{x}	2.21 ± 0.20^{y}	2.68 ± 0.20^{y}
18:2n6c (LA)	10.54 ± 0.80^{a}	13.05 ± 0.80^{b}	10.72 ± 0.90^{a}	13.25 ± 0.80^{b}
20:3n6 (DGLA)	0.19 ± 0.10^{a}	0.83 ± 0.10^{b}	0.07 ± 0.10^{a}	0.77 ± 0.10^{b}
20:4n6 (AA)	$25.39\pm0.80^{a,x}$	$13.05\pm0.80^{b,x}$	$21.96\pm0.80^{a,y}$	$11.61\pm0.80^{b,y}$
20:5n3(EPA)	$0.04+0.28^{a,x}$	$3.55+0.28^{b,x}$	$0+0.28^{a,y}$	$1.58+0.28^{b,y}$
22:6n3 (DHA)	$2.50+0.27^{a,x}$	$5.56+0.27^{b,x}$	$3.31+0.29^{a,y}$	$6.36+0.27^{b,y}$

^{a,b}Different letters signify significant difference between diets
^{x,y}Different letters signify significant difference between strains
All values are means$\pm$SEM, n = 6-8

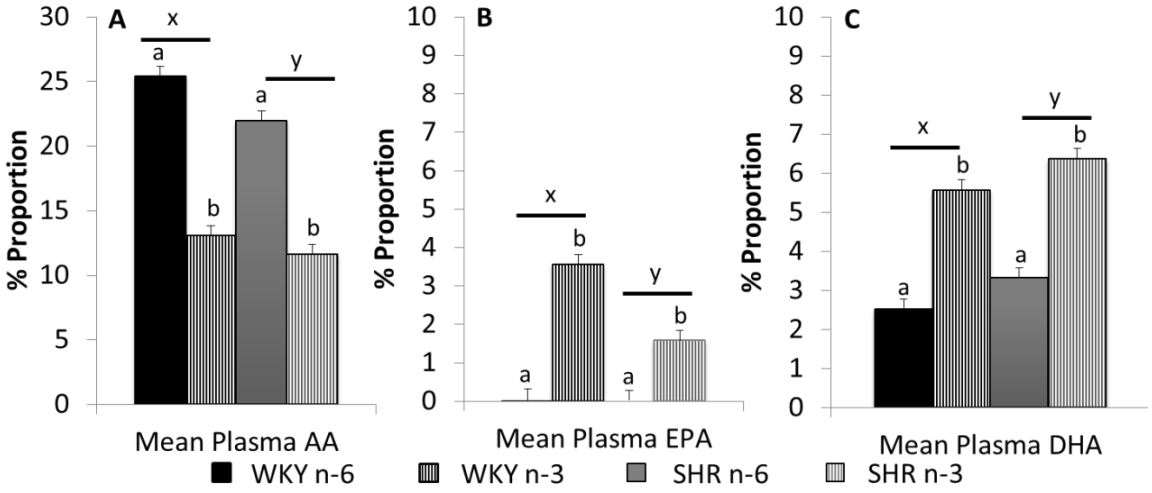

Figure 10: A. Mean plasma phospholipid proportion of arachidonic acid (AA), B. eicosapentaenoic acid (EPA), C. and docosahexaenoic acid (DHA) in the WKY and SHR for both diets, n = 6-8.[x,y]Signify significant differences between strains, [a,b]signify significant impact of diet. Mean + SEM.

Table 5: Fatty acid composition of brain in WKY and SHR on both diets (% proportion)

	Phospholipids			
Fatty Acids	WKY		SHR	
	Omega-6	Omega-3	Omega-6	Omega-3
16:0	$23.51\pm0.30^{a,x}$	$22.30\pm0.28^{b,x}$	$24.20\pm0.30^{a,y}$	$23.67\pm0.30^{b,y}$
18:0	22.79 ± 0.36	23.53 ± 0.34	22.99 ± 0.36	23.36 ± 0.36
16:1n7	0.51 ± 0.02^{a}	0.61 ± 0.02^{b}	0.51 ± 0.02^{a}	0.67 ± 0.02^{b}
18:1n9c	$19.24\pm0.39^{a,x}$	$20.71\pm0.37^{b,x}$	$18.64\pm0.39^{a,y}$	$19.15\pm0.37^{b,y}$
18:1n9t	4.48 ± 0.09^{x}	4.41 ± 0.08^{x}	4.07 ± 0.09^{y}	3.92 ± 0.09^{y}
18:2n6c (LA)	1.01	0.98	1.04	0.97
20:4n6 (AA)	12.54 ± 0.20^{a}	10.23 ± 0.20^{b}	12.06 ± 0.20^{a}	10.57 ± 0.20^{b}
20:5n3(EPA)	0.36 ± 0.02^{x}	0.37 ± 0.02^{x}	0.31 ± 0.02^{y}	0.28 ± 0.02^{y}
22:6n3 (DHA)	15.58 ± 0.36^{a}	16.86 ± 0.36^{b}	16.13 ± 0.36^{a}	17.33 ± 0.36^{b}
AA/DHA	0.80 ± 0.02^{a}	0.61 ± 0.02^{b}	0.75 ± 0.02^{a}	0.61 ± 0.02^{b}

[a,b]Different letters signify significant difference between diets
[x,y]Different letters signify significant difference between strains
All values are mean±SEM, n = 8-9

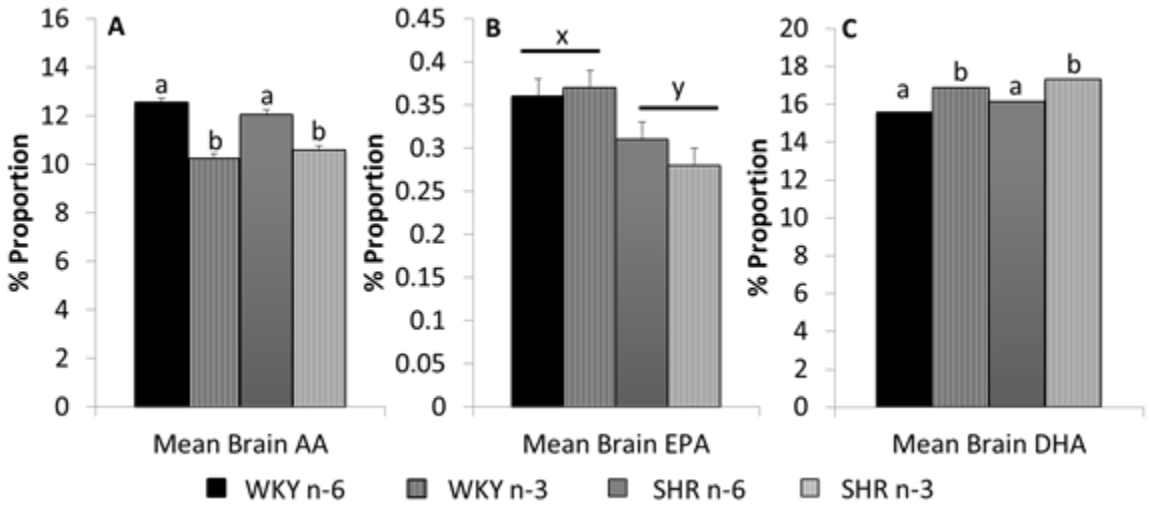

Figure 11: A. Mean brain phospholipid proportion of arachidonic acid (AA), B. eicosapentaenoic acid (EPA), C. and docosahexaenoic acid (DHA) in the WKY and SHR for both diets, n = 8-9.[x,y]Signify significant differences between strains, [a,b]signify significant impact of diet. Mean + SEM.

Table 4 and 5 summarize the fatty acid composition of plasma and whole brain phospholipids for SHR and WKY on both diets. The omega-3 enriched diet was successful in significantly increasing DHA in both the brain and plasma, but EPA was only significantly greater in plasma. Omega-6 PUFA precursor linoleic acid (LA) was significantly higher with the omega-3 enriched diet in plasma, but arachidonic acid was significantly lower in both plasma and brain. Interestingly, there was a significant strain difference for brain EPA and both brain and plasma EPA and DHA, with EPA higher in the WKY strain and DHA higher in the SHR. AA/DHA ratio in the brain was significantly lower in rats on the omega-3 diet.

