Omega-3 polyunsaturated fatty acids induced gender-specific changes in activity, impulsiveness and attention in an animal model of Attention-Deficit/Hyperactivity Disorder

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Abstract

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Title: Omega-3 polyunsaturated fatty acids induced gender-specific changes in activity, impulsiveness and attention in an animal model of Attention-Deficit/Hyperactivity Disorder

Background: It is argued that omega-3 (n-3) polyunsaturated fatty acids (PUFAs) may influence Attention-Deficit/Hyperactivity Disorder (ADHD) symptoms and/or development by affecting the lipid composition of neuromodulator systems such as the dopamine system which is believed to be hypo-functioning in children with ADHD. A validated animal model of ADHD was used to explore whether a long-chain n-3 PUFA-enriched chow would exert any positive effects on behavioral measures of activity, impulsiveness and attention.

Method: A total of 36 spontaneously hypertensive rats (SHRs) were tested in this study. One group (8 males and 7 females) received a long-chain n-3 PUFA-enriched chow with a balanced omega-6/omega-3-ratio and the control group (11 males and 10 females) received standard lab-chow. Both SHR dams and offspring were exposed to the feeding conditions. The offspring were tested using a variable reinforcement schedule designed to measure activity level, impulsiveness and attention. In addition, a measure of general activity was derived from video-recorded data.

Results: PUFA-treatment tended to interact with gender and reduced activity, impulsiveness and improving attention in male-SHRs while female-SHRs showed no effect or an increased activity, impulsiveness and reduced attention on operant measures. Results from video-recorded activity revealed that both genders reduced their general activity in the beginning of each session and that general activity was different across sessions than the results obtained from the operant measure of activity.

Conclusion: Long-chain n-3 PUFA-treatment administered to both SHR dams and offspring induced two distinct sets of behavioral changes in the offspring. One gender-specific effect showed reduced reinforcer-controlled activity, impulsiveness and improved attention in male-SHRs and none or opposite effects in female-SHRs. Another effect showed reduced exploratory activity in both genders. It is hypothesized that the n-3 PUFA-treatment might have exerted gender-specific effects on neural systems regulating reinforcement-controlled behavior and effects on neural systems regulating exploratory activity in both genders.

Author contribution

This study was part of a large interdisciplinary collaboration examining effects of PUFAs in an animal model of Attention-Deficit/Hyperactivity Disorder at the University of Oslo. Christian A. Drevon (Department of Nutrition) designed the content of the enriched polyunsaturated fatty acids-chow. The making of the chow was carried out by Anne Randi Enget (Department of Nutrition) and Torbjørn Helgesen Sandvik (Department of Psychology). Terje Sagvolden (Department of Physiology), Grete Wøien (Department of Physiology), Espen Borgå Johansen (Oslo and Akershus University College, Kjeller, Norway) and Torbjørn Helgesen Sandvik were responsible for the gathering of behavioral data. Torbjørn Helgesen Sandvik did the analyses of behavioral data and is the author of this thesis. Analyses of neurotransmitter levels in the subjects were carried out by a group led by S. Ivar Walaas (Department of Biochemistry) and these results will be published elsewhere and are not discussed in this thesis.

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1 Introduction

1.1 General overview of ADHD

1.1.1 ADHD prevalence

Attention-Deficit/Hyperactivity Disorder (ADHD) (American Psychiatric Association, 2000) is regarded as a heterogeneous behavioral disorder. The symptoms are usually manifest before the child is 7 years old (Applegate et al., 1997). In children under age 18 years, the worldwide prevalence of ADHD has been estimated to be ~5 % (Polanczyk, de Lima, Horta, Biederman, & Rohde, 2007), but epidemiology estimates vary highly across studies, generally from 4 to 12 % among grade school children (Brown et al., 2001). In the general population, ADHD is seemingly more common in males than in females with a ratio about 3:1, and when diagnosed, symptoms are typically found to be more severe in boys than in girls (Gaub & Carlson, 1997).

1.1.2 ADHD behavior and diagnosis

Since no other objective marker has been found, ADHD is diagnosed based on behavioral criteria (Barkley, 2006). ADHD symptoms are characterized by three categorical clusters of age-inappropriate behavior including attention problems, hyperactivity and impulsiveness (American Psychiatric Association, 2000). The *inattention* cluster includes behavioral descriptions such as difficulty in sustaining attention, distractibility, lack of persistence and disorganization. The *hyperactivity* and *impulsiveness* clusters include behavior descriptions such as excessive motor activity (e.g. excessive running, climbing) and impulsive responding (e.g. impatience and difficulty delaying gratification). According to the diagnostic criteria, hyperactive-impulsive or inattentive symptoms must be present before age 7 years, cause significant clinical impairment in two or more settings and not be better accounted for by another mental disorder (American Psychiatric Association, 2000).

1.1.3 Differences in diagnostic taxonomies

The American Psychiatric Association (APA) and World Health Organization (WHO) diagnostic taxonomies differ somewhat in their description of behaviors representing ADHD

and their criteria for diagnosis (Biederman & Faraone, 2005). The main difference is that according to the DSM-IV-TR (APA), it is possible to have ADHD without being inattentive or to have ADHD without being hyperactive-impulsive (American Psychiatric Association, 2000). The ICD-10 (WHO) criteria are more restrictive for the corresponding hyperkinetic disorder (HKD) (World Health Organization, 1993), demanding that all three behavioral clusters need to be affected in order to fulfill the criteria for a diagnosis. Both ICD-10 HKD and combined subtype DSM-IV-TR ADHD appear to exhibit the same predictive validity over a period of at least 6 years (Lahey et al., 2006). However, evidence for the validity and clinical use of the specific DSM-IV-TR subtypes is mixed, and there is still an ongoing controversy about whether a sheer inattentive disorder exists and could be causally different from the combined type of ADHD or HKD (Biederman & Faraone, 2005).

1.1.4 Persistence and longitudinal outcomes of ADHD

ADHD symptoms decline with age, but follow-up studies reveal that some symptoms persist into adulthood for the majority of children with ADHD (Biederman, Petty, Evans, Small, & Faraone, 2010; Faraone, Biederman, & Mick, 2006).

A considerable amount of prospective longitudinal studies show that children diagnosed with ADHD are highly at risk for developing a range of problems related to social adjustment and functioning, as well as several psychiatric disorders in adolescence and adulthood (Biederman et al., 2006; Cantwell, 1985; Hinshaw, Owens, Sami, & Fargeon, 2006; Hoza et al., 2005; Lee, Lahey, Owens, & Hinshaw, 2008; Lee, Humphreys, Flory, Liu, & Glass, 2011). For example, ADHD children are at higher risk for developing antisocial personality disorder (Mannuzza, Klein, Bessler, Malloy, & LaPadula, 1998) and various substance disorders (Rasmussen & Gillberg, 2000). They are also more likely to have poor academic and job career outcomes (Barkley, Fischer, Smallish, & Fletcher, 2006), are more prone to accidents (DiScala, Lescohier, Barthel, & Li, 1998; Woodward, Fergusson, & Horwood, 2000) and have a higher arrest rate as adults (Mannuzza, Klein, & Moulton, 2008). Furthermore, in addition to causing individual suffering, ADHD is believed to have large negative socio-economic implications (Bernfort, Nordfeldt, & Persson, 2008).

1.1.5 Treatment of ADHD

Medical and behavioral treatment regimes have been the most common form of intervention in order to reduce ADHD symptoms (Roth & Fonagy, 2005). The most known study comparing the efficacy of behavioral and medical treatment on ADHD is the Multimodal Treatment study of children with ADHD (MTA) (Roth & Fonagy, 2005). The researchers concluded that medication alone, or a combination of medication and therapy, was more efficient than behavior therapy alone, in reducing ADHD symptoms over a 24 month period (Jensen, Arnold, Severe, Vitiello, & Hoagwood, 2004). Although this might the case, possibly partially because of the profit motive, there is a disproportionate amount of research on pharmacological treatments, with much less known about different behavioral or complementary therapies, such as dietary management, either alone or in combination with medications (Efron, Hazell, & Anderson, 2011). Hence, more interdisciplinary research is needed in order to understand what might cause, prevent and reduce ADHD symptoms (Snowling, 2009).

1.2 Etiology

Consistent with its heterogeneity in clinical presentation, ADHD is seen as a complex multifactoral disorder caused by different types of risk factors (i.e. genetic, biological, psychosocial and environmental) (Biederman & Faraone, 2005). Progress in research has led one to believe that no single causal factor is necessary or sufficient in causing ADHD, but each risk factor has an increasingly small effect in the complex, interactive and causal pathways leading to ADHD (Biederman & Faraone, 2005).

1.2.1 Genes and heritability

Family, twin, and adoption studies provide strong evidence that genes play a considerable role in mediating vulnerability to ADHD (Faraone et al., 2005). Based mainly on American and European population samples, an average heritability of 0.76 has been estimated (Faraone et al., 2005). Candidate genes related to several neurotransmitter systems in the brain have been identified, but no single gene provides a strong effect on mediating the development of ADHD (Faraone & Mick, 2010). Instead, it is believed that the genetic architecture of mental orders like ADHD is complex and mediated by many genes. These genes may indirectly increase the probability for the disorder through an interaction with adverse environmental

factors (Rutter, Moffitt, & Caspi, 2006). However, the high heritability suggests that children with ADHD might have an observable biological makeup different from non-ADHD children, and much research has been directed in finding biological ADHD correlates.

