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The effects of dietary polyunsaturated fatty acids and the *anx* +/- mouse in the activity-based *anorexia* mouse model

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Abstract

Decreased levels of ω-3 long chain polyunsaturated fatty acids (LCPUFA) in blood have recently been linked to *anorexia nervosa* (AN) and behavioral traits related to AN. Hence the main objective in this work was to investigate the role of dietary polyunsaturated fatty acids (PUFA), especially ω-3, on AN-related behaviors in mice. The mice were subjected to a diet restriction and an induced hyperactivity in the activity-based *anorexia* (ABA) model, a rodent model of AN.

It is known that the homozygote *anx* -/- mouse develop spontaneous *anorexia* which leads to death in the homozygotes after 3-5 weeks. Therefore, it was also investigated if the otherwise normal heterozygote *anx* +/- mouse carrying one allele with the mutated *anx* locus is more vulnerable in the ABA model compared to the wild type mouse.

Two experiments were conducted both using the ABA model. The measures done were on activity, body weight (BW), food intake (FI) and body composition. In the first experiment where different diets were studied, 32 female C57BL/J6 mice were divided into three groups of 10-11 animals of which each had a ω-3 enriched diet, a ω-3 deficient diet or a control diet. When the mice were ten weeks old they were subjected to the ABA model, where they got a fixed amount of food scheduled 11.30 a.m. each day for eight days in total. In the second experiment, 29 three months old female B6C3, 18 *anx* +/- and 11 *anx* +/+ were subjected to the ABA model. The food was given a restricted time, 60-90 min each day from 12.00 a.m., for five days. After refeeding they had another four days of food restriction, but now with a fixed amount of food.

Neither supplementation of ω-3 enriched food nor ω-3 deficient food altered body weight or activity in animals exposed to the ABA model. Given that the rodent model used mainly mimics the physiological traits of AN, it is possible there are dietary effects on emotional and/or cognitive functions. In the *anx* +/- mice, a significantly higher food anticipation activity (FAA) was measured during food restriction. Though, there was no difference in food intake (FI) or body weight (BW) for the *anx* +/- mice.

These findings indicate that the heterozygote *anx* +/- mice are more vulnerable to the effects of food restriction in the ABA model compared to wild type mice. These results enable a future use of the heterozygote mice for studying the *anx* locus in relation to *anorexia*.

Keywords: *Anorexia nervosa*, PUFA, ω-3, inflammation, activity-based *anorexia*, food restriction, eating disorder, *anx* +/-, mitochondrial dysfunction.

Contents

List of Abbreviations

AA	Arachidonic acid
ABA	Activity based *anorexia*
ADHD	Attention deficit/hyperactivity disorder
AgRP	Agouti-related protein
ALA	α-Linolenic acid
ALS	Amyotrofisk lateral skleros
AN	*Anorexia nervosa*
ARC	Arcuate nucleus
ASD	Autism spectrum disorders
BN	*Bulimia nervosa*
BW	Body weight
CART	Cocaine- and amphetamine-regulated transcript
COND	Childhood onset neuropsychiatric disorder
COX	Cyclooxygenase
DEXA	Dual-energy X-ray absorptiometry
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DSM-IV	Diagnostic and Statistical Manual IV
ED	Eating disorder
EFA	Essential fatty acid
EFAD	Essential fatty acid deficiency
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAA	Food anticipation activity
FI	Food intake
HPA	Hypothalamic-pituitary-adrenal
KI	Karolinska institutet
LA	Linolenic acid
LCPUFA	Long-chain poly unsaturated fatty acid
LOX	Lipoxygenase
MUFA	Monounsaturated fatty acid
NAc	Nucleus accumbens
NF-κB	Nuclear factor kappa B
NPY	Neuropeptide Y
OXPHOS	Oxidative phosphorylation system
POMC	Pro-opiomelanocortin
PPAR	Peroxisome proliferator-activated receptor
PUFA	Poly unsaturated fatty acid
ROS	Reactive oxygen species
RW	Running wheel
RWA	Running wheel activity
SEM	Standard error of the mean
SSPS	Statistical Package for the Social Sciences
VTA	Ventral tegmental area
WT	Wild type

1. Introduction

This chapter will give a brief description of the importance of the *anorexia nervosa* disease and how the *anx* mouse can be used to study the same disease. From there will the chapter continue to the aim of this work and then end with the limitations made in the study.

1.1 Background and purpose

Eating disorders (ED) are diverse and can be classified in, for example, the form of *anorexia nervosa* (AN), *bulimia nervosa* (BN) or other EDs not otherwise specified. These disorders constitute one of the most common mental health problems afflicting adolescent girls. ED leads to significant impairment of physical and psychological health and an increased risk of premature death, especially in the case of AN [1]. The lifetime prevalence of EDs among Western women is around 10% (~2% for AN) [2], with the great majority of anorectics between 13 and 25 years of age. Treatment success is difficult to achieve and only around 75% of the cases reach full recovery within 10-15 years [3]. The costs for the individual, the family as well as for society are substantial [4]. EDs thus ruins the developmentally important youth years (both in aspects of physiological and cognitive terms) of many young women and constitutes an economic burden on society.

Increasing evidence suggests that supplementation of the ω-3 long chain polyunsaturated fatty acids (LCPUFA) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are of importance for the appetite and body weight regulation during starvation/cachexia [5, 6]. Polyunsaturated fatty acids are also important in other psychiatric conditions that display overlapping behavioral traits with AN such as anxiety and depression [7, 8]. Furthermore, ω-3 PUFA levels are decreased in AN patients and have recently also been shown to correlate negatively with depression scores in AN [9]. It can hence be hypothesized that a deficiency, possibly due to malnutrition, could be involved in the development or the progression of AN and that supplementation with n-3 fatty acids may benefit AN patients.