3.3.3 Total Glutathione

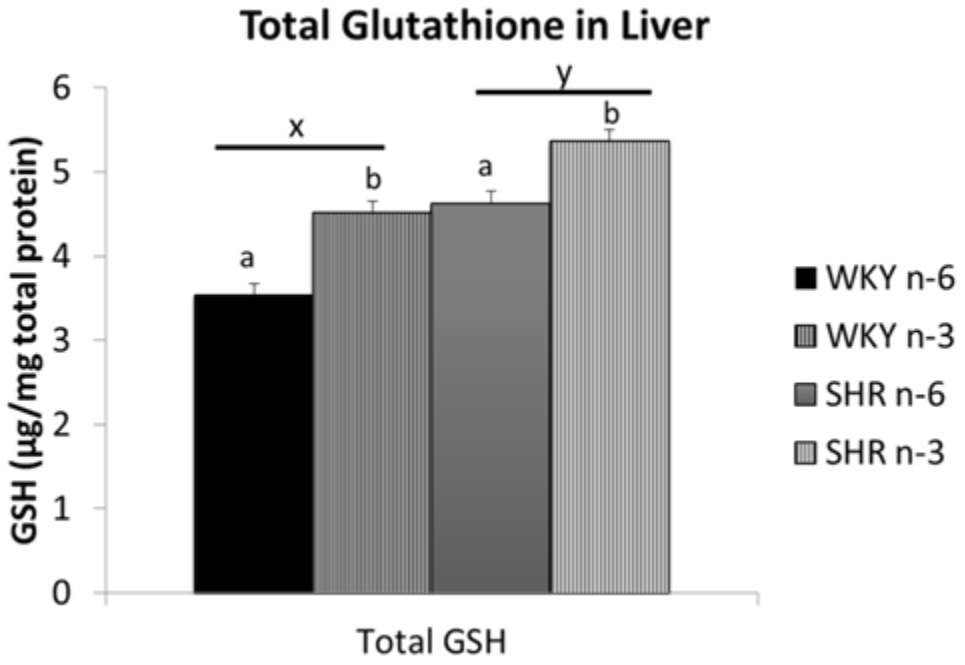

Figure 12: GSH concentration (µg/mg protein) in the liver in SHR and WKY on both diets. [x,y]Signify significant differences between strains, [a,b]signify significant impact of diet. Mean + SEM, n = 8-9.

Liver total GSH concentration (µg/mg protein) was significantly impacted by diet, and differed according to strain. SHR and WKY on the omega-3 enriched diet both displayed higher GSH concentrations, increasing from 4.63 to 5.37 and 3.54 to 4.52, respectively. SHR also had significantly higher liver GSH concentration than WKY, independent of diet. There was not a significant strain x diet interaction (Figure 12).

3.3.4 Neurotransmitters

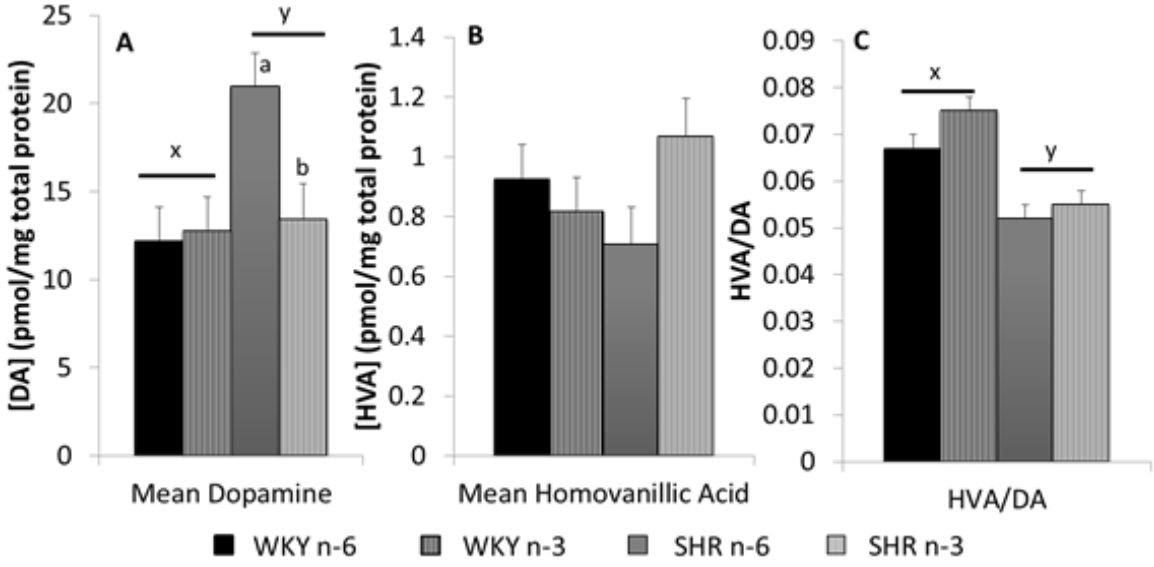

Figure 13: A. Dopamine (DA) concentration, B. homovanillic acid (HVA) concentration, and C. dopamine turnover (HVA/DA) in the neostriatum of SHR and WKY on both diets, n = 6-8. [x,y]Signify significant differences between strains, [a,b]signify significant impact of diet. Mean + SEM.

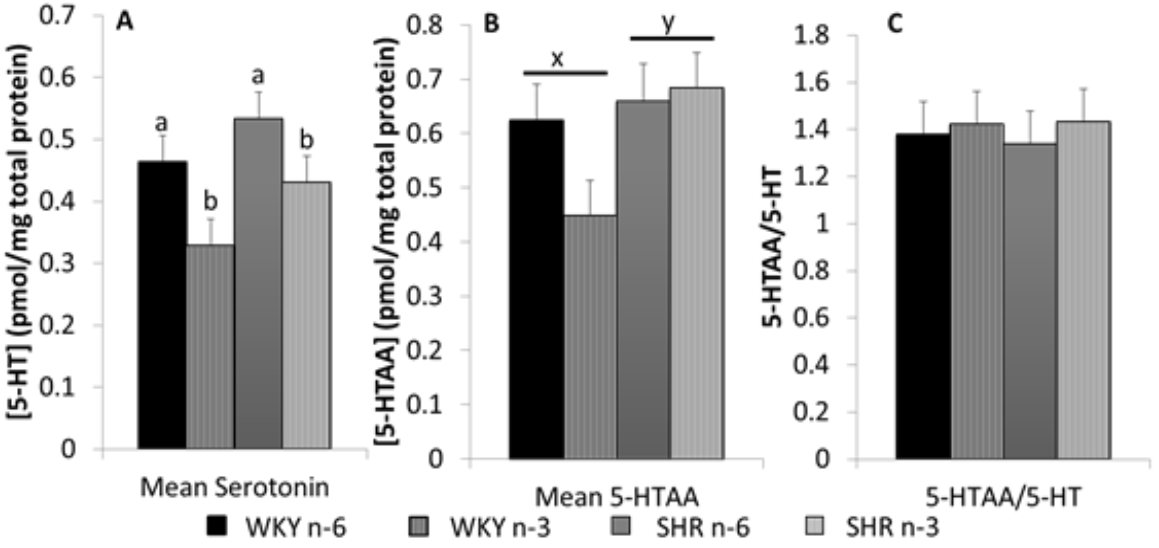

Figure 14: A. Serotonin (5-HT) concentration, B. 5-hydroxyindole-3-acetic acid (5-HIAA) concentration, and C. serotonin turnover (5-HTAA/5-HT) in SHR and WKY neostriatum on both diets, n = 6-8. [x,y]Signify significant differences between strains, [a,b]signify significant impact of diet. Mean + SEM.