1.2.2 Neurobiological correlates and behavioral consequences

A hypo-functioning dopamine system has for a long time been proposed as an underlying neurobiological basis for ADHD (Sagvolden, Johansen, Aase, & Russell, 2005). Stimulant drugs, such as methylphenidate hydrochloride (e.g. Ritalin and Concerta) and d-amphetamine (Dexamine), increase the synaptic availability of dopamine, and reduce ADHD symptoms (Jensen et al., 2001; Volkow et al., 1998; Volkow et al., 2007). The dopamine deficit theory has been further strengthened by genetic studies linking candidate genes to dopamine function (Faraone & Mick, 2010), and studies on animal models showing that abnormal dopamine functioning is linked to ADHD-like behavior (Russell, 2000; Sagvolden, 2000; Schneider, Sun, & Roeltgen, 1994).

The abnormal dopamine systems appear to be linked with altered behavioral learning processes (Sagvolden et al., 2005). It is suggested that the hypo-functioning of dopamine systems produce altered reinforcement of behavior and deficient extinction of previously reinforced behavior (Sagvolden & Sergeant, 1998). This may contribute to an inability of delaying responses, which again might increase the risk of learning maladaptive responses in new situations, and lead to an increased behavioral variability in general (Sagvolden et al., 2005).

Research utilizing neuroimaging techniques has shown several brain abnormalities in children with ADHD (Makris, Biederman, Monuteaux, & Seidman, 2009; Valera, Faraone, Murray, & Seidman, 2007). Structural cortical abnormalities in frontal, temporal and parietal regions linked to attention and behavior inhibition have been found (Sowell et al., 2003). These abnormalities appear early in development and remain roughly parallel during childhood and adolescence, suggesting that early causal agents are involved in the brain development of these children (Castellanos et al., 2002). Furthermore, functional imaging (fMRI) studies of children with ADHD have found that brain activation in areas linked to attention and response inhibition, were hypo-activated relative to controls (Durston et al., 2003).

1.2.3 Environmental risk factors and their relation to heritability

Although ADHD is highly heritable, and several biological correlates have been found, the concordance of monozygotic twins is not perfect, indicating contribution from non-genetic causes (Biederman & Faraone, 2005). Environmental factors may act in an additive or interactive way with genetic influences, or may even represent an independent (i.e. non-genetic) factor in ADHD development (Caspi & Moffitt, 2006). By categorizing explained behavioral variance in a population to either genetic or environmental variance, the concept of heritability might create an artificial dichotomy, which masks variance that in reality can be attributed to gene-environment interactions (Krueger & Johnson, 2008; Rutter et al., 2006). Consistent with this notion, the Journal of Child Psychology and Psychiatry recently expressed an urgent need for longitudinal studies to identify risk factors, and start looking for unknown gene-environment interactions in order to enhance our understanding of ADHD (Snowling, 2009).

Environmental risk factors known to be associated with ADHD include maternal cigarette smoking (Linnet et al., 2003) and alcohol use during pregnancy (Mick, Biederman, Faraone, Sayer, & Kleinman, 2002). Also, birth complications and low birth weight (Mick, Biederman, Prince, Fischer, & Faraone, 2002) are associated with ADHD development. On a general basis, studies have found that psychosocial adversity like marital distress, family dysfunction and low social class are associated risk factors for ADHD in children (Biederman et al., 1995; Biederman, Faraone, & Monuteaux, 2002). Recently, a study examining gene-environment interactions found that the adverse effect of certain psychosocial stressors in children with ADHD might be moderated by having particular dopamine and serotonin transporter genotypes (Sonuga-Barke et al., 2009).

The effect of nutrion on ADHD-development is a gene-environment interaction not yet thoroughly researched (Mysterud, 2006; Schuchardt, Huss, Stauss-Grabo, & Hahn, 2010). It is hypothesized that since our contemporary typical Western diet is far from identical to the hunter-gatherer diet in the Paleolithic period, certain individuals in the population might be genetically vulnerable to a Western dietary pattern (Mysterud, 2003). That a Western diet can contribute to the development of several somatic diseases (e.g. allergies, diabetes, cardiovascular diseases) in some individuals has been shown in a considerable amount of research (Mysterud, 2006). Even though it is plausible, it is more controversial that a modern Western diet can contribute to psychiatric disorders such as ADHD (Biederman & Faraone,

2005; Busch, 2007). Still in an exploratory phase, evidence suggests that a healthy diet regime in children vulnerable to developing ADHD might function as a protective factor or reduce ADHD-symptoms (Carter et al., 1993; Howard et al., 2011; Pelsser et al., 2011). In addition to examining the total effect of a healthy diet, future research needs to determine which nutrients are especially beneficial for children with ADHD (Pelsser et al., 2011). A group of nutrients which have been suggested mediating the development of ADHD-symptoms are polyunsaturated fatty acids (PUFAs) (Schuchardt et al., 2010).

1.3 Polyunsaturated fatty acids and ADHD

1.3.1 Long-chain polyunsaturated fatty acids metabolism

A fatty acid (FA) is a molecule that consists of chains with at least four carbon molecules attached to hydrogen and oxygen atoms (Mysterud, 2006). At one side of the chain, the first carbon atom is attached to three hydrogen atoms in a methyl group (CH_3). At the other side of the chain, there is a carboxyl group (COOH) which consists of a carbon atom, two oxygen atoms and a hydrogen atom. Fatty acids can be either saturated or unsaturated. Unsaturated fatty acids have one or more double bindings between the carbon atoms while saturated fatty acids only have single bindings. When there is more than one double binding, the fatty acid is called polyunsaturated. When there are more than 20 carbon atoms and two or more double bindings, the fatty acid is called long-chain polyunsaturated fatty acid (LC-PUFA).

The most common forms of PUFAs are called omega-3 (n-3) and omega-6 (n-6). Among these, alfa-linolic acid (ALA), an n-3 FA, and linoleic acid (LA), an n-6 FA, are essential nutrients to humans and therefore must to be obtained from food (Schuchardt et al., 2010). Humans are able to synthesize the long-chain n-6 FA arachidonic acid (AA) from LA, and the long-chain n-3 FAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from ALA. However, the process of synthesizing these LC-PUFAs is extremely slow and ineffective in most mammals (Pawlosky, Hibbeln, Novotny, & Salem, 2001). On a phylogenetic explanation level, the reason for this is probably that as predators, humans have evolved to get most of their LC-PUFAs directly from a high intake of meat (Mysterud, 2006). On an ontogenetic explanation level, the conversion process is also influenced by several individual factors such as sex hormones, n-6/n-3 ratio intake, genes, saturated fatty acids intake, stress hormone levels as well as vitamins and mineral cofactors. A non-effective

conversion process has been suggested to create a vulnerability to an unbalanced LC-PUFA intake and further be a risk factor in the development of both somatic and mental disorders such as ADHD (Schuchardt et al., 2010; Simopoulos, 2002).

1.3.2 Polyunsaturated fatty acids and the nervous system

PUFAs play a central role in the brain and the nervous system. They are biochemically involved in the development of the brain, brain gene expression, in neuronal structures and are central in various processes within the nervous system (Uauy & Dangour, 2006). Studies have shown that it is especially important with an adequate supply of PUFAs, especially DHA, during early development. Randomized controlled trials have reported impaired mental performance in healthy infants due to lack of DHA and AA (Willatts, Forsyth, DiModugno, Varma, & Colvin, 1998). n-3 deficiency in rhesus monkeys during gestation and postnatal development caused impaired visual function and cognitive and psychomotor deficits (Neuringer, Connor, Lin, Barstad, & Luck, 1986).

PUFAs also play a central role as structural components in neuronal cell membranes which modify the influence of protein receptors and transporters embedded in the membrane (Chalon, 2006). For example, animal studies using rats have shown that chronic n-3 FAs deficiency caused abnormalities in the dopaminergic and serotoninergic neurotransmitter systems and might be related to the expression of ADHD-symptoms (Delion et al., 1994). The mechanisms explaining this interplay are still not completely understood. A possible mechanism is that incorporation of the n-3 DHA in the neuronal cell membranes seems to increase with its intake (Chalon et al., 1998; Kamada et al., 1986). Furthermore, this causes the cell membrane to maintain a fluid and optimal quality for signal processes within the cell (Mitchell & Litman, 1998; Yehuda, Rabinovitz, & Mostofsky, 1999). Altered cell membrane fluidity might alter the protein structure of membrane receptors which in turn might cause an effect on their function and activity (Yehuda et al., 1999). If this hypothesis is true, then PUFAs function as mediators of signal systems such as the serotoninergic and dopaminergic neuronal modulator systems (Yehuda et al., 1999).

1.3.3 The omega-6/omega-3 ratio

In addition to the effect of adequate PUFA-supply, the n-6/n-3 intake ratio seems to be of central physiological importance. Our contemporary genotype is very similar to the humans

that lived in the Paleolithic period 40.000 years ago (Mysterud, 2006). However, today's diet deviates markedly from the Paleolithic diet. The n-6/n-3 ratio of the Paleolithic period has been estimated to be ~1:1 while today's Western diet has ratios as high as 16:1 (Simopoulos, 2006). Studies have shown that a high n-6/n-3 ratio promotes the pathogenesis of cardiovascular diseases, cancer, inflammatory and autoimmune diseases, while a low ratio exerts a suppressing anti-inflammatory effect on these diseases (Simopoulos, 2002). The proximal, physiological explanation for this is that when LC-PUFAs are synthesized, n-6 and n-3 FAs compete for the same enzyme systems and are able to reciprocally displace each other (Schuchardt et al., 2010). A high n-6/n-3 ratio will therefore create an unbalanced metabolism favoring long-chain n-6 FAs synthesis and exert inflammatory effects, while a low ratio will favor long-chain n-3 FAs synthesis and exert anti-inflammatory effects. In addition to playing a role in mediating somatic diseases, a more controversial hypothesis suggest that a high n-6/n-3 ratio also might influence neural functioning, particularly in individuals vulnerable to neuro-cognitive disorders such as ADHD, by diminishing the availability of long-chain n-3 FAs in the brain (Busch, 2007; Schuchardt et al., 2010).