There has recently been an increasing interest in mitochondrial function and it has been related to numerous disorders such as cancer, cachexia, Parkinson, ALS, Alzheimer's and AN [10, 11]. A mitochondrial dysfunction in mice, caused by a mutation in the anx gene, is believed to lead to neurodegeneration in the brain and especially in the hypothalamus, which is the most important area handling food intake and weight regulation. The mitochondrial function is important for every cell and it has been seen to be especially important for the skeletal muscle and the heart. Though, for the *anx* -/- mice it is more likely the mitochondrial defect in the hypothalamus that causes the anorectic behavior and early death than a decreased motoric function due to muscle weakness and a weaker heart.

A defect in the oxidative phosphorylation system (OXPHOS) is a typical mitochondrial dysfunction that lessens the ATP production [12]. A genetic mutation/variation in *anx* locus, which has been narrowed down to base pairs, gives rise to a very strong anorexic phenotype in the homozygote *anx* -/- mice. Recently has the *Ndufaf1* gene been identified within the *anx* locus and is thought to be implicated in the function of the OXPHOS. It has been coupled to decreased mitochondrial function in the *anx* -/- strain of mice. The AN like phenotypes of the *anx* -/- mouse are poor apatite, low body weight and increased activity [13].

Not much is known about the heterozygote *anx* +/- mouse. Under normal circumstances the heterozygote does not show any difference in phenotype to the wild type. This lead to the hypothesis that triggering an anorectic behavior in the rodent ABA model will help to see differences between the wild type and the heterozygote mouse.

1.2 Specific aims

- Investigate the effect of dietary PUFAs on AN like behaviors in mice that are exposed to the ABA model.
- Explore the importance of the *anx* locus by subjecting the heterozygote *anx* +/- mouse to food restriction in the ABA model.

1.3 Delimitations

The project is focused on the effects of different dietary alteration of PUFA on AN-like behaviors in rodents. Furthermore, PUFAs could potentially be of importance for some other of the different EDs presented in the introduction and it may be possible to draw relevant conclusions from the current experiments with regard to these other EDs. However, the primary focus of this work was to study behaviors/features of relevance for AN.

Concerning the *anx* +/- mice, there was only one experiment conducted with these animals, testing for difference in the heterozygote and the wild type mice mainly by comparing body weight, food intake (FI) and running wheel activity (RWA). A future study outside of the scope of this work will investigate the possible protective effects of PUFAs in the *anx* mouse model.

2. Theory

This chapter will discuss the theoretical background behind the experiments conducted. At first is the *anorexia* disease described and then the theory behind hunger and body regulation is explained before a general description how the ABA model works. At last the diets/mice that were tested in ABA model are discussed.

2.1 *Anorexia nervosa* in a historical perspective

The etiology of *anorexia nervosa* (AN) is still largely unknown, but there seems to be a mix of biological, psychological and cultural factors involved. It is obvious that there are crucial physiological changes in AN, but the question is if these changes are primary causes or just secondary causes resulting from the psychological and cultural factors [14].

The older theoretical models for the development of AN are vague and basically stating that the etiology of AN is multifactorial and includes sociocultural, psychological/familial and biological/genetic factors. During the second half of the 20th century, research on AN has shifted from a sociocultural/psychosocial perspective to including more biological and genetic factors. Nevertheless, the processes behind the development of AN are still largely unknown [14].

Although twin studies show that AN has a high genetic component [15], there are many things that disqualify the onset of the disease to pure genetic factors. When looking back at the hist ory of the disease the first reported cases were Catholic ascetics and they had a similar disorder related to AN called *anorexia mirabilis*. The diseased from this disorder were seen as saints and were worshipped for their ability to survive on almost no food at all. These anorectics had a bit different onset of the disease compared to the anorectics of today [16].

The “modern” criteria describing *anorexia nervosa* were first defined in the late 19th century by William Gull, but it was not until the beginning of the 20th century that the disease started to flourish with a fashion trend of a slimmer body ideal like the flappers (a slender and liberated woman at this time) had in the 1920s [16].

Furthermore, scientific reports from this time started showing that people with normal weight had a longer life expectancy than overweight. Hence to be lean was associated to a healthy lifestyle and people started to count calories [14]. This combined fashion and healthy trend has since then varied a bit over the century and today the ideal body shape is slimmer than ever before and it has been suggested to be due to how the media has changed the public view of an ideal body [17]. There has been an overall increase in the number of diagnosed AN patients in the recent century in the western countries but given the changes in the diagnostic criteria there are some problems comparing the prevalence over time. However, since the 70s the prevalence of AN has been quite stable [18].

A common view is to see AN progress in different phases. The first phase is that what triggers the disease and “recruit” the anorectic to starvation. The second phase is when the patient gets stuck in the disease like an addiction. The first phase has varied depending on the environment in the society, while the second phase always seems to proceed the same way. In that view, it is reasonable to connect the first phase mainly with environmental factors and the second phase with physiological factors [14].

2.1.1 The definition and characteristics of *anorexia nervosa*

The fourth edition of the American Psychiatric Association's Diagnostic and Statistical Manual (DSM-IV) describes the criteria relevant for diagnosing AN. If all except one criteria are satisfied, the patient is diagnosed for AN. For male patients only the first three criteria are applied [19].

- Consistent weight under 85% of normal body weight
- Intense fear of gaining weight or become fat
- A distorted body image
- Amenorrhea

Two subtypes of AN have been identified. One is the restricting type, in which severe weight is lost by reducing the food intake and very often increasing the activity level substantially. The other type is the binge-eating-purging type that uses laxatives, diuretics or enemas and self-induced vomiting to keep down the body weight [20].

Commonalities for AN patients is that the disease mostly starts in adolescent girls and only 10% of those with AN are male. The anorectics often have comorbid symptoms/disorders such as concentration problems, cognitive decline, depression, compulsive disorders, muscle weakness, lowered metabolism, lower pulse, lower blood pressure and osteoporosis. There are also some typical personal traits associated with the vulnerability for *anorexia*. Typical traits described are pedantic behavior, high motivation and social phobia [19]. Hyperactivity is also found in many cases of AN patients which makes the disorder even more severe and hard to recover from.