DA, HVA, 5-HT, and 5-HTAA were measured using HPLC and normalized to internal standard (pmol/mg). DA concentration differed significantly between strains, with WKY having lower neostriatal DA concentration on both diets (17.18±1.41 SHR vs 12.48±1.36 WKY, p = 0.023). DA concentration was lower in SHR with the n-3 enriched diet, and this difference was found to be significant using one-way ANOVA. There was also a significant diet x strain interaction (p=0.047) (figure 13). There was not a significant impact of strain or diet on HVA concentration. Interestingly, SHR had significantly lower dopamine turnover than WKY on both diets (0.053+0.002 SHR vs. 0.071+0.002 WKY, p = 0.000). Serotonin concentration was significantly lower with the n-3 enriched diet for both strains (0.463±0.043 vs. 0.328±0.041 omega-6 vs omega-3 WKY and 0.533±0.043 vs. 0.430±0.046 omega-6 vs omega-3 SHR, p = 0.011) but there was no strain difference or strain x diet interaction. The diet did not have an impact on the serotonin degradation product, 5-HTAA, but there was a significant strain difference with WKY having a lower concentration of 5-HIAA than SHR (0.536±0.045 WKY vs. 0.671±0.048 SHR, p = 0.049) (Figure 14). Despite these differences there was no between strain, diet, or strain x diet interaction on serotonin turnover.

3.4 Discussion

The current 2 x 2 design study provides insight into the impact of an omega-3 enriched diet on the behavior and biochemistry of the SHR. To the authors' knowledge, it is also one of few studies that compare an omega-3 enriched diet to a diet typical of a western diet – a more realistic scenario than comparing to an omega-3 deficient diet. The SHR and WKY received a diet with either a 2:1 or 13.3:1 ratio of omega-6:omega-3 fatty

acids. Our goal was to test if altering the omega-6:omega-3 ratio would be enough to impact behavior and neurobiology. Thus, the diets only differed in their fat source and fatty acid ratio. We found that the omega-3 diet was successful in changing phospholipid proportions and altering a marker of oxidative stress in both strains but not in modulating behavior or neurotransmission. We also further confirmed both biochemical and behavioral strain differences between the SHR and WKY, including inherent differences in plasma and brain phospholipid composition, neurotransmission and oxidative stress markers.

Rats in both dietary groups remained healthy and grew as expected throughout the study, with no differences between groups. As predicted, the SHR was significantly more hyperactive than the WKY. However, our dietary intervention had no impact on behavior in either strain. That being said, the lack of impact on behavior is consistent with our neurotransmitter results.

The omega-3 diet was successful in improving omega-3 PUFA proportion in both plasma and brain phospholipids. Of particular interest, strain differences were observed in plasma AA, EPA, and DHA. Unexpectedly, SHR had higher plasma DHA than WKY while WKY had higher plasma and brain EPA. The dietary intervention was effective in changing overall plasma and brain phospholipid composition, with the exception of EPA in the brain. This differs from our observations in previous human studies, where a subgroup of children and adults with ADHD had low proportions of plasma phospholipid DHA. A recent study on SHR and hyperactivity also reported higher DHA proportion in the brain compared to WKY. The researchers investigated the correlation between AA/DHA ratio and hyperactive behavior, and found that a higher AA/DHA ratio in the

brain was significantly correlated with greater hyperactivity. Thus, altering AA/DHA ratio could have a greater impact on hyperactivity than changing DHA alone[154]. In our study, the omega-3 enriched diet was successful in lowering the AA/DHA ratio in brain, but this did not translate into a change in behavior.

Disturbances in the dopaminergic system can lead to ADHD-like behavior such as lack of attention, impulsivity, and hyperactivity and are thus thought to be associated with ADHD. However, focusing on dopamine alone may be an oversimplification of the disorder, as successful stimulant treatments can also block other monoamine transporters[162,163] and subsequently increase concentrations of DA, norepinephrine (NE), and 5-HT[164,165]. That is why we evaluated serotonin turnover in addition to dopamine turnover. Dopamine concentration was lower for SHR on the omega-3 diet, while serotonin concentration was lower for both strains. Nonetheless, as no impact was found on serotonin or dopamine turnover, it can be concluded that our intervention did not have an overall impact on dopamine and serotonin utilization. However, of note is that the SHR had lower DA turnover than the WKY, which is consistent with other studies on DA turnover in SHR[126,133]. In an omega-3 dietary intervention study conducted by Dervola et al.[133] DA turnover did not differ between SHR on the control diet and WKY, which differed from our results. On the other hand, their intervention was successful in improving DA turnover. A noteworthy difference between our studies is the length and strength of intervention. Dervola et al. supplemented rats with a high dose of omega-3 fatty acids for two generations, which was successful in improving behavior and modulating neurotransmission[133]. We chose to focus on juvenile SHR in order to narrow

down the most effective time period for intervention. We also maintained consistent fat proportion for both diets to eliminate extra calories as a confounding factor.

Previous studies have provided evidence supporting that those with ADHD may have increased oxidative stress[143,144]. There are also multiple papers demonstrating that adult SHR have higher oxidative stress than their WKY counterparts[166,167]. Thus, we hypothesized that we also would find evidence of higher oxidative stress in the SHR than the WKY. We used GSH as a marker of oxidative stress, with higher concentration indicative of an improved redox balance and less oxidative stress while very high concentrations could be indicative of increased oxidative stress. Surprisingly, the WKY had lower GSH concentration. However, oxidative stress is best understood by evaluating multiple markers. A secondary oxidative stress marker, such as F_2-isoprostanes or oxidized glutathione (GSSG), will be measured in the future as an internal standard. Nonetheless, our preliminary data hints that the omega-3 diet was effective in reducing oxidative stress.

While there was a marked behavioral difference between the SHR and WKY, our dietary intervention did not have our desired impact on hyperactive behavior. These results are not surprising, however, given that dopamine neurotransmission was also not affected. While brain DHA proportion was increased, the dosage or length of time for the intervention was apparently not enough to have an impact on dopamine or serotonin turnover. There are few other studies that have tested an omega-3 intervention on SHR behavior. One such study compared SHR on an omega-3 adequate or omega-3 deficient diet, and found that the omega-3-adequate diet resulted in less locomotion/hyperactivity than the deficient diet. However, many studies have already confirmed the detrimental

impact of omega-3 deficiency on behavior, independent of ADHD status. A very recent study was published that also looked at an omega-3 enriched diet in comparison to a primarily omega-6 diet and similarly did not observe a change in hyperactive behavior[168]. Studies with our timeline are important because they narrow down the timeframe and dosage for efficacy of an omega-3 intervention. As a dietary intervention during prenatal and postnatal growth was successful[133], whereas studies during 3-10 weeks of age were not[168], a pre-weaning age range should be investigated in the future.

3.5 Conclusion

In summary, our omega-3 PUFA enriched diet did not improve hyperactive behavior, consistent with the lack of impact on DA and 5-HT turnover. However, our intervention successfully increased omega-3 PUFA proportion in plasma and brain, and may have improved redox balance and decreased oxidative stress in both strains. Further studies should be conducted investigating the impact of an omega-3 intervention pre-weaning. Additionally, our study further highlights the inherent behavioral and biochemical differences between SHR and WKY.

CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTIONS

ADHD is a multifactorial disorder with conflicting evidence pertaining to its etiology. Over 11% of children in the United States, and around 5% of both children and adults worldwide, are affected[6]. The symptoms of the disorder can negatively impact school performance, personal relationships, and can lead to greater risk taking behaviors. Medicinal treatment is effective for around 70% of those with ADHD, but it comes with the risk of side effects such as decreased appetite and insomnia. Clearly, these side effects could have unfavorable effects on both children and adults. Consequently, alternative treatments with fewer side effects are of great interest.

Although the origin of ADHD is not fully understood, what we do know can help elucidate potential treatments. Our lab has identified a subgroup of children with ADHD that display essential fatty acid (EFA) deficiency symptoms such as increased thirst, frequent urination, and dandruff. Subsequently a number of studies, ours included, reported that as high as 40% of subjects with ADHD had lower proportions of plasma docosahexaenoic acid than healthy controls and others with ADHD. Omega-3 polyunsaturated fatty acids, EPA and DHA especially, are known to be vital for healthy brain development and function. In fact, an omega-3 PUFA deficiency can induce ADHD-like behavior in otherwise healthy animals. In cases of omega-3 PUFA induced learning deficits and hyperactivity, postnatal omega-3 PUFA supplementation was

effective in reversing the effects. The reason why DHA proportions are lower in a subgroup of those with ADHD, despite similar diets to healthy controls, is currently unknown. However, oxidative stress could be a potential factor, since long chain PUFAs are easily oxidized. There exists some evidence of increased oxidative stress in those with ADHD[143], and the animal model for ADHD is known to have heightened states of oxidative stress[166,167].