1.3.4 Research examining the effect of PUFA-supply on children with ADHD

Observational studies have shown that n-3 levels of FAs such as ALA and DHA are lower in the membrane of red blood cells and plasma in children and adults with ADHD symptoms relative to healthy controls (Antalis et al., 2006; Colter, Cutler, & Meckling, 2008). Also, some studies show that low n-3 levels are associated with a higher level of ADHD symptoms (Stevens et al., 1995; Stevens, Zentall, Abate, Kuczek, & Burgess, 1996). Even though PUFA levels in blood cells and plasma correlate well with the levels of the brain (Yehuda et al., 1999), the causal interpretation of these findings is complicated by the possible influence of several other confounding factors. However, these studies suggest a positive association between ADHD-symptoms and low levels of n-3 PUFAs.

Randomized controlled trials (RCTs) are the golden standard for efficacy research which goal is to establish causal links between treatment and outcome while controlling for several confounding factors such as placebo effects (Roth & Fonagy, 2005). As mentioned above, there is a disproportionate amount of RCT research on pharmacological interventions relative to dietary interventions (Efron et al., 2011). The sample of RCT PUFA studies is therefore too

small to conduct an objective meta-analysis. Hence, only literature reviews of the available research exist (Raz & Gabis, 2009; Schuchardt et al., 2010; Transler, Eilander, Mitchell, & van de Meer, 2010).

In general, reviews state that the exploration of the effects of PUFAs on ADHD symptoms is in a premature phase. The results are mixed and more rigorous research designs are needed in order to infer whether PUFA-supply is beneficial for either all ADHD children or a subgroup of them. It should also be noted that there is a lack of longitudinal designs exploring the relationship between PUFA-supplementation and ADHD development. All studies to date are short-term, lasting for about three months. If there is a long-term developmental relationship between PUFA-intake and ADHD, then short-term RCT studies might not be the proper design to examine such a causal link. On the other hand, longitudinal studies are very expensive and possibly unethical to conduct on children when considering that one group can be deprived of beneficial nutrition. A possibly fruitful way of exploring the longitudinal relationship between PUFAs and ADHD-like behavior is through the use of a validated animal model of ADHD tested in a controlled experimental setting.

1.4 An animal model of ADHD

1.4.1 Advantages and disadvantages of animal models

Most neuroscientists use animal models to examine the mechanisms they want to understand in humans (Bear, Connors, & Paradiso, 2007). Nervous systems of different species have evolved from common ancestors and therefore may also have common mechanisms. For example, rats become addicted if they are given the chance to self-administer cocaine repeatedly (Bear et al., 2007). Hence, rodent models illuminate how psychoactive drugs affect the nervous system in humans.

There are several advantages of using animal models. Animal models have simpler nervous systems and their behavior is easier to interpret than human clinical cases (Sagvolden, Russell, Aase, Johansen, & Farshbaf, 2005). They also often provide stronger internal validity over human research because the models are often more genetically homogenous, the environment is easier to control, and more invasive interventions can be conducted compared to humans (Sagvolden et al., 2005).

A common critique raised about research using laboratory animals is that the high internal validity comes with the price of loosing external validity (Bordens & Abbott, 2005). Generalizations from the highly controlled research setting to other populations and settings, where uncountable confounding and interacting variables exist, is therefore a problematic issue. However, the main purpose of conducting laboratory experiments is not necessarily to generalize directly to a human population, but rather to generalize indirectly by testing hypothesis derived from a theory (Bordens & Abbott, 2005). Over time, the accumulated empirical findings will lead to the support or revision of a theory which goal is to explain and predict human behavior (Bordens & Abbott, 2005).

1.4.2 The spontaneously hypertensive rat as a validated animal model of ADHD

The spontaneously hypertensive rat (SHR) is bred from the Wistar Kyoto rat (WKY) and is currently the most frequently used model of ADHD (Sagvolden, 2000). The utility of SHR as an animal model of ADHD can be assessed by three validation criteria: face validity, construct validity, and predictive validity (Sagvolden et al., 2005). Face validity is the ability to mimic the behavioral symptoms of the disorder. Construct validity is whether the model conforms to the theoretical rationale of the disorder. Predictive validity is the ability to predict previously unknown aspects of behavior, genetics, and neurobiology of the disorder.

SHRs should mimic inattention, hyperactive and impulsive behavior in children with ADHD in order to have face validity. When SHRs and children with ADHD were tested with the same reinforcement schedule, similar results were obtained on operationalized measures of sustained attention, motor impulsiveness and hyperactivity (Sagvolden, 2000). Thus within a behavioral framework, research supports the face validity of the SHR as a model of ADHD (Sagvolden et al., 2005).

The issue of construct validity is more problematic because of the lack of agreement on the theoretical rationale for ADHD (Sagvolden et al., 2005). It is therefore premature to conclude on the construct validity of the SHR-model (Sagvolden et al., 2005). However, results with SHRs are consistent with the key processes within a behavioral theory of ADHD which are thought to be associated with hypo-functioning dopamine systems (Sagvolden, 2000; Sagvolden et al., 2005).

Finally, the SHR-model has already provided insights on the behavioral level on several processes thought to affect the development of hyperactivity, impulsiveness and inattention (Sagvolden, 2000). Thus far, the SHR-model therefore fulfills the criteria of predictive validity to a sufficient extent (Biederman & Faraone, 2005; Sagvolden et al., 2005). The model is believed to be useful not only in understanding behavioral processes linked to ADHD, but also genetics, neurobiology and pharmacology.

1.4.3 Research examining the effect of PUFA-supply on SHRs

A literature search on PubMed, PSYCINFO and ISI Web of Knowledge using the keywords "Spontaneously hypertensive rat", "Polyunsaturated fatty acids" and "ADHD" reveals that to this date, only one study has examined the behavioral effects of PUFA-supply on SHRs. The study investigated whether SHRs would differ from WKYs on a version of the Morris water maze performance task when given PUFA-supply (Clements, Girard, Xing, & Wainwright, 2003). Their task was thought to measure short-term memory deficit in rats. The results showed no performance effect of PUFA-supplementation. However, this does not necessarily imply that PUFA-supply does not exert any effects on other measures of ADHD. The researchers used a measure of short-term memory not linked to the validation of the SHR as an animal model of ADHD (Sagvolden et al., 2005). It might prove more fruitful to use already validated measures of ADHD in the SHR-model as part of an explorative design testing the effects of PUFA-supply.

1.5 The aim of this study

The aim of this study was to investigate whether long-chain n-3 PUFA-enriched chow with a balanced n-6/n-3 ratio exerted any positive behavioral effects on SHRs relative to controls receiving standard lab chow. It was also an aim to explore gender-specific differences in relation to the experimental manipulation of the feeding regime. The animals were tested using a reinforcement schedule designed to measure reinforcement-controlled level of activity, impulsiveness and attention (Sagvolden, 2000). In addition, the animals were videotaped and their level of movement from frame to frame was used as a measure of general activity.

2 Method

2.1 Subjects

Spontaneously Hypersensitive Rodents (SHR/NCrl) purchased from Charles River, Sulzfeld, Germany were used as breeding subjects in this study. During the first three weeks of birth, the rats were under the care of a veterinarian at the Norwegian Defense Research Establishment in Kjeller. The veterinarian administered feeding of either n-3 PUFA-enriched food or standard lab chow to dams before breeding, under pregnancy and after birth. The pups were therefore constantly exposed to the experimental manipulation.

At postnatal day (PND) 25, a total of 36 rats were shipped to the University of Oslo for behavioral testing. The rats were experimentally naïve on arrival and young rats were used in order to mimic the early manifestation and development of ADHD (Sagvolden & Xu, 2008).

During habituation and shaping, the rats were housed together in twos or threes in 41 x 25 x 25 (height) cm transparent cages. Following acquisition of lever pressing and throughout the rest of the experiment, the rats were housed individually in the same type of cages. The rats had unlimited access to either n-3 PUFA-enriched food or standard lab chow depending on their assigned experimental condition. They had unlimited access to water at all times prior to the dipper training sessions. During the experimental procedure, the rats were given water as reinforcers and therefore deprived from water for 21 hours a day to ensure sufficient motivation (Sagvolden & Xu, 2008). In addition to water obtained through reinforcers, in their home cage they had unlimited access to water for 45 minutes immediately after each day's session.

The temperature in the housing area was approximately 22^{o}C, and the light was on from 06.00 to 18.00 hours. The behavioral training took place between 9.00 and 14.00 hours 7 days a week and lasted for 34 days.

The study was approved by the Norwegian Animal Research Authority (NARA), and conducted in agreement with the laws and regulations controlling experiments with live animals in Norway.