Today there is not really any successful treatment for AN. About 30% of the recovered patients relapse to the disease within a year [21], only 50% get fully recovered over a longer period of time [22]. Many different drugs have been used to cure AN pathology like anti-depressants and hunger inducer, but none of these has been very effective. The most promising drug is Olanzapine, which has been successful in helping anorectic patients gain weight and also have positive effects on psychological symptoms associated with AN like reducing depression and obsessive behavior. It is an atypical antipsychotic drug and works as an antagonist for serotonin and dopamine receptors. [23].

2.2 Homeostatic and hedonic regulation in *anorexia nervosa*

How food intake and body weight is regulated is a very complex issue and has here been divided into the systems of homeostatic and hedonic regulation. Despite the fact that these two often are separated theoretically, they are indeed highly integrated.

2.2.1 The central homeostatic regulation

To keep the body weight normal, there are many regulating mechanisms. The hypothalamus has been found to be the main control center of the brain for energy homeostasis. In the arcuate nucleus (ARC, a part of the hypothalamus) there are two subsets of neurons that are especially important for the homeostasis. One of them synthesizes the anorectic peptide pro-opiomelanocortin (POMC) [24] and the cocaine- and amphetamine-regulated transcript (CART) [25]. The other subset of neurons synthesizes the orexigenic agouti-related peptide (AgRP) [26] and neuropeptide Y (NPY) [27].

2.2.2 The peripheral homeostatic regulation

Then there are some hormones that regulate food intake and hunger signals. One of them is insulin that the AN patients have low levels of. The major function of insulin is to induce the uptake of glucose from the blood to peripheral target tissue after a meal. In short-term it acts centrally to reduce body weight and food intake, but in long-term it increases hunger and body fat storage [28].

Another important peripheral hormone is ghrelin. It is produced in the gut and is thought to be involved in meal initiation. Ghrelin levels increase pre-prandial (that is before a meal) and is negatively correlated to body weight. In agreement, AN patients have high plasma levels of ghrelin. However, these high levels of ghrelin does not seem to be able to promote a necessary increase in food intake in AN patients and hence there are some theories that state that AN patients have got ghrelin resistant [29].

Among other peripheral hormones are the two adiopokines, leptin and adioponectin, which are produced by white adipocytes. While the amount of leptin is proportional to the amount of body fat percentage, the amount of adioponectin is inversely correlated to the amount of body fat percentage [30]. Consequently, in AN leptin levels are low and adioponectin levels are high [31, 32]. In rodents, leptin has been found to be anorectic while it stimulates the ARC by up-regulating POMC/CART and down-regulating NPY/AgRP [33, 34]. The role of adioponectin is still a bit unclear. It seems to have a close relation to insulin and modulates insulin sensitivity in peripheral target tissues [35].

2.2.3 The hedonic regulation

While the energy homeostasis induces eating when people are energy deficient and makes them eat less when they have a surplus of energy stored, the hedonic system concerns eating for pleasure or reward, which can continue although they are satisfied/full. The hedonic system is mostly concentrated to the mesolimbic system (a dopamine pathway) in the brain. This part of the brain has been suggested to be implicated to food reward and food addiction as well as other addictions (such as chemical drugs or sex) [36].

The mesolimbic system have dopaminergic projections, one of those goes from the ventral tegmental area (VTA) to lateral hypothalamus via nucleus accumbens (NAc) [37]. The ventral tegmental area is in the central part of the brain and consists of many neurons. It is the origin of the mesolimbic system and the center of dopamine signaling.

The main neurotransmitter in the reward pathway is dopamine. The hedonic regulation is highly associated with dopamine signaling. The common theory is that palatable and pleasurable food raise dopamine levels and that obese are addicted to high dopamine levels and therefore also eat more rewarding food beyond the food that makes them satisfied. This creates an imbalance in their long term energy expenditure and intake [36]. In contrast, AN subjects are thought to be dopamine deficient. This is part of what is called the hypo-dopamine theory which is something that is used to explain several reward related diseases. Low dopamine lead to anhedonia which people then try to reverse by using drugs, eating palatable food or getting kicks from other things. This leads to addiction. In AN it can be the hyperactivity or the obsessive-compulsiveness giving these kicks [37].

In both obesity and AN, the dopamine signal seems to be blunted and this could reflect an imbalance in the dopamine receptor expression/signaling [38, 39]. The obvious difference is that the imbalance in the dopamine signal leads to higher intake in obese and less intake in AN patients.

2.3 Animal models and *anorexia nervosa*

It is hard to create an animal model for a psychiatric disorder. A model does not have to cover all the symptoms of a disorder but can still be useful in looking at a specific aspect of a disorder (often called an endophenotype) [40]. The characteristics of human *anorexia nervosa* that typically are studied (and possible to study) in the available animal models are increased sensitivity in the female sex, decreased food intake, body weight, temperature and increased activity levels as well as similar neuroendocrine adaptations [41]. A model could also be valid if the animals show the same effects in response to treatments as do humans.

For AN different models have been suggested [40]. One proposed model is the stress-induced appetite loss model that causes the animal to eat less. However, stress mainly affects the appetite, which is not considered to be a major problem in AN. Then there are also separation models, in which the animals are separated from each other while they are still aware of each other by sight and smell. The food is then eaten together. The model causes depression and reduced food intake and body weight loss [42].

Another model is food restriction where the animals are starved. This model makes the animals eat less and loose in weight, but reduction in food intake is not voluntary as can be seen in AN patients. However, in the activity-based *anorexia* (ABA) model a kind of voluntary intake is seen. The ABA model has been suggested to be the best animal model for AN [42]. This model combines food restriction with hyperactivity, which in turn makes the animals show symptoms resembling of AN, such as lower food intake, loss of body weight, hyperactivity and hypothermia.

2.3.1 The activity-based *anorexia* model

The rodents in the model are housed separately with access to a running wheel (RW) and water all the time. Food is restricted for 2-4 hours during daytime when they are normally inactive which will make them loose weight rapidly. If the experiments are not stopped after a couple of days, the rodents will die of starvation. If the animals have lost 30% of their body weight, it is difficult for them to recover and usually the ethical stop is when they have reached around 75% of their original weight. Animals that have food *ad libitum* (access to food all the time) and access to a RW, or have no access to a RW but are food restricted will keep their weight and survive. Consequently, it is the combination of food restriction and RW in the ABA model that makes it suitable to study effects of AN [40].