In our study we investigated the hypothesis that an omega-3 PUFA enriched diet would decrease hyperactivity, modulate neurotransmission, decrease oxidative stress, and increase omega-3 PUFA proportions in the brain and plasma. One concern was that our intervention may not have been long enough to modulate brain phospholipid proportions, but we were successful in increasing DHA proportion, and decreasing arachidonic acid proportion, in brain. Both EPA and DHA proportions were increased in the plasma. For the most part, the impact of the omega-3 diet was similar for plasma and brain. However, some strain differences were only seen in plasma. As reported in a previous study, DHA proportion in the brain was higher in the SHR than the WKY[154]. These results went against our predication that the SHR would also display lower DHA proportions, as seen in our previous human studies.

Another surprising result was that the SHR seem to have less incidence of oxidative stress than the WKY based on liver total glutathione concentrations. There is a possible explanation for this occurrence, however, as GSH alone is not enough to evaluate redox balance and oxidative stress. In the future, we plan to analyze oxidized glutathione (GSSG) for a more complete picture of redox balance. F_2-isoprostanes can be analyzed as well as an internal standard. Together, these analyses will provide more reliable

information on the redox state of the animals. Nonetheless, the omega-3 diet slightly improved GSH levels, suggesting decreased oxidative stress, which is consistent with the reports of other studies[169]. In addition, our diets contained greater vitamin E than in a typical rodent diet. The increased antioxidant content in the diet could also be a potential confound to the oxidative stress data, although it was the same for both diets.

Despite the increase in brain DHA proportion, dopamine (DA) and serotonin (5-HT) neurotransmission was not modulated. DA and 5-HT turnover were similar for both diets, but DA turnover was lower in the SHR. This is consistent with other studies on the SHR[111,123,124,133], and also consistent with the validation for its use as a model for ADHD[105] .

While our intervention did not elicit a behavioral or neurotransmitter change, it has provided information on the best time for dietary interventions. As a previous omega-3 intervention animal study has been successful in reducing ADHD symptoms, it seems that the greatest impact occurs pre-weaning or prenatally via maternal supplementation. In the future it would be beneficial to test the impact of supplementation during isolated developmental periods, such as solely maternal and prenatal intervention.

Our pilot study discussed in Chapter 2 was done in order to develop behavioral testing protocols that elucidated the difference in behavior between the SHR and WKY. Our single-lever operant test was not difficult enough to show any differences between strains. Other studies have confirmed a behavioral difference when using nose-poke tests or a dual-lever discrimination task[105,133,170]. Future studies should utilize these operant chamber tests when evaluating the impact of treatments or interventions on ADHD-like behavior in an animal model. On the other hand, the open field maze test was appropriate

for identifying hyperactivity in the SHR and the number of box crossings and rearings was significantly higher than for the WKY. Thus, we chose to open field maze test in our main study.

There is still much to understand in regards to ADHD treatment, etiology, and neurobiology. As there exists a subgroup of those with ADHD who display EFA deficiency symptoms, human intervention studies should evaluate that subgroup separately from the rest. The disparity between the results of human intervention trials could be due to omega-3 interventions impacting those with low omega-3 PUFA proportions more so than others. In terms of animal studies, future research should narrow the age-range for when intervention is most effective. It would also be beneficial to test high doses of omega-3 EPA or DHA supplementation, such as what is used in human studies. Furthermore, if it established that an increase in omega-3 PUFA proportions modulates dopamine neurotransmission in the SHR, research should focus on which part of the dopaminergic pathway is being affected. Specifically, more research should be done on dopamine active transporter (DAT) availability and activity, and if omega-3 fatty acids have an impact. Finally, oxidative stress should continue to be investigated in the subgroup of those with ADHD who display EFA deficiency symptoms, as a possible rationale for the lower omega-3 PUFA proportions.

REFERENCES

REFERENCES

1. American Psychiatric Association. . *Diagnostic and statistical manual of mental health disorders: DSM-5*. 5th ed. American Psychiatric Publishing; 2013.

2. Biederman J, Mick E, Sc D, Faraone S V, Ph D. Age-Dependent Decline of Symptoms of Attention Deficit Hyperactivity Disorder : Impact of Remission Definition and Symptom Type. 2000;(May):6–8.

3. Bird HR, Gould MS, Staghezza BM. Patterns of diagnostic comorbidity in a community sample of children aged 9 through 16 years. *J. Am. Acad. Child Adolesc. Psychiatry*. 1993;32(2):361–8.

4. Wolraich ML, Wibbelsman CJ, Brown TE, et al. Attention-deficit/hyperactivity disorder among adolescents: a review of the diagnosis, treatment, and clinical implications. *Pediatrics*. 2005;115(6):1734–46.

5. Polanczyk G V, Willcutt EG, Salum GA, Kieling C, Rohde LA. ADHD prevalence estimates across three decades: an updated systematic review and meta-regression analysis. *Int. J. Epidemiol.* 2014.

6. Center for Disease Control.*ADHD Stat.* 2013. Available at: www.cdc.gov/ncbddd/adhd/data.html. Accessed July 20, 2014.

7. Visser SN, Danielson ML, Bitsko RH, et al. Trends in the parent-report of health care provider-diagnosed and medicated attention-deficit/hyperactivity disorder: United States, 2003-2011. *J. Am. Acad. Child Adolesc. Psychiatry*. 2014;53(1):34–46.e2.

8. Fan X, Xu M, Hess EJ. D2 dopamine receptor subtype-mediated hyperactivity and amphetamine responses in a model of ADHD. *Neurobiol. Dis.* 2010;37(1):228–36.

9. Swanson JM, Kinsbourne M, Nigg J, et al. Etiologic subtypes of attention-deficit/hyperactivity disorder: brain imaging, molecular genetic and environmental factors and the dopamine hypothesis. *Neuropsychol. Rev.* 2007;17(1):39–59.

10. Castellanos FX, Lee PP, Sharp W, et al. Developmental trajectories of brain volume abnormalities in children and adolescents with attention-deficit/hyperactivity disorder. *JAMA*. 2002;288(14):1740–8.

11. Volkow ND, Wang G-J, Newcorn J, et al. Depressed dopamine activity in caudate and preliminary evidence of limbic involvement in adults with attention-deficit/hyperactivity disorder. *Arch. Gen. Psychiatry*. 2007;64(8):932–40.

12. Wigal SB, Nemet D, Swanson JM, et al. Catecholamine response to exercise in children with attention deficit hyperactivity disorder. *Pediatr. Res.* 2003;53(5):756–61.

13. Goldman LS, Genel M, Bezman RJ, Slanetz PJ. Diagnosis and Treatment of Attention-Deficit / Hyperactivity Disorder in Children and Adolescents. 1998;279(14).

14. Banaschewski T, Roessner V, Dittmann RW, Santosh PJ, Rothenberger A. Non-stimulant medications in the treatment of ADHD. *Eur. Child Adolesc. Psychiatry*. 2004;13 Suppl 1:I102–16.

15. Kratochvil CJ, Vaughan BS, Harrington MJ BW. tomoxetine: a se- lective noradrenaline reuptake in- hibitor for the treatment of attention- deficit/hyperactivity disorder. *Expert Opin Pharmacother*. 2003;4:1165–1174.

16. Faraone S V. Genetics of adult attention-deficit/hyperactivity disorder. *Psychiatr. Clin. North Am.* 2004;27(2):303–21.

17. Sprich S, Biederman J, Crawford MH, Mundy E, Faraone S V. Adoptive and biological families of children and adolescents with ADHD. *J. Am. Acad. Child Adolesc. Psychiatry*. 2000;39(11):1432–7.

18. Volkow ND, Wang G-J, Kollins SH, et al. Evaluating dopamine reward pathway in ADHD: clinical implications. *JAMA*. 2009;302(10):1084–91.

19. Faraone S V, Perlis RH, Doyle AE, et al. Molecular genetics of attention-deficit/hyperactivity disorder. *Biol. Psychiatry*. 2005;57(11):1313–23.

20. Faraone S V, Doyle AE. The nature and heritability of attention-deficit/hyperactivity disorder. *Child Adolesc. Psychiatr. Clin. N. Am.* 2001;10(2):299–316, viii–ix.

21. Faraone S V, Biederman J. Neurobiology of attention-deficit hyperactivity disorder. *Biol. Psychiatry*. 1998;44(10):951–958.