2.2 Behavioral apparatus

The behavioral apparatus without cameras has been described before (Johansen et al., 2011; Sagvolden & Xu, 2008). Sixteen Campden Instruments operant chambers were used. The chambers were located in two separate rooms each containing eight chambers that were run by two separate computers. Each chamber was enclosed in a sound-resistant outer housing, equipped with a grid floor and well ventilated. The animal's working space in eight of the chambers was 25 x 25 x 30 (height) cm, and 25 x 25 x 20 (height) cm in the other eight chambers. A fan producing a low masking noise and a 2.8-Watt house light were on during the entire experimental session. Each chamber was equipped with two retractable levers requiring a dead weight of at least 3 g to activate a micro-switch, and with a 2.8-Watt cue light located above each lever.

The reinforcers (0.05 ml tap water) were delivered by a liquid dipper located in a small recessed cubicle where a 2.8-Watt cue light lit up when a reinforcer was presented. A 7 x 5 cm transparent plastic top-hinged flap separated the cubicle from the animal's working space. The rat could easily open the lid with a light push with the nose or paw.

The two computers and the program LabView 7.1 (National Instruments, 2004) recorded the behavior, scheduled reinforcers and lights.

In order to compare the general activity in the operant chamber with the activity on the levers, activity in the operant chambers was videotaped and digitized by Mini Color Hidden Cameras (420TVL, 0,1lux) from Tracer Technology Co., Ltd, Taiwan (http://www.tracer.com.tw). One camera was permanently installed in every operant chamber and positioned in the upper rear corner at an angle of 45°. The cameras captured virtually the entire working space of the animal (through a 2.5mm wide angle lens with a F2.0 aperture): front wall with levers and signal lights, the entire floor, side and rear walls. The cameras were controlled by the DVR Live Capture program (Novus Security, 2009) designed for recording up to 40 cameras simultaneously. The output file from each camera had 15 fps (frames/s) and was stored as Audio Video Interleave files (AVI).

2.3 Procedure

2.3.1 Group assignment

All PUFA-exposed pups from the breed were behaviorally tested. The controls were selected from a pool of SHRs used as normal control subjects in another study (Johansen et al., 2011). The testing took place on two different times and assignment to the experimental chamber took place in a semi-randomized manner. The PUFA-group consisted of 15 rats (8 males and 7 females) and the control group consisted of 21 rats (11 males and 10 females).

2.3.2 Feeding of the experimental group

The experimental group was given a semi-synthetic PUFA-enriched feeding regime high on the long-chain omega-3 polyunsaturated fatty acids, C20:5n-3 (EPA) and C22:6n-3 (DHA). The n-6/n-3 ratio was about 1:2.7, slightly lower than the ~1:1 ratio that has been theorized as an evolutionary optimal ratio for humans (Simopoulos, 2006).

The n-3 enriched food was made in accordance to the same procedure used by other n-3 experiments with rats (Rokling-Andersen et al., 2009). The n-3 fatty acid supplement (n-3 FA) consisted of Triomar™ (EPAX6000 TG); EPAX AS, Lysaker, Norway). Triomar contained >60 % of total n-3 FA as triacylglycerols (TAG): Eicosapentaenoic acid (EPA), 300 mg/g; Docosahexaenoic acid (DHA), 200 mg/g; total n-3 FA, 600 mg/g (total n-3: EPA, DHA, 18:3, 18:4, 20:4, 21:5, 22:5). In addition, lard (Erica Lard; Ten Kate Vetten BV, Musselkanaal, The Netherlands) consisting of several FAs was used. To avoid essential FA undernourishment, soybean oil (Mills Soyaolje; Denofa Lilleborg, Fredrikstad, Norway) was also used.

The dietary composition (g/100 g) was: total fat, 21; sucrose, 20; maize starch, 31.5; casein, 20; methionine, 0.4; cellulose, 1; vitamin mixture, 1.5; salt mixture, 5. The food was kept at − 20°C and given to the rats in portions sufficient for 1 day supply in order to avoid oxidation.

2.3.3 Feeding of the control group

The controls were given standard lab chow (RM3 (E) from Special Diet Services, Witham, Essex CM8 3AD, UK). This product covers the essential nutrition needed for rats, is low on

EPA and DHA, and has an n-6 to n-3 ratio about 7:1. An estimate of the n-6/n-3 ratio in today's Swedish diets was reported to be around 4.7:1 (Ambring et al., 2006), and other estimates of contemporary Western diets have been reported to have a ratio as high as 15:1 (Simopoulos, 2006). Therefore, the relative high ratio of 7:1 in the control chow was considered appropriate for comparison to the experimental group which had a low intake ratio of 1:2.7.

2.3.4 Operant testing: Habituation, dipper training and response acquisition

An established procedure of operant behavioral testing of the SHR as ADHD model was used and has been described before (Johansen et al., 2011; Sagvolden & Xu, 2008). Prior to behavioral testing, the rats were assigned an operant chamber and a time of testing in a semi-randomized and balanced way. Habituation to the operant chambers started two days after arrival (PND 27) and lasted for 30 minutes. During the habituation session, the flap between the working space and the reinforcement cubicle was held open by tape. No levers were present, the cue lights above the levers were off, and no reinforcers were delivered. The habituation session was followed by two 30 minutes dipper training sessions. The flap was taped open, no levers were present, and the cue lights above the levers were off. The computer delivered water every 10 second irrespective of the animal's behavior using a variable-time schedule. The cue light in the small recessed cubicle was lit during each water delivery.

In the following two sessions, the rats were trained to open the flap to gain access to the drop of water. The tape was removed from the flap, no levers were present, and the cue lights located above the levers were off. Each flap-opening turned on the cue light in the water cubicle and produced the presentation of a single drop of water. The water-dipper was lowered after 5 seconds irrespective of the animal's behavior.

During the next two sessions, lever pressing was shaped according to the method of successive approximations (Catania, 1998). During the first of these sessions, the animals learned to press the left lever in order to receive a reinforcer immediately following every press. The cue light above the left lever was lit for the entire session except during presentation of the reinforcer when only the light in the water cubicle was lit. The right lever was removed and the light above the right lever was off. On the second session, the right lever was inserted and the left lever retracted. During this session, the light above the right lever

was lit the entire session except during presentation of the reinforcer when the light in the water cubicle was lit. Immediately following response shaping on each lever, the rat was monitored to make sure the response was learned, and then left in the chamber for an additional 15 minutes to further strengthen the newly learned behavior. During this time, every press on the lever produced a reinforcer.

2.3.5 The variable interval 3 seconds schedule

A variable interval schedule is a schedule of reinforcement where the delivery of a reinforcer depends on the passage of a variable time and then the emission of a single response (Catania, 1998). Response acquisition was followed by six 30 minutes long training sessions (session 8 to 13) using a variable interval 3 seconds reinforcement schedule (VI 3 s). During the VI 3 s sessions and throughout the rest of the study, both levers were present. At the start of the session and following each reinforcer, the computer program semi-randomly selected which lever to produce the reinforcer. Lever selection was limited to a maximum of 4 consecutive reinforcers on the same lever to avoid the development of a lever-preference.

The lever associated with the reinforcement schedule was signaled (discriminative stimulus) by the lit cue light located above the respective lever. The light stayed lit for as long as the lever was associated with reinforcement, but was turned off during reinforcer presentation. The timer for the next interval started when the dipper was presented. Both scheduled reinforcers and reinforcers produced but not collected, were accumulated and scheduled for the next correct response.

From now on reinforcers were accessible for 3 seconds after the flap into the water cubicle was opened by the rat. Then, the dipper was lowered and the cubicle light was turned off. If the rat did not open the flap within 5 seconds after a reinforcer presentation, the water dipper was lowered and the cubicle light was turned off. A concurrent extinction schedule was in effect on the alternative lever. Concurrent schedules of reinforcement are schedules of reinforcement that are simultaneously available to an animal subject or human participant, so that the subject or participant can respond on either schedule (Catania, 1998). An extinction schedule means that responding to the lever produces no reinforcer. The light above the alternative lever was always off. Thus, the present task can be described as a simultaneous visual discrimination task where the rat had to learn which lever to press in accordance to which light was lit.

2.3.6 The variable interval 180 seconds schedule

The final schedule, a variable interval 180 seconds schedule (VI 180 s) was in effect for 90 minutes from session 14 and until the end of the study (see Table 1). The schedule alternated semi-randomly between the two levers, but was always signaled by a lit light above the lever. A concurrent extinction schedule was in effect on the alternative lever.

Table 1.
Summary of the experimental procedure.

Behavioral procedure	Session number	Reinforcement schedule
Habituation	1	
Magazine training	2 – 3	FT 10 seconds
Flap training	4 – 5	CRF
Shaping of lever-pressing	6 – 7	
30 minutes session	8 – 13	VI 3 seconds
90 minutes session, 16 chambers	14 – 41	VI 180 seconds

Note. Summary of the experimental procedure. FT: Fixed time schedule of reinforcement. CRF: Continuous reinforcement schedule. VI: Variable interval schedule of reinforcement.

The program Variable Interval Generator 1.2 developed by Mark P. Reilly, 2001, was used by courtesy of Peter Killeen to generate Catania-Reynolds distribution of intervals (Catania & Reynolds, 1968). Inter-reinforcer intervals during the VI 180 s schedule ranged from 6 to 719 seconds and were distributed in a semi-randomized fashion. There was neither any external stimulus signaling that a reinforcer was programmed nor any external stimulus signaling the time since the last response.

2.3.7 Operant behavior

The computer recorded number of presses on the lever producing reinforcers and on the alternative lever. Furthermore, it recorded the number of flap openings to the cubicle, number of reinforcers produced and the number of reinforcers collected, and finally it recorded the time of the events.