The mice are habituated to the new environment for three days before put on a food restriction protocol. During the restriction period, the mice can be manipulated in various ways and the effect is measured by changes in food intake, body weight and running wheel activity. The temperature in which the animals are housed also determines how the ABA progresses. The temperature at which the rodents are housed is important in the ABA model due to the fact that mice easier maintain their body weight at temperatures a bit higher than room temperature than at lower temperatures, which improves survival [40].

2.3.1.1 The mechanisms in the activity-based *anorexia* model

The ABA model usually results in body weight loss, increased activity and reduced food intake (see figure 1). How this is connected is not yet well established. Animals given access to running wheels will increase their general activity levels compared to sedentary animals. This means expending more energy and hence increase food intake to counterbalance the increased energy expenditure, given that food is present in excess.

However, in the ABA model, food is restricted and given in a specific scheduled manner which leads to a further increase in activity compared to animals with access to running wheels and food *ad libitum*. In a normal system of body homeostasis this increased activity would be followed by an increased food intake to compensate for the extra energy lost. Paradoxically food intake in the ABA model is even more reduced compared to sedentary animals put on a similar food restricted schedule. The initial body weight loss due to food restriction causes an increased activity which in turn increases the energy expenditure and the corresponding food intake is not enough to compensate for this.

The decline of the food intake can be related to various factors such as fatigue, loss of hunger signals triggered by the lower weight or less time to eat because of the higher activity levels. In human AN, the reduction in FI is not likely to be a direct effect of a higher activity. It is probably due to a psychological change caused mainly by a loss in BW and that could be the reward of being able to follow a restricted diet to get slim.

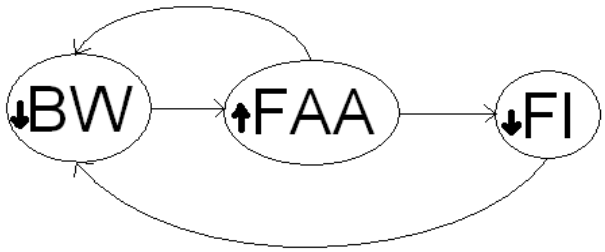

Figure 1: A hypothetical relation between body weight (BW), food anticipation activity (FAA) and food intake (FI) in the ABA model.

In the ABA model it is reasonable to believe the regulation act as in figure 1, but the body weight does not have to be influenced by either FI (figure 2) or FAA (figure 3) and then there is a simpler relation. In all three cases it is interesting to know which factor that starts the process.

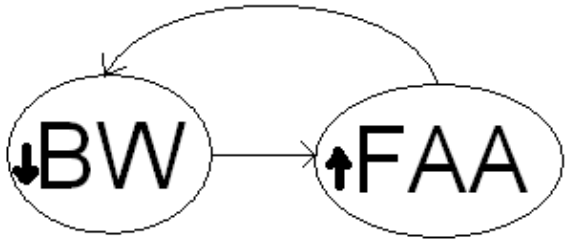

Figure 2: A hypothetical relation between body weight (BW) and food anticipation activity (FAA) in the ABA model.

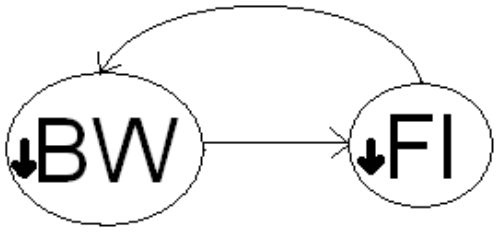

Figure 3: A hypothetical relation between body weight (BW) and food intake (FI) in the ABA model.

2.3.1.2 The food anticipation activity

During the time just before the feeding, 2-3 hours, there is an increase in locomotor activity [43]. This is the food anticipatory activity (FAA) that is crucial for the

development of the anorectic behavior in ABA and has been identified in many different mammals. If the running wheels are locked during the time just before feeding, the animals will be able to keep their weight. When the animals loose in weight their FAA paradoxically increases. The main theory that explains this is that during scarcity of food, it is important to find new food quickly for starving animals and for that they have to be more active to search and find new food [44]. Similar trends of FAA in rodents have also been found in AN patients [37].

2.3.1.3 Validation of the ABA model

The aspects that give the ABA model predictive value is that the mice with the traits of low weight, low age and female gender are more susceptible to develop anorectic behavior in ABA and can model for self-starvation, amenorrhea, hypothermia and hyperactivity [40, 45].

2.3.2 Genetic rodent models for AN

The regulation of food intake has been studied in diverse genetic models. It has been used extensively for research in obesity, like in the leptin deficient Ob/Ob mouse which has a very obese phenotype. Genetic models are not as common in AN and has probably to do with the negative evolutionary impact of those mutations compared to those mutations for obesity [41].

2.3.2.1 The *anx* -/- mouse model

There is only one spontaneous mutation that has been extensively studied for AN and that is the mutation in the *anx* -/- mouse. This recessive mutation gives rise to mice with the characteristics of poor appetite, reduced body weight, emaciated appearance, body tremors, head weaving, hyperactivity, and uncoordinated gait [41]. The *anx* -/- mouse has its mutation in a mitochondrial protein-encoded gene that induce a decreased food intake, loss in body weight and an increase in energy expenditure much in the same way as in AN. The *anx* gene is recessive and the homozygotes *anx* -/- dies within 20-30 days of life [13].

The *anx* mutation has been found to be a mutation that causes impairment of the OXPHOS complex I (CI) and in some degree also an impairment of complex III (CIII) in the hypothalamus [12]. This impairment of CI has been related to the *Ndufaf1* gene [13]. Complex I oxidizes NADH to NAD+ so that the electrons transferred with different carriers are released. This is how the electrochemical gradient is built up, which is used to drive ATP synthesis in the mitochondria. Defect in the CI function cause electrons to leak and produce reactive oxygen species (ROS). In *anx* -/-, an increase of ROS in the hypothalamus has been detected and this had a negative effect on CI functioning and which further increased the oxidative load in the hypothalamus. Still, the cause of a defective CI derives probably from the mutation in the *Ndufaf1* gene and is only reinforced by the ROS. Reactive oxygen species can cause inflammation and damage to the function of the NPY/AgRP neurons [13], which has also been reported in studies of the *anx* -/- mouse [46, 47]. However, little is known whether the heterozygote *anx* +/- mouse is affected by this mutation.