22. Kustanovich V, Ishii J, Crawford L, Yang M, McGough JJ, McCracken JT. Transmission disequilibrium testing of dopamine-related candidate gene polymorphisms in ADHD: Confirmation of association of ADHD with DRD4 and DRD5. *Mol Psychiatry*. 2003;9:711–717.

23. Appleton KM, Rogers PJ, Ness a R. Is there a role for n-3 long-chain polyunsaturated fatty acids in the regulation of mood and behaviour? A review of the evidence to date from epidemiological studies, clinical studies and intervention trials. *Nutr. Res. Rev.* 2008;21(1):13–41.

24. Barr CL, Wigg KG, Bloom S, et al. Further evidence from haplotype analysis for linkage of the dopamine D4 receptor gene and attention-deficit hyperactivity disorder. *Am. J. Med. Genet.* 2000;96(3):262–7.

25. Payton A, Holmes J, Barrett JH, et al. Examining for association between candidate gene polymorphisms in the dopamine pathway and attention-deficit hyperactivity disorder: a family-based study. *Am. J. Med. Genet.* 2001;105(5):464–70.

26. Comings DE, Gade R, Muhleman D, Sverd J. No association of a tyrosine hydroxylase gene tetranucleotide repeat polymorphism in autism, Tourette syndrome, or ADHD. *Biol. Psychiatry*. 1995;37(7):484–6.

27. Cook EH, Stein MA KM. Association of attention deficit disorder and the dopamine transporter gene. *Am J Hum Genet*. 1995;56:993–998.

28. Lim M-H, Kim HW, Paik K-C, Cho SC, Yoon DY, Lee H-J. Association of the DAT1 polymorphism with attention deficit hyperactivity disorder (ADHD): a family-based approach. *Am. J. Med. Genet. B. Neuropsychiatr. Genet.* 2006;141B(3):309–11.

29. Dougherty DD, Bonab AA, Spencer TJ, Rauch SL, Madras BK, Fischman AJ. Dopamine transporter density in patients with attention deficit hyperactivity disorder. *Lancet*. 354(9196):2132–3.

30. Swanson JM, Kinsbourne M, Nigg J, et al. Etiologic subtypes of attention-deficit/hyperactivity disorder: brain imaging, molecular genetic and environmental factors and the dopamine hypothesis. *Neuropsychol. Rev.* 2007;17(1):39–59.

31. Millichap JG. Etiologic classification of attention-deficit/hyperactivity disorder. *Pediatrics*. 2008;121(2):e358–65.

32. Linnet KM, Dalsgaard S, Ph D, et al. Reviews and Overviews Maternal Lifestyle Factors in Pregnancy Risk of Attention Deficit Hyperactivity Disorder and Associated Behaviors : Review of the Current Evidence. 2003;(June):1028–1040.

33. Braun J, Kahn RS, Froehlich T, Auinger P, Lanphear BP. Exposures to Environmental Toxicants and Attention Deficit Hyperactivity Disorder in US Children. *Environ. Health Perspect.* 2006;11261(12):1904–1909.

34. Mick E, Biederman J, Faraone S V, Sayer J, Kleinman S. Case-control study of attention-deficit hyperactivity disorder and maternal smoking, alcohol use, and drug use during pregnancy. *J. Am. Acad. Child Adolesc. Psychiatry*. 2002;41(4):378–85.

35. Knopik VS, Heath AC, Jacob T, et al. Maternal alcohol use disorder and offspring ADHD: disentangling genetic and environmental effects using a children-of-twins design. *Psychol. Med.* 2006;36(10):1461–71.

36. Eppright TD, Vogel SJ, Horwitz E TH. Results of blood lead screening in children referred for behavioral disorders. *Mo Med*. 1997;94(6):295–297.

37. Prentice A. Vitamin D deficiency: a global perspective. *Nutr. Rev*. 2008;66(2):S153–S64.

38. Bener A, Al-Hamaq AO, Saleh NM. Association between vitamin D insufficiency and adverse pregnancy outcome: global comparisons. *Int. J. Womens. Health*. 2013;5:523–31.

39. Eyles DW, Feron F, Cui X, et al. Developmental vitamin D deficiency causes abnormal brain development. *Psychoneuroendocrinology*. 2009;34 Suppl 1:S247–57.

40. Vinh Quôc Luong K, Thi Hoàng Nguyên L. Vitamin D and Parkinson's disease. *J. Neurosci. Res.* 2012;90(12):2227–36.

41. Bener A, Kamal M. Predict attention deficit hyperactivity disorder? Evidence -based medicine. *Glob. J. Health Sci.* 2014;6(2):47–57.

42. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, Murad MH, Weaver CM ES. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab.* 2011;96(7):1911–30.

43. Burr, GO. Burr M. On the nature and role of the fatty acids essential in nutrition. *Biol. Chem.* 1930;86:587–621.

44. Stevens J, Deck L, Lipp R. Essential fatty acid metabolism in boys with. 1995.

45. Chen J-R, Hsu S-F, Hsu C-D, Hwang L-H, Yang S-C. Dietary patterns and blood fatty acid composition in children with attention-deficit hyperactivity disorder in Taiwan. *J. Nutr. Biochem.* 2004;15(8):467–72.

46. Bekaroğlu M, Aslan Y, Gedik Y, et al. Relationships between serum free fatty acids and zinc, and attention deficit hyperactivity disorder: a research note. *J. Child Psychol. Psychiatry.* 1996;37(2):225–7.

47. Golmirzaei J, Namazi S, Amiri S, et al. Evaluation of attention-deficit hyperactivity disorder risk factors. *Int. J. Pediatr.* 2013;2013:953103.

48. Carlson S. Long-chain polyunsaturates fatty acids and development of human infants. *Acta Paediatr Suppl*. 1999;88:72–7.

49. Simopoulos A. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed. Pharmacother.* 2002;56(8):365–379.

50. Horrobin D. Fatty acid metabolism in health and disease: the role of delta-6-desaturase. *Am J Clin Nutr*. 1993;57:732S–736S.

51. Simopoulos AP, Leaf A, Salem N. Workshop on the Essentiality of and Recommended Dietary Intakes for Omega-6 and Omega-3 Fatty Acids. *J. Am. Coll. Nutr.* 1999;18(5):487–9.

52. Jumpsen J, Lien EL, Goh YK CM. Small changes of dietary (n-6) and (n-3)/ fatty acid content ratio alter phosphatidylethanolamine and phosphatidylcholine fatty acid composition during development of neuronal and glial cells in rats. *J Nutr*. 1997;127:724–731.

53. Jumpsen JA, Lien EL, Goh YK CM. During neuronal and glial cell development diet n-6 to n-3 fatty acid ratio alters the fatty acid composition of phosphatidylinositol and phosphatidylserine. *Biochim Biophys Acta*. 1997;1347:40–50.

54. Administration USF and D. A to Z Subject Index. *FDA News Release*. 2004.

55. Burdge GC WS. Conversion of alpha-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. *Br J Nutr*. 2002;88:411–420.

56. Burdge GC, Jones AE WS. Eicosapentaenoic and docosapentaenoic acids are the principle products of alpha-linolenic acid in young men. *Br J Nutr*. 2002;88:355–363.

57. Bourre JM. Roles of unsaturated fatty acids (especially omega-3 fatty acids) in the brain at various ages and during ageing. *J. Nutr. Health Aging*. 2004;8(3):163–74.

58. Marin-Perez P, Betancourt A, Lamota M, Lo Menzo E, Szomstein S, Rosenthal R. Outcomes after laparoscopic conversion of failed adjustable gastric banding to sleeve gastrectomy or Roux-en-Y gastric bypass. *Br. J. Surg.* 2014;101(3):254–60.

59. N. SJ. Omega-3 fatty acids: molecular and biochemical aspects. In: *New Protective Roles of Selected Nutrients in Human Nutrition*.; 1989:109–228.

60. Uauy R, Calderon F, Mena P. Essential fatty acids in somatic growth and brain development. *World Rev. Nutr. Diet.* 2001;89:134–60.

61. Shaikh SR, Edidin M. Polyunsaturated fatty acids, membrane organization, T cells, and antigen presentation. *Am J Clin Nutr*. 2006;84(6):1277–1289.