Building on earlier ADHD research on SHRs (Johansen et al., 2011; Sagvolden, 2006; Sagvolden & Xu, 2008), the following four operationalized measures of ADHD-like behavior were calculated from the recorded data across all sessions:

Attention (stimulus control)

We speak of stimulus control when a stimulus becomes an effective signal (Catania, 1998). To produce a reinforcer the animal had to press the lever with the corresponding lit light above. Hence, the light above the reinforcer-producing lever worked as a discriminative stimulus for reinforced behavior. Two measures of stimulus control were derived from the recorded behavior:

Firstly, the total percentage correct of presses on the lever associated with reinforcement was used as a measure of stimulus control. If there was no stimulus control, then the total percentage correct would be at chance level ~ 50 %. Thus, this measure of stimulus control existed on a continuum from 50 % to 100 %. In a neuropsychological conceptual tradition, this measure might be seen as an ability to *sustain attention* because the rats had to pay attention to which lever the light was lit above in order to receive a reinforcer following a response (Sagvolden, 2006; Sagvolden & Xu, 2008; Sagvolden, DasBanerjee, Zhang-James, Middleton, & Faraone, 2008).

Secondly, total percentage of first press on the reinforcer-producing lever after a new VI-schedule began was used as another measure of stimulus control. This measure of stimulus control also existed on a continuum from 50 % to 100 %. Again, in a neuropsychological conceptual tradition, this measure might capture some other aspect of controlling attention by focusing on the ability to *shift attention* to relevant stimuli (Lezak, Howieson, & Loring, 2004). After reinforcement delivery, the rats either shifted their attention to where the light was lit and responded to the reinforcement producing lever, or they persevered to the previously reinforced response without refocusing on the new reinforcement conditions.

Hyperactivity

Level of activity was measured by the total number of presses on the two levers combined.

Impulsiveness

Inter-response times (IRTs), the time interval passing between two lever presses, were recorded and divided into short IRTs (<0.67 s) and long IRTs (>0.67 s). Responses with IRTs shorter than 0.67 s will hardly ever produce a reinforcer and can thus be considered as a measure of impulsiveness ("e.g. inability to delay gratification" or "premature responding".).

2.3.8 Video recording of general activity in operant chambers

The animals were video-taped during the operant task for three sessions. The three sessions were chosen to represent behavior early, middle and late in the experimental testing. As a supplement, each of the three sessions was divided into five segments to analyze within-session changes in locomotion in both the experimental and control group.

The cameras recorded 15 frames per second, and frame-to-frame analyses of changes in pixels were done by the computer program Musical Gestures Toolbox, developed for audio and video analysis (Jensenius, A.R., Godøy, & Wanderley, 2005). To measure animal activity more reliably, pixel-changes were averaged across frames (15 frames) and a noise reduction threshold (0.25) and filter (length of 8) were used.

General activity was calculated by subtracting values in two consecutive video frames (Jensenius, 2007). The parts of the animal that moved from one frame to the next were shown as a blob of active pixels in the motion image. The motion image is therefore a measure of the movement by the animal between frames. The quantity of motion is calculated as the number of active pixels in a frame divided by the total number of pixels in the screen. The quantity of motion is 0 if there are no active pixels in a frame and 1 if all pixels available on the screen are active. Hence, the quantity of motion is an estimate of *general activity*.

2.4 Data analysis

All statistical analyses were done in Statistica 6.0 (StatSoft, 2005). Data were evaluated by multivariate analyses using Wilks lambda (MANOVAs) when the degrees of freedom relative to the number of levels of the repeated factor permitted this approach, or by univariate analyses of variance (ANOVAs) adjusting the degrees of freedom with the Huynh–Feldt epsilon (Myers & Well, 2003). In the operant task, in order to compare recorded operant behavior with video-data, the three corresponding sessions (early, middle, late session) were

used as within-subject factor. In the video analyses, the same three sessions were used as within-subject factor, and as a supplement, segments were used as a within-subject factor. PUFA-treatment and sex were used as between-subjects factor in both analyses. Post-hoc comparisons following ANOVAs were performed using the Unequal N HSD test. This procedure is a modification of the Tukey HSD test and can be used in the analysis of unequal sample sizes (StatSoft, 2011).

3 Results

3.1 Operant behavior

3.1.1 Attention (stimulus control)

Overall, PUFA-treatment tended to interact with sex, improving male-SHRs' performance on both measures of stimulus control, while PUFA-treated female-SHRs showed no effect on total percentage correct and poorer performance on first press percentage correct (see Figure 1 and 2).

Analyses of the percentage of total number of lever presses on the lever producing reinforcers showed a statistically significant main effect of session, $F(2,31) = 11.31$; $p < 0.01$, where all SHRs improved their performance from the early to the late session (not shown). In addition there were three trend effects that fell shy of significance: A main effect of sex, $F(1,32) = 3.45$; $p = 0.07$, a sex x feeding condition interaction effect, $F(1,32) = 3.66$; $p = 0.06$ (see Figure 1), and a session x sex interaction effect, $F(2,31)=3,0$; $p = 0.07$.

The analyses of the percentage of the first lever press following reinforcer delivery on the reinforcer-producing lever, showed a statistically significant sex x feeding condition interaction effect, $F(1,32) =5.99$; $p = 0.02$ (see Figure 2). No other significant effects were found.

Unequal N HSD post-hoc analyses of the significant session effect on total number of correct lever presses, revealed a difference between the early and the late session, $p < 0.01$. The post-hoc analyses found no significant effects of the sex x feeding condition interaction effect on the measure of first press correct after reinforcer-delivery.

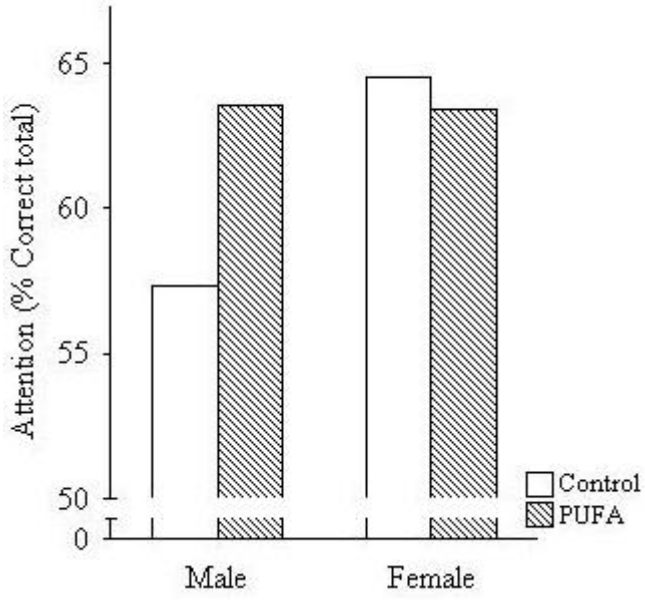

Figure 1. Percentage of responses on the reinforcer-producing lever as a function of sex and feeding condition.

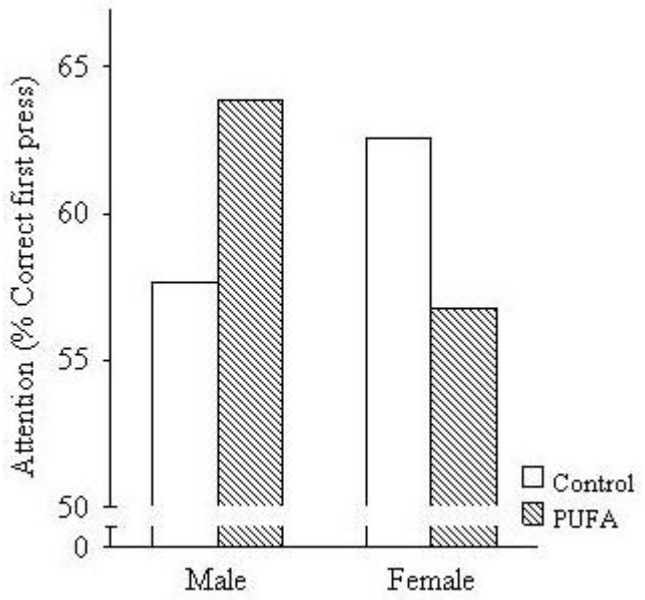

Figure 2. Following reinforcer delivery, percentage of first press on the reinforcer-producing lever as a function of sex and feeding condition.

3.1.2 Hyperactivity (total lever presses)

In general, PUFA-treatment interacted with sex reducing total lever presses in male-SHRs and increasing total lever presses in female-SHRs (see Figure 3).

The analyses showed statistically significant main effects of sex, $F(1,32) = 6.23$; $p = 0.02$, and session, $F(2,31) = 8.62$; $p < 0.01$. In total, male-SHRs emitted more lever presses than female-

SHRs (not shown) and all animals reduced their number of lever presses from the middle session to the late session (not shown). Furthermore, there was a statistically significant sex x feeding condition interaction effect (see Figure 3), $F(1,32) = 4.94$; $p = 0.03$. No other effects were found.

Unequal N HSD post-hoc analyses on the significant main effect of session showed that all SHRs reduced their lever presses from the middle session to the late session, $p < 0.01$. Post-hoc analyses of the significant sex x feeding condition interaction effect showed that male controls emitted significantly more lever-presses than female controls, $p < 0.01$.