2.4 Polyunsaturated fatty acids

The amount and composition of polyunsaturated fatty acids (PUFAs) in our diet has increasingly become a health issue because more attention has been drawn to research involving PUFAs. The LCPUFAs are important in a range of different disorders or

physiological functions and are most known for reducing the incidence of cardiovascular diseases [48]. The PUFAs have also been shown to have some effect on AN related diseases such as attention-deficit/hyperactivity disorder (ADHD), depression and other mental disorders [7-9]. One possible mechanism why LCPUFAs would be healthy is that they tend to be anti-inflammatory.

2.4.1 The structure of polyunsaturated fatty acids

PUFA is the term for fatty acids with more than one double bond. There is a convenient way to write it in a short form. First is the number of carbons in the carbon chain which gives the length. Then here is a colon before the next number describing how many double bonds there are in total. Next follows "n-" before the last number that describes how many carbons there are from the methyl end of the carbon chain to the first carbon connected to any double bond. A fatty acid with 22 carbon atoms in length, with six double bonds and the last double bond at carbon number three will consequently be noted 22:6n-3. The last double bond gives also the category name like an **ω**-3 fatty acid or an **ω**-6 fatty acid [49, 50].

The location of this double bond has also implications for the properties of the fatty acids. The ω-3 and ω-6 fatty acids often have contrary inflammatory responses. While ω-3 is generally regarded as anti-inflammatory, ω-6 is regarded as pro-inflammatory. Some use the ratio of ω-6 and ω-3 (ω-6/ω-3) in the food as a marker for health. Eating food with a low value around 1 can be considered to be healthy. Though, with a modern western diet, high in ω-6 and low in ω-3, this ratio has elevated to much higher values [51].

Humans are not able to synthesize every fatty acid they need, so they also have to get some of them from the food they eat. The fatty acids that cannot be synthesized in humans are called essential fatty acids (EFAs). The two main EFAs needed are α-linolenic acid (ALA, 18:3n-3) and linolenic acid (LA, 18:2n-6). From these two FAs, many different long-chain polyunsaturated fatty acids (LCPUFA) are synthesized by different elongation and desaturation steps [52].

The two ω-3 fatty acids docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5-n3) can both be synthesized from ALA, but the conversion of ALA to EPA is not very efficient and the uptake of EPA and DHA is much better directly via the food. These two fatty acids in particular are associated with health benefits and their main source in the diet is from seafood and fatty fish [50].

2.4.2 Polyunsaturated fatty acids and its relation to *anorexia nervosa*

Ayton *et al.* conducted a clinical study with seven study objects, in which they used EPA as a treatment for AN. This study had a very positive outcome. Three of the patients recovered fully and the rest all made some improvements in the recovery process. The treatment had in general a positive effect on the mood of the patients but also beneficial effects on body weight and food consumption were found. This promising evidence supports the idea of treating AN patients with ω-3 PUFAs [53].

The phospholipids are the main components of the cell membrane and they consist of a diglycerid of two fatty acids linked with an ester bond to a polar phosphate group. The two fatty acyl chains usually consist of a saturated fatty acid and a monounsaturated fatty acid (MUFA) or PUFA [49].

By analyzing the cell membrane of AN patients, researcher found out that the AN patients phospholipids have changed to shorter length of saturated and monounsaturated fat in their carbon chain and that their LCPUFAs have decreased in amount [54-56].

2.4.3 The mechanisms how PUFA can influence inflammation and dopamine/serotonin signaling

There are various mechanisms proposed how PUFAs molecules signal to change regulation of intracellular metabolites and hormones which can influence inflammation. Some of them are more direct like change in the intracellular signaling and change in gene expression [57]. One mechanism that is believed to be the most influential in inflammation is the biosynthesis of eicosanoids that are derived from ω-3 and ω-6 fatty acids.

A diet high in ω-3 PUFAs is considered to increase the amount of ω-3 PUFAs incorporated in the phospholipids. This will be on the cost of arachidonic acid (AA, 20:4n-6) that consequently will decrease in amount in the cell membrane. When there is an inflammatory response, some EFAs (AA, EPA and dihomo-γ-linolenic acid (DGLA 20:3n6)) will be cleaved off from the phospholipids and become free fatty acids. From there on they can be oxygenated, either by lipoxygenases (LOX) or cyclooxygenase-2 (COX-2) to different eicosanoids [58].

The eicosanoids derived from AA are generally considered pro-inflammatory, while the ones derived from EPA are considered anti-inflammatory. DGLA has not a big impact as it is not frequent in the cell membrane, even though it is mostly considered anti-inflammatory. Both EPA and DHA are equally good in reducing AA in the membrane, but EPA seems to be more effective in treatment of mood disorders [8].

When the intake of ω-3 fatty acids is increased, there will initially be a linear increase of these in the cell membrane. Therefore the composition of the cell membrane will be changed and get more fluid. This has a profound effect on the cell signaling and the gene expression [50] that is important both for inflammation and dopamine and serotonin transmission [59].

The drug treatment for mood disorders is usually made up of drugs that enhance the dopaminergic and serotonergic neurotransmission. If LCPUFAs show the same positive effect as these drugs, there is some potential to use LCPUFA as a treatment for the same disorder [59].

2.4.4 Comorbid diseases of *anorexia nervosa* and its relation to PUFA

Childhood onset of neuropsychiatric disorders (CONDs) is more common among those with EDs [60]. To CONDs include autism spectrum disorders (ASD), ADHD and tic disorders. Attention-deficit/hyperactivity disorder is a disorder that shares some pathology with AN including hyperactivity, depression and anxiety. It has been found out that ADHD patients are deficient in LCPUFAs [61] and that EFA supplementation has elevated their levels of EFA, but no other important positive effect has been found [62].