62. Mascio D, Murphy E. Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols. *Am J Clin Nutr*. 1991;53:194S–200S.

63. Du Z-X, Zhang H-Y, Meng X, Guan Y, Wang H-Q. Role of oxidative stress and intracellular glutathione in the sensitivity to apoptosis induced by proteasome inhibitor in thyroid cancer cells. *BMC Cancer*. 2009;9:56.

64. Anderson ME. Glutathione: an overview of biosynthesis and modulation. *Chem. Biol. Interact.* 1998;111-112:1–14.

65. Hammond CL, Lee TK, Ballatori N. Novel roles for glutathione in gene expression, cell death, and membrane transport of organic solutes. *J. Hepatol.* 2001;34(6):946–54.

66. Cadenas E, Ginsberg M, Rabe U SH. Evaluation of a-tocopherol antioxidant activity in microsomal lipid peroxidation as detected by low-level chemiluminescence. *Biochem J*. 1984;223(32):755–9.

67. Lehman J, Rao DD, Canary JJ JJ. Vitamin F and relationships among tocopherols is human plasma, platelets, lymphocytes, and red blood cells. *Am J Clin Nutr*. 1988;47(25):470–4.

68. Yehuda S. Omega-6/omega-3 ratio and brain-related functions. *World Rev. Nutr. Diet.* 2003;92:37–56.

69. Yehuda S, Rabinovitz S, Carasso RL, Mostofsky DI. Fatty acids and brain peptides. *Peptides*. 1998;19(2):407–19.

70. Denisova NA, Cantuti-Castelvetri I, Hassan WN, Paulson KE, Joseph JA. Role of membrane lipids in regulation of vulnerability to oxidative stress in PC12 cells: implication for aging. *Free Radic. Biol. Med.* 2001;30(6):671–8.

71. Joseph JA, Denisova N, Fisher D, et al. Membrane and receptor modifications of oxidative stress vulnerability in aging. Nutritional considerations. *Ann. N. Y. Acad. Sci.* 1998;854:268–76.

72. Urano S, Sato Y, Otonari T, et al. Aging and oxidative stress in neurodegeneration. *Biofactors*. 1998;7(1-2):103–12.

73. Choi JH, Yu BP. Brain synaptosomal aging: free radicals and membrane fluidity. *Free Radic. Biol. Med.* 1995;18(2):133–9.

74. Joseph JA, Denisova N, Fisher D, Bickford P, Prior R, Cao G. Age-related neurodegeneration and oxidative stress: putative nutritional intervention. *Neurol. Clin.* 1998;16(3):747–55.

75. Meehan E, Beaugé F, Choquart D, Leonard BE. Influence of an n-6 polyunsaturated fatty acid-enriched diet on the development of tolerance during chronic ethanol administration in rats. *Alcohol. Clin. Exp. Res.* 1995;19(6):1441–6.

76. Bickford PC, Shukitt-Hale B, Joseph J. Effects of aging on cerebellar noradrenergic function and motor learning: nutritional interventions. *Mech. Ageing Dev.* 1999;111(2-3):141–54.

77. Bland JS. The use of complementary medicine for healthy aging. *Altern. Ther. Health Med.* 1998;4(4):42–8.

78. Villeponteau B, Cockrell R, Feng J. Nutraceutical interventions may delay aging and the age-related diseases. *Exp. Gerontol.* 2000;35(9-10):1405–17.

79. Benarroch EE, Na T. Implications of neurologic disease. 2011:287–293.

80. Waxman SG, Ritchie JM. Molecular dissection of the myelinated axon. *Ann. Neurol.* 1993;33(2):121–36.

81. Bourre JM, Francois M, Youyou a, et al. The effects of dietary alpha-linolenic acid on the composition of nerve membranes, enzymatic activity, amplitude of electrophysiological parameters, resistance to poisons and performance of learning tasks in rats. *J. Nutr.* 1989;119(12):1880–92.

82. Seo D, Patrick CJ, Kennealy PJ. Role of Serotonin and Dopamine System Interactions in the Neurobiology of Impulsive Aggression and its Comorbidity with other Clinical Disorders. *Aggress. Violent Behav.* 2008;13(5):383–395.

83. Delion S, Chalon S, Guilloteau D, Besnard JC, Durand G. alpha-Linolenic acid dietary deficiency alters age-related changes of dopaminergic and serotoninergic neurotransmission in the rat frontal cortex. *J. Neurochem.* 1996;66(4):1582–91.

84. Chalon S, Delion-Vancassel S, Belzung C, et al. Dietary fish oil affects monoaminergic neurotransmission and behavior in rats. *J. Nutr.* 1998;128(12):2512–9.

85. De la Presa Owens S, Innis SM. Docosahexaenoic and arachidonic acid prevent a decrease in dopaminergic and serotoninergic neurotransmitters in frontal cortex caused by a linoleic and alpha-linolenic acid deficient diet in formula-fed piglets. *J. Nutr.* 1999;129(11):2088–93.

86. Zimmer L, Delpal S, Guilloteau D, Aïoun J, Durand G, Chalon S. Chronic n-3 polyunsaturated fatty acid deficiency alters dopamine vesicle density in the rat frontal cortex. *Neurosci. Lett.* 2000;284(1-2):25–8.

87. Zimmer L, Durand G, Guilloteau D, Chalon S. n-3 polyunsaturated fatty acid deficiency and dopamine metabolism in the rat frontal cortex. *Lipids*. 1999;34 Suppl:S251.

88. Cao D, Xue R, Xu J, Liu Z. Effects of docosahexaenoic acid on the survival and neurite outgrowth of rat cortical neurons in primary cultures. *J. Nutr. Biochem.* 2005;16(9):538–46.

89. Palozza P, Sgarlata E, Luberto C, et al. n-3 fatty acids induce oxidative modifications in human erythrocytes depending on dose and duration of dietary supplementation. *Am. J. Clin. Nutr.* 1996;64(3):297–304.

90. Kubo K, Saito M, Tadokoro T, Maekawa A. Changes in susceptibility of tissues to lipid peroxidation after ingestion of various levels of docosahexaenoic acid and vitamin E. *Br. J. Nutr.* 1997;78(4):655–69.

91. Valk EE, Hornstra G. Relationship between vitamin E requirement and polyunsaturated fatty acid intake in man: a review. *Int. J. Vitam. Nutr. Res.* 2000;70(2):31–42.

92. Levant B, Radel JD, Carlson SE. Decreased brain docosahexaenoic acid during development alters dopamine-related behaviors in adult rats that are differentially affected by dietary remediation. *Behav. Brain Res.* 2004;152(1):49–57.

93. Bayer SA AJ. Neurogenesis and neuronal migration. In: *The Rat Nervous System*.; 1995:1041–78.

94. Carrié I, Clément M, De Javel D, Francès H, Bourre J. Learning deficits in first generation OF1 mice deficient in (n-3) polyunsaturated fatty acids do not result from visual alteration. *Neurosci. Lett.* 1999;266(1):69–72.

95. Carrié I, Clément M, de Javel D, Francès H, Bourre JM. Phospholipid supplementation reverses behavioral and biochemical alterations induced by n-3 polyunsaturated fatty acid deficiency in mice. *J. Lipid Res.* 2000;41(3):473–80.

96. Bloch MH, Qawasmi A. Omega-3 fatty acid supplementation for the treatment of children with attention-deficit/hyperactivity disorder symptomatology: systematic review and meta-analysis. *J. Am. Acad. Child Adolesc. Psychiatry*. 2011;50(10):991–1000.

97. Milte CM, Parletta N, Buckley JD, Coates AM, Young RM, Howe PRC. Increased Erythrocyte Eicosapentaenoic Acid and Docosahexaenoic Acid Are Associated With Improved Attention and Behavior in Children With ADHD in a Randomized Controlled Three-Way Crossover Trial. *J. Atten. Disord.* 2013.

98. Cao J, Schwichtenberg KA, Hanson NQ, Tsai MY. Incorporation and clearance of omega-3 fatty acids in erythrocyte membranes and plasma phospholipids. *Clin. Chem.* 2006;52(12):2265–72.

99. Milte CM, Parletta N, Buckley JD, Coates AM, Young RM, Howe PRC. Increased Erythrocyte Eicosapentaenoic Acid and Docosahexaenoic Acid Are Associated With Improved Attention and Behavior in Children With ADHD in a Randomized Controlled Three-Way Crossover Trial. *J. Atten. Disord.* 2013.