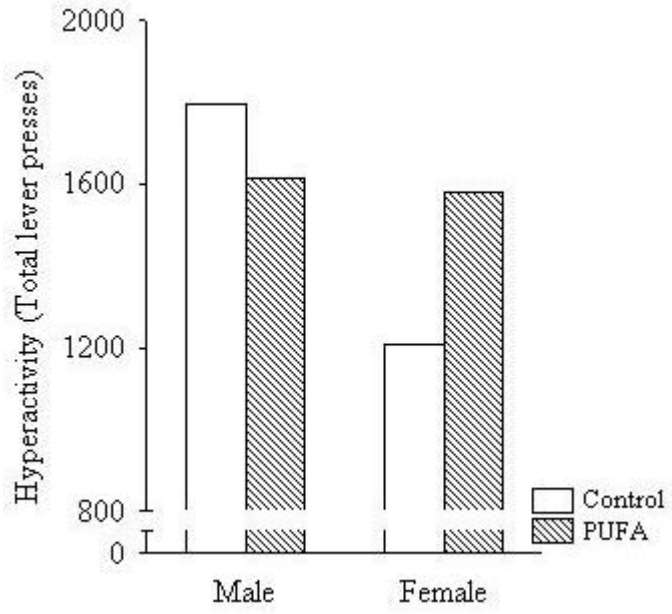

Figure 3. Total lever presses as a function of sex and feeding condition.

3.1.3 Impulsiveness (short IRTs)

Overall, PUFA-treatment interacted with sex reducing short IRTs in male-SHRs while increasing it in female-SHRs (see Figure 4).

The analyses showed a statistically significant main effect of session, $F(2,31) = 4.23$; $p = 0.02$, increasing the number of short IRTs between the early and middle session in all SHRs (not shown). Further, there was a significant sex x feeding condition interaction effect, $F(1,32) = 5.81$; $p = 0.02$ (see Figure 4). Also, a statistically significant three way interaction effect between sex x feeding condition x session was found, $F(2,31) = 5.60$; $p < 0.01$.

Unequal N HSD post-hoc analyses showed an increased number of short IRTs in PUFA-treated females between early and middle session, $p < 0.04$. No significant effects were found in the post-hoc analyses of the significant sex x feeding condition interaction effect.

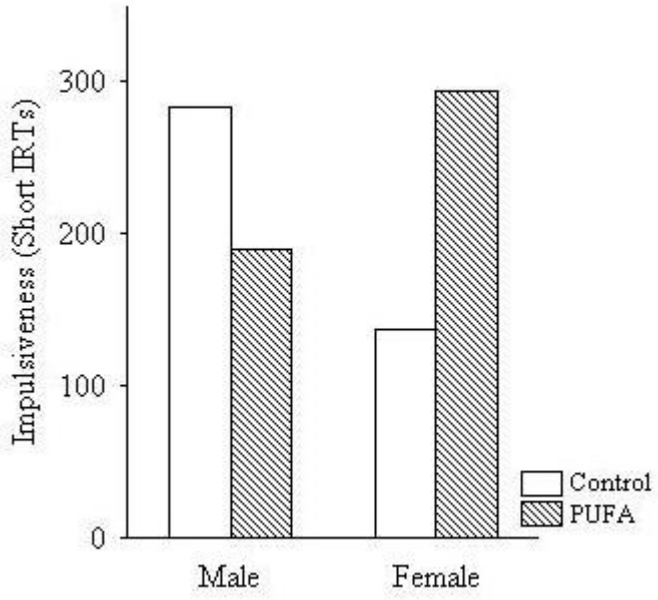

Figure 4. Short IRTs as a function of sex and feeding condition.

3.1.4 Reinforcers collected

No statistically significant effects were found for analyses of number of reinforcers collected. Out of the possible 30 reinforcers available per session, the mean number of reinforcers collected was closely similar in all groups across sessions, and ranged from 28.3 (PUFA-treated males, session 27) to 29 (male controls, session 6).

3.2 Video-recorded general activity in the operant chambers

Overall, PUFA-treatment reduced general activity in the initial segment in both genders (see Figure 5). Until the late session, over segments across sessions, both PUFA-treated genders showed a linear pattern, while the control SHRs showed a falling steep slope of general activity (see Figure 6). Further, while the operant measure of activity (total lever presses) showed no three-way interaction effect of feeding condition x sex x session, there was a

discontinuous behavioral pattern dependent on both PUFA-treatment and sex on the video measure of general activity across sessions (see Figure 7).

The analyses showed statistically significant main effects of session, $F(2,30) = 8.63$; $p < 0.01$, and segment, $F(4,28) = 14.77$; $p < 0.01$. All SHRs increased their general activity between early and middle session and within sessions there was a decrease in general activity across segments (not shown). Furthermore, there was a statistically significant sex x segment interaction effect, $F(4,28) = 3.29$; $p < 0.03$, where male-SHRs showed more general activity across segments relative to female-SHRs (not shown). There were also a significant feeding condition x segment, $F(4,28) = 4.63$; $p < 0.01$ (see Figure 5), and a session x segment interaction effect, $F(8,24) = 6.31$; $p < 0.01$. From session to session, all SHRs increased their general activity in the initial segment (not shown). Additionally, as already described, the analyses showed statistically significant sex x feeding condition x session, $F(2,30) = 3.54$; $p = 0.04$ (see Figure 6), and feeding condition x session x segment three-way interaction effects, $F(8,24) = 2.44$; $p = 0.04$ (see Figure 7).

Unequal N HSD post-hoc analyses showed that the main effect of session were statistically significant between early and middle session, $p < 0.01$. Post-hoc analyses of the feeding condition x segment interaction effect showed that the first segment of the control group differed significantly from the first segment of the experimental group, $p = 0.05$. Moreover, analyses of the session x segment interaction effect showed that all SHRs increased their activity in the initial segment between early and middle session, $p < 0.01$, and early and late session, $p < 0.01$, and there was a statistical trend effect pointing in the same direction between middle and late session, $p = 0.08$. Additionally, post-hoc analyses of the three-way feeding condition x session x segment interaction effect revealed that PUFA-treated SHRs did not differ across the initial segments in the early and middle session. This finding confirms the linear pattern of general activity seen in Figure 6. Finally, post-hoc analyses of the three-way interaction effect of feeding condition x sex x session showed no significant effects.

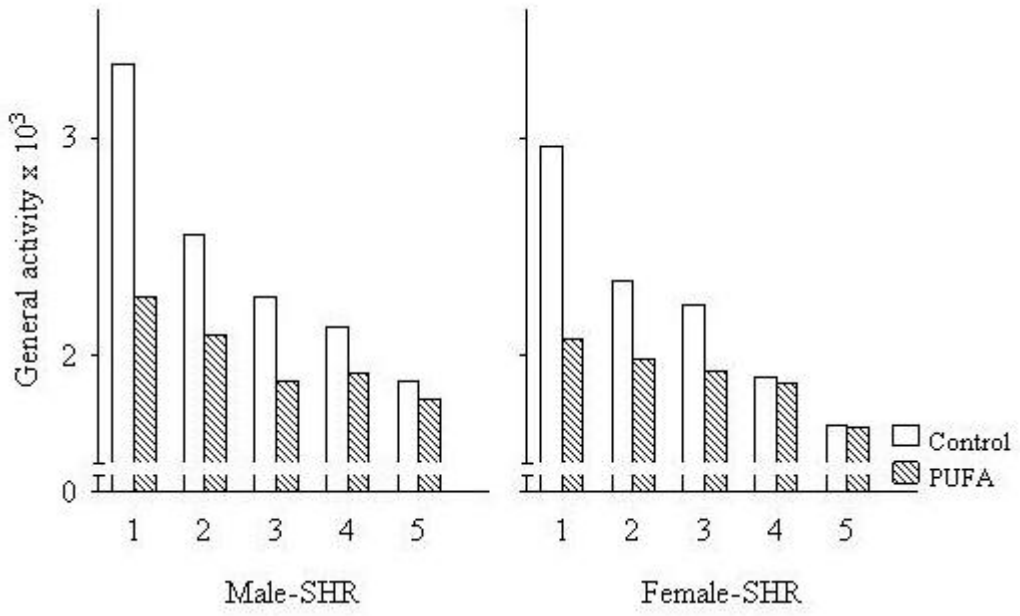

Figure 5. General activity as a function of sex, segment and feeding condition (not significant). Both genders showed reduced general activity in the initial segment.

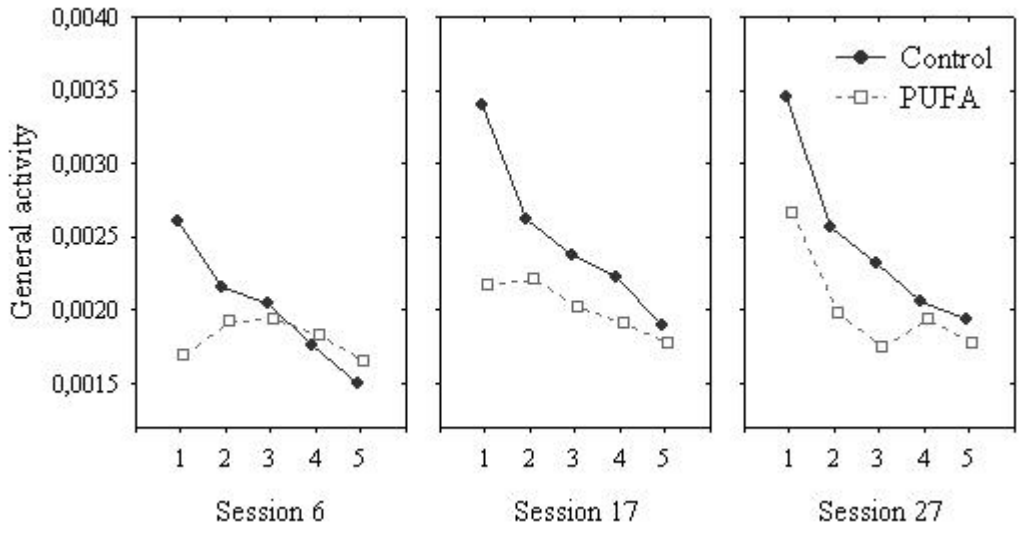

Figure 6. General activity as a function of session, segment and feeding condition.