There is some evidence that ω-3 LCPUFAs might be beneficial for the mood. There is also some weaker evidence that ω-3 PUFA supplementation also helps with attention deficit disorder and anxiety, which are typical comorbid traits common for AN patients [59]. These two characteristics of AN have been proposed to be caused by inflammation in the brain. Depression in AN adolescents seems to be related to their low amount of ω-3 PUFAs in their body [9]. Supplementation of ω-3 PUFAs and EPA in particular has indicated improvements in depressed people by lowering their anxiety and depression [63].

When cancer patients have lost much weight, they often reach an anorectic state called cachexia. Cachexia resemble AN in the sense of malnutrition and low weight. Nutritional supplementation with EPA has shown a positive effect in cachexia patients

[64]. A systematic review of treatments with ω-3 PUFAs in cachexia has shown that it reduces the body weight loss and improves the appetite [5].

3. Method

This chapter describes how the two different experiments with the use of the ABA model were conducted, what measures that were taken and how it was statistically analyzed.

3.1 The ABA model with different PUFA diets

The first experiment was performed on mice given diets enriched or deficient in ω-3 in the ABA model.

3.1.1 Animals

Female mice (n=36) (Harlan, Horst, The Nederlands) three weeks old C57BL/6J background weighing 14-17 gram upon arrival. They were housed in cages of six and separated after habituation to 32 cages with one running wheel (ENV-044) in each cage. The ethics was approved by the University of Gothenburg.

3.1.2 Diets

Three different isocaloric diets were used, each diet intended for 12 mice. The control diet and the PUFA enriched diet mimicked those used in the Avraham study [65] in the aspect of ω-6/ω-3 ratio. The one extra diet that Avraham did not use was the one containing safflower oil which had a blue color. This food had a high ω-6/ω-3 ratio and was essential fatty acid deficient (EFAD). Another group of twelve mice were fed on a diet with 5% fish oil added with a low ω-6/ω-3 ratio which had a red color. The last group was a control group that had a diet with yellow color which was more neutral in the ω-6/ω-3 ratio. To this diet soybean oil has been supplemented. More about the specific fatty acid content and ingredients for all diets can be found in Appendix A-D. One time when the animals were refeeded they got “normal chow”. That is a lab food especially adapted to suit rodents. The “normal chow” used here is the R3 diet from Lantmännen in Vadstena.

3.1.3 Study design

The mice were divided into three diet groups consisting of twelve animals in each group. The diets were given in *ad libitum* for sex weeks of habituation. The room kept a temperature of 20°C±1, had a humidity of 55%±5 and had a 12-hour dark-light cycle with lights on at 6 a.m. The weights of the mice were measured every Monday and the food intake for each cage was measured every Monday, Wednesday and Friday.

After the first six weeks of habituation, the 32 mice with the highest weight were taken to individual housing, ten animals from the EFAD group and eleven mice from the other two groups. They were put in cages in mixed order and in each cage there was a running wheel. Then there was one week of habituation to the new environment with food in *ad libitum* before the restriction in food intake. During the habituation for the running wheel the activity of the wheels was continuously measured. The intake was calculated by searching how much food was left in each cage and subtracting it from what was given two or three days earlier. The weight was measured from the start of habituation to individual housing until the end just before food restriction. Before the mice were put on food restriction they were scanned with Dual-Energy X-ray Apsorptiometry (DEXA) for bone mass density, bone mineral content, lean mass and fat mass. Furthermore, the length of the animals was measured.

Next, the food restriction started for a total of eight days. All mice got a fixed amount of weighed food each day at 11.30 a.m. seen in table 1 below. The amount of food given was based on the earlier experiment with the time restricted feeding, using the average intake from that experiment. The weight of the mice was also measured at the same time each day. The feeding and weighing took about half an hour. The RWA was measured all the time.

On day four, the food was given for a restricted time of 85 minutes. Afterwards the animals were fed with additional food so they got 1.5 gram food in total for the day. On day nine when the ABA experiment finished, a similar refeeding was done during 90 minutes. Although for this time, the diets were changed for all groups to a normal chow diet.

Table 1: Food given each day during the restriction.

	Weight [g]
Day 1	1
Day 2	1.2
Day 3	1.2
Day 4	1.5
Day 5	1.1
Day 6	1.1
Day 7	1.1
Day 8	1.1

3.2 The ABA model with the *anx* +/- mice

In the second experiment, the *anx* +/- and the wild type mice *anx* +/+ were investigated using the ABA model.

3.2.1 Animals

Female mice (n=29) (Karolinska Institutet, Stockholm, Sweden [originally obtained from The Jackson Laboratory, Ben Harbor, USA]) 3 months old B6C3 *anx* +/+ and *anx* +/- background. There were no homozygotes, 18 heterozygotes and 11 wild types. They were housed individually and were equipped with a running wheel (ENV-044). The ethics was approved by the University of Gothenburg.

3.2.2 Diet

The animals were fed with normal chow, see section 3.1.2 above.

3.2.2 Study design

The mice were housed individually for habituation in five days where they had access to a running wheel and normal chow in *ad libitum.* The weight of the mice was measured at the beginning, after three days and in the end of the habituation. The intake over the whole habituation was measured. The RWA was measured throughout the whole experiment. This was a combination of a genetic (the *anx* model) and an environment model (the ABA model).

After the habituation they were put on a restricted diet with a time restriction of 90 min from 12 a.m. to 13.30 a.m. After some days the restriction period was cut down to 60 min to get a tougher fasting of the animals.

When the first restriction week was finished, the animals were put on refeeding for two days, during which the mice had food in *ad libitum*. After the refeeding there was another food restriction period, now with a fixed amount of food of 0.9 gram, given for four days at 11.30 a.m. The weights of the mice were measured every day during the food restrictions and the refeeding. The animals that showed too poor performance at some point of the food restriction were removed and were considered “dead” in the study.