100. Vaisman N, Kaysar N, Zaruk-Adasha Y, et al. Correlation between changes in blood fatty acid composition and visual sustained attention performance in children with inattention: effect of dietary n-3 fatty acids containing phospholipids. *Am. J. Clin. Nutr.* 2008;87(5):1170–80.

101. Appleton KM, Rogers PJ, Ness AR. Is there a role for n-3 long-chain polyunsaturated fatty acids in the regulation of mood and behaviour? A review of the evidence to date from epidemiological studies, clinical studies and intervention trials. *Nutr. Res. Rev.* 2008;21(1):13–41.

102. Hirayama S, Hamazaki T, Terasawa K. Effect of docosahexaenoic acid-containing food administration on symptoms of attention-deficit/hyperactivity disorder - a placebo-controlled double-blind study. *Eur. J. Clin. Nutr.* 2004;58(3):467–73.

103. Voigt RG, Llorente AM, Jensen CL, Fraley JK, Berretta MC, Heird WC. A randomized, double-blind, placebo-controlled trial of docosahexaenoic acid supplementation in children with attention-deficit/hyperactivity disorder. *J. Pediatr.* 2001;139(2):189–96.

104. Bélanger SA, Vanasse M, Spahis S, et al. Omega-3 fatty acid treatment of children with attention-deficit hyperactivity disorder: A randomized, double-blind, placebo-controlled study. *Paediatr. Child Health*. 2009;14(2):89–98.

105. Sagvolden T. Behavioral validation of the spontaneously hypertensive rat (SHR) as an animal model of attention-deficit/hyperactivity disorder (AD/HD). *Neurosci. Biobehav. Rev.* 2000;24(1):31–9.

106. Sagvolden T, Johansen EB. Rat models of ADHD. *Curr. Top. Behav. Neurosci.* 2012;9:301–15.

107. Berger DF, Sagvolden T. Sex differences in operant discrimination behaviour in an animal model of attention-deficit hyperactivity disorder. *Behav. Brain Res.* 1998;94(1):73–82.

108. Boix F, Qiao SW, Kolpus T, Sagvolden T. Chronic L-deprenyl treatment alters brain monoamine levels and reduces impulsiveness in an animal model of Attention-Deficit/Hyperactivity Disorder. *Behav. Brain Res.* 1998;94(1):153–62.

109. Holene E, Nafstad I, Skaare JU, Sagvolden T. Behavioural hyperactivity in rats following postnatal exposure to sub-toxic doses of polychlorinated biphenyl congeners 153 and 126. *Behav. Brain Res.* 1998;94(1):213–24.

110. Sagvolden T, Hendley ED, Knardahl S. Behavior of hypertensive and hyperactive rat strains: hyperactivity is not unitarily determined. *Physiol. Behav.* 1992;52(1):49–57.

111. Russell VA, de Villiers A, Sagvolden T, Lamm M TJ. Differences between electrically-, ritalin- and D-amphetamine- stimulated release of [3H]dopamine from brain slices sug- gest impaired vesicular storage of dopamine in an animal model of Attention-Deficit Hyperactivity Disorder. *Behav Brain Res*. 1998;94:163–171.

112. Sagvolden T, Russell V a, Aase H, Johansen EB, Farshbaf M. Rodent models of attention-deficit/hyperactivity disorder. *Biol. Psychiatry*. 2005;57(11):1239–47.

113. Denenberg VH. OPEN-FIELD BEHAVIOR IN THE RAT: WHAT DOES IT MEAN? *Ann. N. Y. Acad. Sci.* 1969;159(3 Experimental):852–859.

114. Bobb AJ, Castellanos FX, Addington AM, Rapoport JL. Molecular genetic studies of ADHD: 1991 to 2004. *Am. J. Med. Genet. B. Neuropsychiatr. Genet.* 2005;132B(1):109–25.

115. Kirley A, Lowe N, Hawi Z, et al. Association of the 480 bp DAT1 allele with methylphenidate response in a sample of Irish children with ADHD. *Am. J. Med. Genet. B. Neuropsychiatr. Genet.* 2003;121B(1):50–4.

116. Krause KH, Dresel SH, Krause J, Kung HF, Tatsch K. Increased striatal dopamine transporter in adult patients with attention deficit hyperactivity disorder: effects of methylphenidate as measured by single photon emission computed tomography. *Neurosci. Lett.* 2000;285(2):107–10.

117. Watanabe Y, Fujita M, Ito Y, Okada T, Kusuoka H, Nishimura T. Brain dopamine transporter in spontaneously hypertensive rats. *J. Nucl. Med.* 1997;38(3):470–4.

118. Leo D, Sorrentino E, Volpicelli F, et al. Altered midbrain dopaminergic neurotransmission during development in an animal model of ADHD. *Neurosci. Biobehav. Rev.* 2003;27(7):661–9.

119. De Jong W, Linthorst AC, Versteeg HG. The nigrostriatal dopamine system and the development of hypertension in the spontaneously hypertensive rat. *Arch. Mal. Coeur Vaiss.* 1995;88(8):1193–6.

120. Linthorst AC, van Giersbergen PL, Gras M, Versteeg DH, de Jong W. The nigrostriatal dopamine system: role in the development of hypertension in spontaneously hypertensive rats. *Brain Res.* 1994;639(2):261–8.

121. Linthorst AC, De Lang H, De Jong W, Versteeg DH. Effect of the dopamine D2 receptor agonist quinpirole on the in vivo release of dopamine in the caudate nucleus of hypertensive rats. *Eur. J. Pharmacol.* 1991;201(2-3):125–33.

122. Bendel P, Eilam R. Quantitation of ventricular size in normal and spontaneously hypertensive rats by magnetic resonance imaging. *Brain Res.* 1992;574(1-2):224–8.

123. Russell VA, de Villiers A, Sagvolden T, Lamm M TJ. Altered dopaminergic function in the prefrontal cortex, nucleus accumbens and caudate-putamen of an animal model of Attention- Deficit Hyperactivity Disorder - the spontaneously hypertensive rat. *Brain Res*. 1995;676:343–351.

124. Ernst M, Zametkin AJ, Matochik JA, Pascualvaca D, Jons PH, Cohen RM. High midbrain [18F]DOPA accumulation in children with attention deficit hyperactivity disorder. *Am. J. Psychiatry*. 1999;156(8):1209–15.

125. Ernst M, Zametkin AJ, Matochik JA, Jons PH, Cohen RM. DOPA decarboxylase activity in attention deficit hyperactivity disorder adults. A [fluorine-18]fluorodopa positron emission tomographic study. *J. Neurosci.* 1998;18(15):5901–7.

126. De Villiers AS, Russell VA, Sagvolden T, Searson A, Jaffer A, Taljaard JJ. Alpha 2-adrenoceptor mediated inhibition of [3H]dopamine release from nucleus accumbens slices and monoamine levels in a rat model for attention-deficit hyperactivity disorder. *Neurochem. Res.* 1995;20(4):427–33.

127. Sagvolden T, Metzger MA, Sagvolden G. Frequent reward eliminates differences in activity between hyperkinetic rats and controls. *Behav. Neural Biol.* 1993;59(3):225–9.

128. Sagvolden T, Aase H, Zeiner P, Berger D. Altered reinforcement mechanisms in attention-deficit/hyperactivity disorder. *Behav. Brain Res.* 1998;94(1):61–71.

129. VI D. Attentional and cognitive problems. In: *Developmental neuropsychiatry*.; 1983:280–329.

130. Russell V a, Sagvolden T, Johansen EB. Animal models of attention-deficit hyperactivity disorder. *Behav. Brain Funct.* 2005;1:9.

131. McCarty R, Kopin IJ. Patterns of behavioral development in spontaneously hypertensive rats and Wistar-Kyoto normotensive controls. *Dev. Psychobiol.* 1979;12(3):239–43.

132. Mook DM, Jeffrey J, Neuringer A. Spontaneously hypertensive rats (SHR) readily learn to vary but not repeat instrumental responses. *Behav. Neural Biol.* 1993;59(2):126–35.