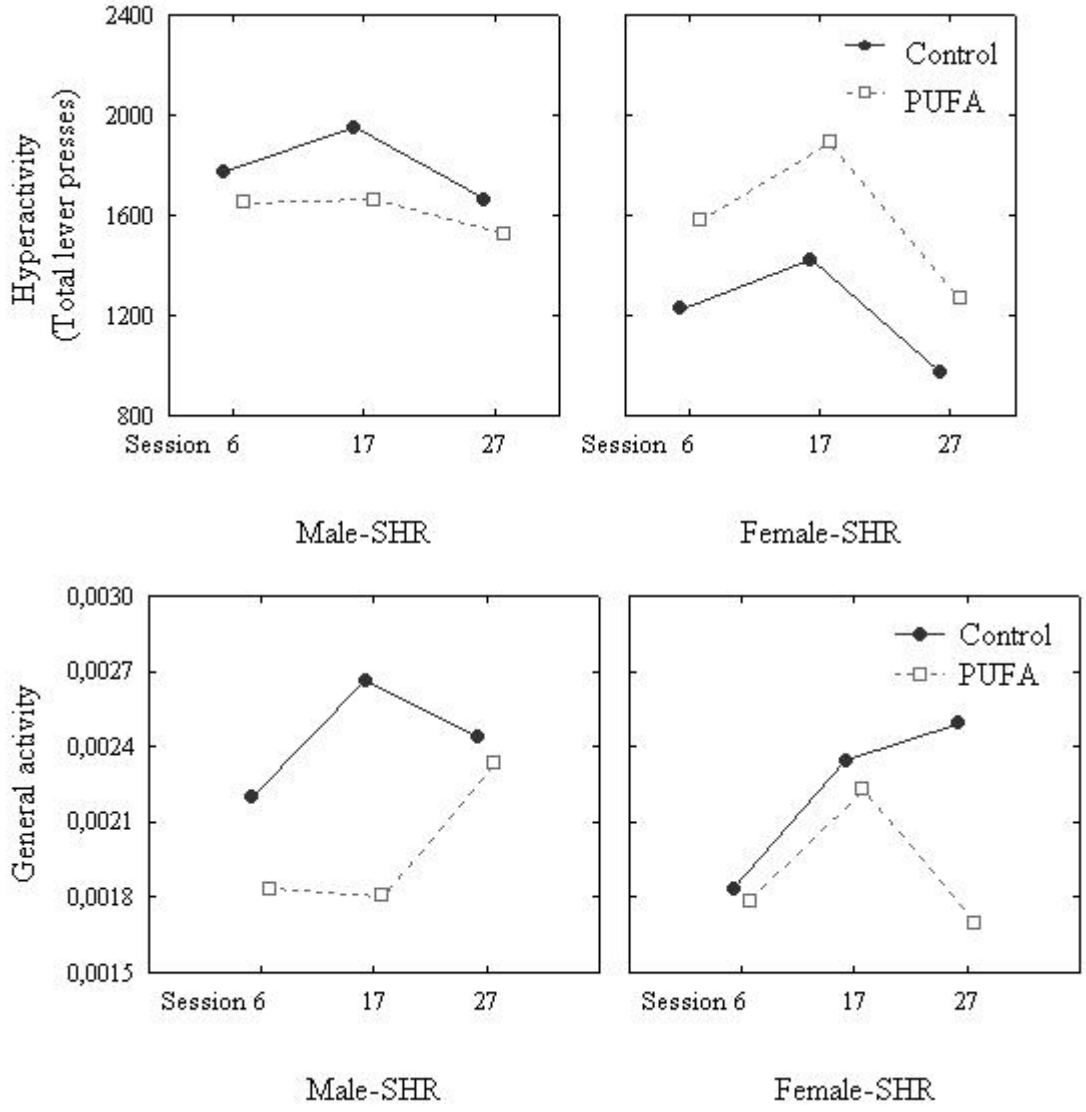

Figure 7. The operant measure of activity (upper two panels) as a function of session, sex and feeding condition (not significant) compared to the video measure of general activity (lower two panels) as a function of session, sex and feeding condition (significant).

4 Discussion

It is argued that a rich PUFA intake and/or a balanced n-6/n-3 ratio can affect the development and/or expression of mental disorders such as ADHD (Schuchardt et al., 2010; Simopoulos, 2002). By using the SHR as an animal model of ADHD, it was possible to provide a controlled experimental setting and explore whether a long-chain n-3 PUFA-enriched chow with a balanced n-6/n-3 ratio had any effects on operant measures of attention, hyperactivity and impulsiveness relative to controls receiving standard lab chow. Effects of PUFA-treatment were also explored by analyzing video-recorded general activity during the operant task.

Overall, PUFA-treatment tended to interact with sex, reducing activity, impulsiveness and improving attention in male-SHRs, while PUFA-treated female-SHRs showed no effect or an increased activity, impulsiveness and reduced attention on measures of reinforcement-controlled behavior (see Figure 1 – 4). Video-recorded general activity showed that PUFA-treatment reduced general activity in the initial segment of sessions in both PUFA-treated males and females (see Figure 5) and that PUFA-treated SHRs showed a linear behavioral pattern across segments in the early and middle session relative to controls which showed a falling steep slope (see Figure 6). Over sessions, the results also revealed a striking difference between reinforcement-controlled and video-recorded activity as a function of PUFA-treatment and sex (see Figure 7).

4.1 The gender-specific effects of PUFA-treatment on reinforcement-controlled behavior and the possible role of gender differences in LC-PUFA-metabolism

All measures of reinforcement-controlled behavior showed the same overall pattern as a function of sex and feeding condition. PUFA-treatment tended to improve male-SHRs ADHD-like reinforcement-controlled behavior while female-SHRs showed no effect or an aggravation of ADHD-like reinforcement-controlled behavior.

It has been argued that the incorporation of the long-chain n-3 DHA in the neuronal cell membranes increases with intake and has a central role in maintaining cell membrane fluidity

for optimal signal processing within the neuron (Chalon et al., 1998; Kamada et al., 1986; Mitchell & Litman, 1998; Yehuda et al., 1999). Altered cell membrane fluidity might alter the protein structure of membrane receptors which in turn might cause an effect on their function and activity (Yehuda et al., 1999). It is therefore hypothesized that LC-PUFAs function as mediators of signal systems such as the dopaminergic neuronal modulator system (Chalon, Vancassel, Zimmer, Guilloteau, & Durand, 2001; Levant, Radel, & Carlson, 2004). The dopaminergic system is believed to be altered and hypo-functioning in children with ADHD (Sagvolden et al., 2005).

The process of synthesizing the LC-PUFAs n-6 AA and the LC-PUFAs n-3 EPA and DHA is extremely slow and ineffective in most mammals and therefore most of these LC-PUFAs need to be obtained from food (Pawlosky et al., 2001). However, the efficiency of this process is not fixed but influenced by several factors such as sex hormones, n-6/n-3 ratio intake, genes, stress hormone levels, saturated fatty acids intake, vitamins and mineral cofactors (Childs, Romeu-Nadal, Burdge, & Calder, 2008; Schuchardt et al., 2010). The SHRs in this study were genetically homogenous, exposed to the same environment and did not differ except for the manipulation of feeding regime. Thus, although this is only speculation, one possible mechanism explaining the gender-specific effects of PUFA-treatment on reinforcement-controlled behavior is gender differences in the metabolism of PUFAs.

In vivo studies examining this metabolism process have shown that young men convert ALA into EPA and DHA much less efficient than young women (Burdge, Jones, & Wootton, 2002; Burdge & Wootton, 2002). Also, results from experiments on rats have shown that male rodents were especially at risk for developing PUFA-deficiency (Huang & Horrobin, 1987). These effects are believed to be caused by testosterone which inhibits the synthesis of LC-PUFAs (Marra & Dealaniz, 1989). Hence, lower metabolism efficiency in male-SHRs relative to female-SHRs might explain why male-SHRs benefitted from the treatment of this study. However, this mechanism does not explain why female-SHRs increased their ADHD-like behavior on several of the operant measures.

One study examining the effect of either a high n-3 or n-6 enriched chow on maternal behavior in mice, found that the n-3 treated dams behaved abnormally by cannibalizing their pups, not building a nest and appearing more stressed than the n-6 treated mice (Fountain et al., 2008). The authors speculated whether the dams could have been exposed to an over-supplementation of n-3 and that this might have affected their nervous system adversely.

Although this might be the case, so far there exists no systematic theorizing or research examining adverse effects of over-supplementation of n-3. Hence, the increased ADHD-like behavior in PUFA-treated female-SHRs on the measures of reinforcement-controlled behavior is left unexplained and needs to be addressed in future studies.