3.3 Statistical analysis

With the Statistical Package for the Social Sciences (SSPS) program, calculation of running wheel activity was made with the repeated measures ANOVA and one way ANOVA or Studen´s t-test for food and body weight measures.

4. Results

In this chapter the result is explained and presented in graphs. Before showing the results of the *anx* mice experiment, the experiment with different PUFA diets will be presented.

4.1 The PUFA experiments

In total there have been two experiments done with different PUFA diets. The first was done by another group using a restricted time for the mice to eat in the ABA model and the second was done the same except giving a fixed amount of food instead of a time restricted intake.

4.1.1 Different PUFA diets in ABA with time restricted feeding

From earlier experiments of another group, which only differed from this one in the use of a time restricted feeding instead of a fixed weight of the food given, there was a significant effect on the food intake between the diet groups. In spite of this, there were no difference in RWA or BW hence it created an interest to investigate whether normalizing the amount of food ingested between the different diets would result in different RWA and BW.

4.1.2 Different PUFA diets in ABA with fixed food intake

During the habituation period for the food and later for the running wheel, no difference in body weight, body weight gain, food intake or running wheel activity was noted between the diet groups (fig 4, BW prior to running wheel access and data not shown, repeated measures ANOVA).

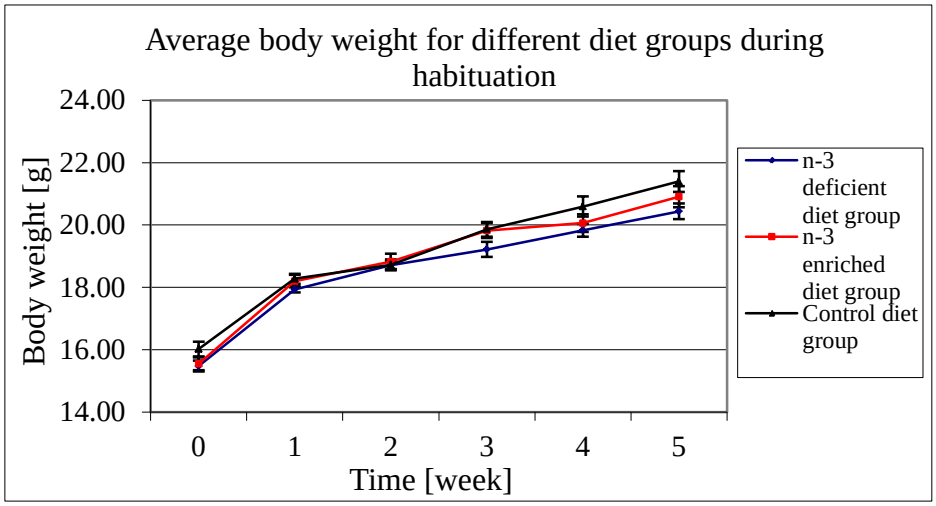

Figure 4: The graph shows the weight for the mice from the start on Monday the first week when the mice were three weeks old, until the Monday on the last of all the six weeks of habituation to the diets. The error bars are the standard error of the mean (SEM). For the n-3 deficient group n=10, for the n-3 enriched diet group n=11 and for the control group n=11.

When the habituation period was over, all mice were scanned in a DEXA scanner analyzing body composition. No differences in body composition were found between the groups (data not shown, one way ANOVA).

Food restriction decreased absolute body weight equally in all groups and there was no effects of diet on body weight loss (change in body weight) during the whole restriction period (fig 5). On day 5 there is a twist in the figure. This is because the mice were fasted harder to induce a stronger body weight loss in the hope of finding a difference in weight between the groups. Nevertheless, in the end of the restriction period the weights converge again.

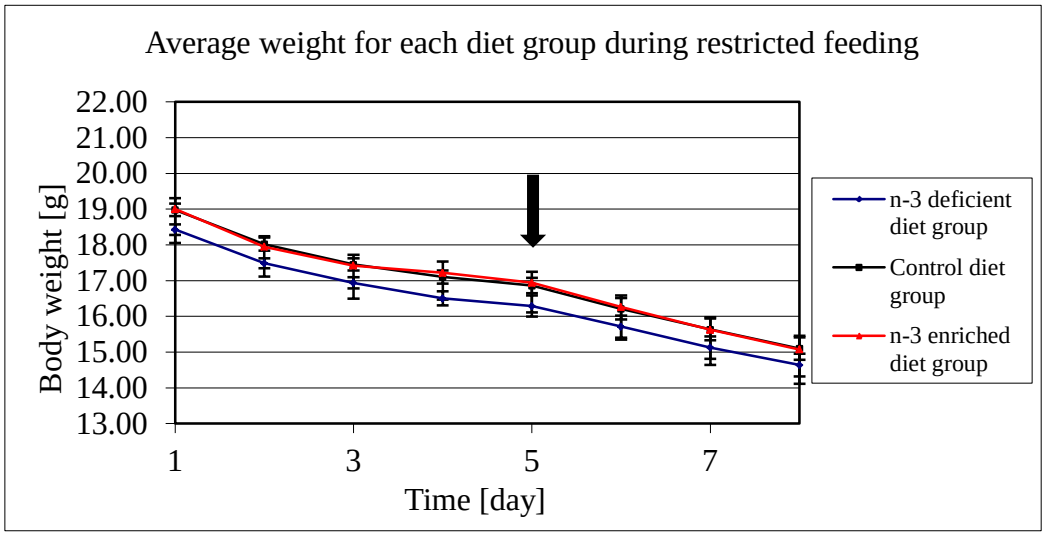

Figure 5: The graph shows how the body weight for the mice decreases relatively to their initial body weight. At the fifth day, there was a twist in the change of body weight and that is pointed out with the black arrow. The error bars are the standard error of the mean (SEM). For the n-3 deficient group n=10, for the n-3 enriched diet group n=11 and for the control group n=11.

On the fourth day the intake over a specific time period of 85 minutes was measured and a significant difference in intake was found between the control diet group the and n-3 enriched diet group (see figure 6 below).