133. Dervola KS, Roberg BA, Wøien G, et al. Marine O-3 polyunsaturated fatty acids induce sex-specific changes in reinforcer-controlled behaviour and neurotransmitter metabolism in a spontaneously hypertensive rat model of ADHD. *Behav. Brain Funct.* 2012;8:56.

134. Hauser J, Makulska-Gertruda E, Reissmann A, Sontag T-A, Tucha O, Lange KW. The effects of nutritional polyunsaturated fatty acids on locomotor activity in spontaneously hypertensive rats. *Atten. Defic. Hyperact. Disord.* 2014.

135. Rahman I, Kode A, Biswas SK. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat. Protoc.* 2006;1(6):3159–65.

136. Pastore A, Federici G, Bertini E, Piemonte F. Analysis of glutathione: implication in redox and detoxification. *Clin. Chim. Acta.* 2003;333(1):19–39.

137. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature*. 2000;408(6809):239–47.

138. Bharath S, Hsu M, Kaur D, Rajagopalan S, Andersen JK. Glutathione, iron and Parkinson's disease. *Biochem. Pharmacol.* 2002;64(5-6):1037–48.

139. Capitan P, Malmezat T, Breuillé D, Obled C. Gas chromatographic-mass spectrometric analysis of stable isotopes of cysteine and glutathione in biological samples. *J. Chromatogr. B. Biomed. Sci. Appl.* 1999;732(1):127–35.

140. Navarro J, Obrador E, Carretero J, et al. Changes in glutathione status and the antioxidant system in blood and in cancer cells associate with tumour growth in vivo. *Free Radic. Biol. Med.* 1999;26(3-4):410–8.

141. Mills BJ, Weiss MM, Lang CA, Liu MC, Ziegler C. Blood glutathione and cysteine changes in cardiovascular disease. *J. Lab. Clin. Med.* 2000;135(5):396–401.

142. Comhair SA, Lewis MJ, Bhathena PR, Hammel JP, Erzurum SC. Increased glutathione and glutathione peroxidase in lungs of individuals with chronic beryllium disease. *Am. J. Respir. Crit. Care Med.* 1999;159(6):1824–9.

143. Ross BM, McKenzie I, Glen I, Bennett CPW. Increased levels of ethane, a non-invasive marker of n-3 fatty acid oxidation, in breath of children with attention deficit hyperactivity disorder. *Nutr. Neurosci.* 2003;6(5):277–81.

144. Stevens L, Zhang W, Peck L, et al. ARTICLES EFA Supplementation in Children with Inattention , Hyperactivity , and Other Disruptive Behaviors. 2003;38(10).

145. Antalis CJ, Stevens LJ, Campbell M, Pazdro R, Ericson K, Burgess JR. Omega-3 fatty acid status in attention-deficit/hyperactivity disorder. *Prostaglandins. Leukot. Essent. Fatty Acids*. 2006;75(4-5):299–308.

146. Wolraich ML, Wibbelsman CJ, Brown TE, et al. Attention-deficit/hyperactivity disorder among adolescents: a review of the diagnosis, treatment, and clinical implications. *Pediatrics*. 2005;115(6):1734–46.

147. Wigal SB, Nemet D, Swanson JM, et al. Catecholamine response to exercise in children with attention deficit hyperactivity disorder. *Pediatr. Res.* 2003;53(5):756–61.

148. Volkow ND, Wang G-J, Newcorn J, et al. Brain dopamine transporter levels in treatment and drug naïve adults with ADHD. *Neuroimage*. 2007;34(3):1182–90.

149. Mick E, Biederman J, Faraone S V, Sayer J, Kleinman S. Case-control study of attention-deficit hyperactivity disorder and maternal smoking, alcohol use, and drug use during pregnancy. *J. Am. Acad. Child Adolesc. Psychiatry*. 2002;41(4):378–85.

150. Martin RE, Rodriguez de Turco EB BN. lopmental maturation of hepatic n-3 polyunsaturated fatty acid metabolism: supply of docosahexaenoic acid to retina and brain. *J Nutr Biochem*. 1994;5:151–60.

151. Innis SM. Dietary (n-3) fatty acids and brain development. *J. Nutr.* 2007;137(4):855–9.

152. Logan AC. Omega-3 fatty acids and major depression: a primer for the mental health professional. *Lipids Health Dis.* 2004;3(1):25.

153. Burgess JR, Stevens L, Zhang W, Peck L. Long-chain polyunsaturated fatty acids in children with attention-deficit hyperactivity disorder. *Am. J. Clin. Nutr.* 2000;71(1 Suppl):327S–30S.

154. Cao A, Yu L, Wang Y, Wang G, Lei G. Composition of long chain polyunsaturated fatty acids (LC-PUFAs) in different encephalic regions and its association with behavior in spontaneous hypertensive rat (SHR). *Brain Res.* 2013;1528:49–57.

155. Benarroch EE, Na T. Implications of neurologic disease. 2011:287–293.

156. Young GS, Maharaj NJ, Conquer JA. Blood phospholipid fatty acid analysis of adults with and without attention deficit/hyperactivity disorder. *Lipids*. 2004;39(2):117–23.

157. Carrié I, Clément M, de Javel D, Francès H, Bourre JM. Phospholipid supplementation reverses behavioral and biochemical alterations induced by n-3 polyunsaturated fatty acid deficiency in mice. *J. Lipid Res.* 2000;41(3):473–80.

158. Bloch MH, Qawasmi A. Omega-3 fatty acid supplementation for the treatment of children with attention-deficit/hyperactivity disorder symptomatology: systematic review and meta-analysis. *J. Am. Acad. Child Adolesc. Psychiatry*. 2011;50(10):991–1000.

159. Sagvolden T, Dasbanerjee T, Zhang-James Y, Middleton F, Faraone S. Behavioral and genetic evidence for a novel animal model of Attention-Deficit/Hyperactivity Disorder Predominantly Inattentive Subtype. *Behav. Brain Funct.* 2008;4:56.

160. Juaneda P, Rocquelin G. Rapid and convenient separation of phospholipids and non phosphorus lipids from rat heart using silica cartridges. *Lipids*. 1985;20(1):40–1.

161. Ohta A, Mayo MC, Kramer N, Lands WE. Rapid analysis of fatty acids in plasma lipids. *Lipids*. 1990;25(11):742–7.

162. Taylor D, Ho BT. Comparison of inhibition of monoamine uptake by cocaine, methylphenidate and amphetamine. *Res. Commun. Chem. Pathol. Pharmacol.* 1978;21(1):67–75.

163. Jones SR, Gainetdinov RR, Wightman RM, Caron MG. Mechanisms of Amphetamine Action Revealed in Mice Lacking the Dopamine Transporter. *J. Neurosci.* 1998;18(6):1979–1986.

164. Genro JP, Kieling C, Rohde LA, Hutz MH. Attention-deficit/hyperactivity disorder and the dopaminergic hypotheses. *Expert Rev. Neurother.* 2010;10(4):587–601.

165. Gainetdinov RR. Role of Serotonin in the Paradoxical Calming Effect of Psychostimulants on Hyperactivity. *Science (80-.).* 1999;283(5400):397–401.

166. Suzuki H, Swei A, Zweifach BW, Schmid-Schonbein GW. In Vivo Evidence for Microvascular Oxidative Stress in Spontaneously Hypertensive Rats : Hydroethidine Microfluorography. *Hypertension*. 1995;25(5):1083–1089.

167. Lee M-C-I, Shoji H, Miyazaki H, et al. Assessment of oxidative stress in the spontaneously hypertensive rat brain using electron spin resonance (ESR) imaging and in vivo L-Band ESR. *Hypertens. Res.* 2004;27(7):485–92.

168. Liso Navarro AA, Sikoglu EM, Heinze CR, et al. Effect of diet on brain metabolites and behavior in spontaneously hypertensive rats. *Behav. Brain Res.* 2014;270:240–7.

169. Mas E, Woodman RJ, Burke V, et al. The omega-3 fatty acids EPA and DHA decrease plasma F(2)-isoprostanes: Results from two placebo-controlled interventions. *Free Radic. Res.* 2010;44(9):983–90.

170. Adriani W, Caprioli A, Granstrem O, Carli M, Laviola G. The spontaneously hypertensive-rat as an animal model of ADHD: evidence for impulsive and non-impulsive subpopulations. *Neurosci. Biobehav. Rev.* 2003;27(7):639–51.