4.2 Effects of PUFA-treatment on video-recorded general activity

4.2.1 PUFA-treatment reduced exploratory activity in both genders

The reduced general activity in PUFA-treated SHRs in the initial segment of each session gives us a more detailed impression of behavior than the mere sum of activity across sessions. By looking at the activity level on Figure 5 and 6, one can observe that it is at its steepest from segment 1 to segment 2, and then it is gradually falling during the session. The falling activity in the initial segments could possibly be related to the reaction of the organism by changing context from being in its cage, to that of being tested in the chamber. This situation can be interpreted as a relatively novel situation when considering the fact that the SHRs only spent about 90 minutes a day in the testing chamber and the rest of the day in their cage. The results might therefore reflect reduced exploratory activity in PUFA-treated SHRs relative to control-SHRs when introduced to a novel environment.

Exploratory activity has been proposed to reflect the emotional reactivity in the animal to a novel environment (Archer, 1973). This notion of the phenomenon has been supported by studies showing that rats administered anxiolytic drugs increase exploratory activity (Pellow & File, 1986). However, alteration in the dopamine system also affects exploratory activity. Lesions in the rodent mesolimbic and mesocortical dopaminergic systems reduced exploratory activity while administration of dopamine agonists increased it (Fink & Smith, 1980). Moreover, increased glutamatergic signal transmission seems to decrease exploratory activity in rats (Granger et al., 1993). A difference in exploratory activity due to PUFA-treatment may therefore possibly be explained by alteration of several neurotransmitter systems, and a single system is not identified (Enslen, Milon, & Malnoe, 1991).

One recent study examined the relationship between brain levels of n-3 PUFAs and ADHD-like behavior in Sprague-Dawley rats (Vancassel et al., 2007). The rats received standard lab-

chow and it was hypothesized that inter-individual differences in attention, impulsiveness and motor activity would correlate with levels of n-3 PUFAs in the frontal cortex. They found no relationship between n-3 PUFAs and measures of impulsiveness and attention. Interestingly, their main finding was that the levels of the long-chain n-3 DHA were negatively correlated with the locomotory reactivity in a novel situation. Hence, individuals categorized as having a low-activity were likely to have higher levels of n-3 DHA than individuals categorized as having a high-activity. The authors hypothesized that future studies might find that PUFA-supply increases the level of n-3 DHA and reduces the locomotory reactivity in a novel situation. The findings in the present study support this hypothesis and in addition, the presented results also show that PUFA-supply interacting with sex influences other reinforcement-controlled ADHD-like behaviors.

In sum, PUFA-treatment induced two distinct sets of behavioral changes in the animals. One effect showed reduced exploratory activity in both genders. A second gender-specific effect showed reduced reinforcement-controlled activity, impulsiveness and improved attention in male-SHRs, while having none or an opposite effect in female-SHRs. PUFA-treatment might therefore have altered certain neuromodulator systems in both genders central to exploratory activity while exerting gender-specific effects in certain other neuromodulator systems central to reinforcement-controlled behavior.

4.2.2 Video-recorded general activity – a promising tool in exploring the face validity in animal models of ADHD

The reinforcement-controlled measure of activity showed a beneficial effect on male-SHRs and an adverse effect on female-SHRs as a function of PUFA-treatment (see Figure 3), but no difference over sessions (see Figure 7). By comparing these results to that of video-recorded general activity, we observe a strikingly different pattern over sessions as a function of sex and PUFA-treatment (see Figure 7).

General activity is a measure of both reinforced and non-reinforced behavior and it probably captures a broader aspect of activity than behavior linked to lever pressing alone which is motivated by reinforcement. The different results obtained from these two different operalizations show that the measure of general activity has captured something qualitatively different or non-related to the measure of lever pressing activity. Exclusive focus on

reinforcement-controlled activity might therefore miss the observation of beneficial treatment effects in other domains of activity.

The SHR-model should mimic hyperactive behavior in children with ADHD in order to have face validity (Sagvolden et al., 2005). The diagnostic criteria of hyperactivity in ADHD includes descriptions such as "often fidgets with hands or feet or squirms in seat" or "is often on the go or often acts as driven by a motor" (American Psychiatric Association, 2000). None of these criteria represents specific behavior related to the impatience of gaining certain reinforcers, but is rather related to aspects of showing a high level of restlessness and many gross body movements (Taylor, 1998). Hence, the measure of general activity might be a good supplement to reinforcement-controlled activity in gaining a better impression of overall restlessness and body movement in the SHR-model.

The face validity of hyperactivity in the SHR-model is restricted to comparing reinforcement-controlled measures of activity of children with ADHD with the reinforcement-controlled measures of activity in different rat-strains (Sagvolden, 2000). The results of PUFA-treatment obtained by the video-recorded measure of activity reveal that other measures of activity are needed in order to capture other aspects of activity. The video-recorded measure of general activity is a new promising tool and can possibly lead to an increased understanding of similarities and differences in the concept of hyperactivity between animal models of ADHD and children with ADHD.

4.3 Limitations and suggestions for future research

4.3.1 Limitations with this study

This study used a semi-synthetic PUFA-treatment high on the long-chain n-3s DHA and EPA (Rokling-Andersen et al., 2009) and had a theoretical balanced n-6/n-3 ratio (Simopoulos, 2006), while standard lab chow served as a control variable. Ideally, to maximize the internal validity, one would prefer to use two similar chows that would only differ in PUFA-composition. This would have strengthened the inference that it was the long-chain n-3 PUFA-supply and not some other dietary difference that caused the effects on the dependent variables. One way of dealing with this problem would have been to analyze the lipid composition of the subjects' brains and relate that to their behavior (Vancassel et al., 2007).

This would have gained insight into the differences of lipid composition in the subject's brain tissue and it would have confirmed whether there was a significant difference between the different experimental groups and the two genders. For economical reasons this was not possible to do in the present study.

Furthermore, the exploratory aim of this study did not permit a large scale design using many subjects and rat strains. Normally, results from SHRs are compared to results from the Wistar Kyoto rat obtained from Harlan - UK (WKY/NHsd), which is the proper control for the SHR-model (Sagvolden et al., 2009). However, the results from the SHRs in this study justify a more comprehensive design examining if the effects of PUFA-treatment were specific to the SHRs or can be generalized to other rat strains such as the WKY/NHsd-controls.

Some caution is advised in the interpretation of the statistical strength of some of the results. First, one of the attention measures, total percentage correct, fell shy of significance (p = 0.06). However, the overall trend across all reinforcement-controlled measures was the same suggesting a real effect. Second, the Unequal HSD post-hoc analyses of reinforcement-controlled results showed no statistical significant differences between each of the genders when comparing them to their own respective experimental condition. This means that the PUFA-treatment x sex interaction effects were dependent on the results from both feeding conditions of both genders in order to be statistical significant.

A final limitation was that the testing of the experimental conditions took place at two different times. All offspring from PUFA-receiving dams constituted the experimental group. The control group consisted of SHRs selected from a pool of normal control subjects used in another study (Johansen et al., 2011). When testing and randomization are not done at the same time, the design is more vulnerable to unknown confounding variables. To our knowledge this was not the case and both groups were exposed to the same environment except from the different feeding conditions.

4.3.2 Suggestions for future research

Future research should use similar feeding conditions only manipulating the PUFA-composition. This will strengthen the internal validity of the design. After operant testing, lipid composition in brain tissue should be examined in order to physically observe the

experimental manipulation[1]. This will further open for the possibility of correlating brain PUFA-composition with behavior. Moreover, to strengthen the design one should assign subjects to the experimental conditions by randomization and test both the experimental and control group simultaneously in time. Also, a more comprehensive design using a higher number of subjects and both WKY/NHsd-controls and SHRs is needed in order to see if the observed results from this study were specific to that of the SHRs or can be generalized to WKYs.

[1] It should be noted that analyses of neurotransmitter levels in the SHRs participating in this study were conducted by a group led by S. Ivar Walaas (Department of Biochemistry, University of Oslo). The results from these analyses will be published elsewhere and a presentation of these results was considered beyond the scope of this thesis.

5 Conclusion and implications

Long-chain n-3 PUFA-treatment administered to both SHR dams and offspring induced two distinct sets of behavioral changes in the offspring. One gender-specific effect showed reduced reinforcement-controlled activity, impulsiveness and improved attention in male-SHRs while having none or an opposite effect in female-SHRs. The second effect showed a reduced exploratory activity in both genders. Hence, the video-recorded measure of activity and the operant measure of activity captured different aspects of activity.

Gender differences in LC-PUFA metabolism may account for the gender-specific results of reinforcement-controlled behavior. Several studies have reported that males are particularly vulnerable to low LC-PUFA-intake and may benefit from such a treatment. However, this explanation does not account for the adverse results in female-SHRs. When considering the other distinct effect reducing exploratory activity in both genders, the limitation of the metabolism explanation becomes apparent. More likely, LC-PUFA-supply induced two distinct changes in the SHR-brains. First, neural systems central to exploratory activity might have been altered equally in both genders. Secondly, gender-specific alterations in neural systems central to reinforcement-controlled behavior might have occurred.

The discrepant finding between the reinforcement-controlled measure of activity and video-recorded measure of activity across sessions shows that the two measures capture different aspects of activity. The video-recorded measure is probably closer to some of the diagnostic criteria of hyperactivity in ADHD by measuring general activity and not just reinforcement-related behavior. The measure can therefore prove fruitful in the examination of face validity of different rat-strains and possibly in detecting beneficial effects of treatments in other domains of activity than reinforcement-controlled activity.

The results from this study raise the need to further explore the effects of PUFA-treatment in animal models of ADHD and several improvements of this study are advised for future research. Gained insight into the relationship between PUFAs and ADHD-like behavior might illuminate the mechanisms and role of PUFAs in the nervous system as well as in the development of ADHD.

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