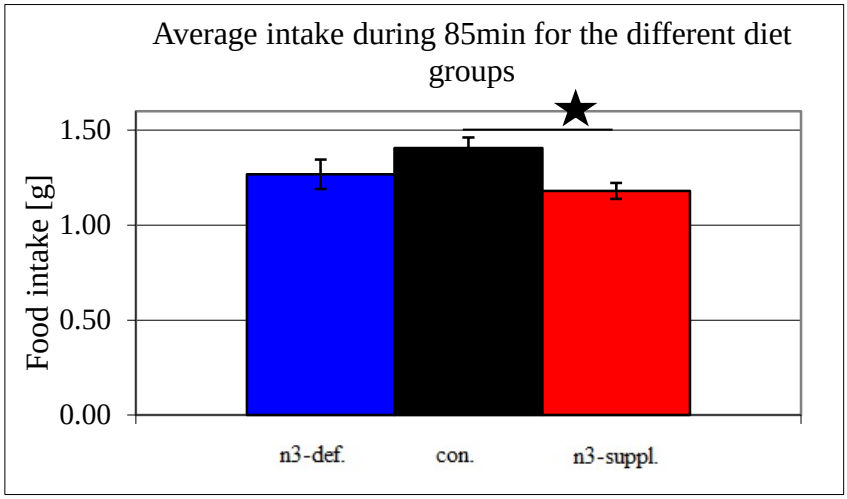

Figure 6: This diagram shows how much in average a mouse in each diet groups ate during a restricted time interval on the fourth day of food restriction. The amount of food consumed were decreased in the n-3 enriched diet group (n3-suppl.) compared to the control diet (con.) group ($p<0.05$, one way ANOVA followed by LSD post hoc). The error bars are the standard error of the mean (SEM). For the n-3 deficient group (n3-def.) n=10, for the n-3 enriched diet group n=11 and for the control group n=11.

The same experiment was repeated on day 9, with the exception that now the groups all had the same diets in form of normal chow (see specification in the methods 3.1.2) and now no difference between the groups was found (fig. 7).

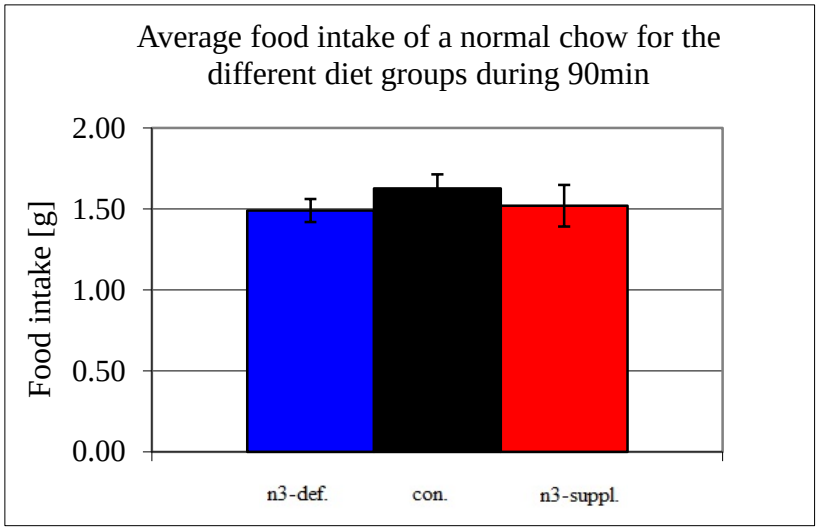

Figure 7: The diagram show how much the mice in the diet groups ate on average of a normal chow diet during a restricted time interval at the ninth day after the food restriction. The error bars are the standard error of the mean (SEM). For the n-3 deficient group (n3-def.) n=10, for the n-3 enriched diet group (n3-suppl.) n=11 and for the control group (con.) n=11.

There was no difference between the groups in RWA during the habituation period (data not shown) or during the restriction period (fig. 8 and 9). Both night time activity, which are the larger peaks as well as the FAA, which are the smaller peaks in between the night time peaks, become higher over time.

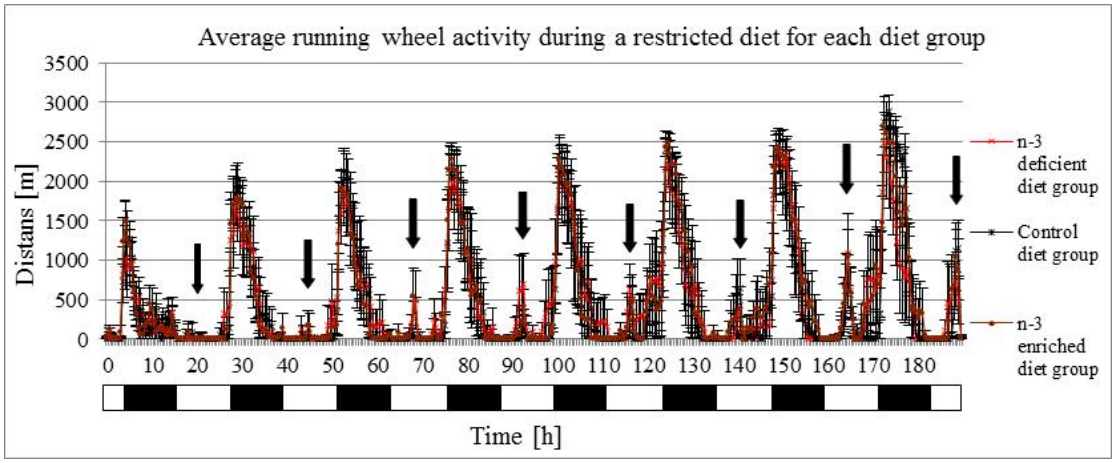

Figure 8: The figure shows the RWA during food restriction. The activity is measured in chunks of 30 minutes. The white and black blocks represent day time and night time respectively. The largest peaks are during the night when the mice are most active. As longer the restriction goes, the higher the peaks get. The small peaks in between that, which gets more distinct as the fasting goes on are the FAA peaks, which peak just before the mice get their meal. The activity is measured in chunks of 30 minutes. The error bars are the standard error of the mean (SEM).

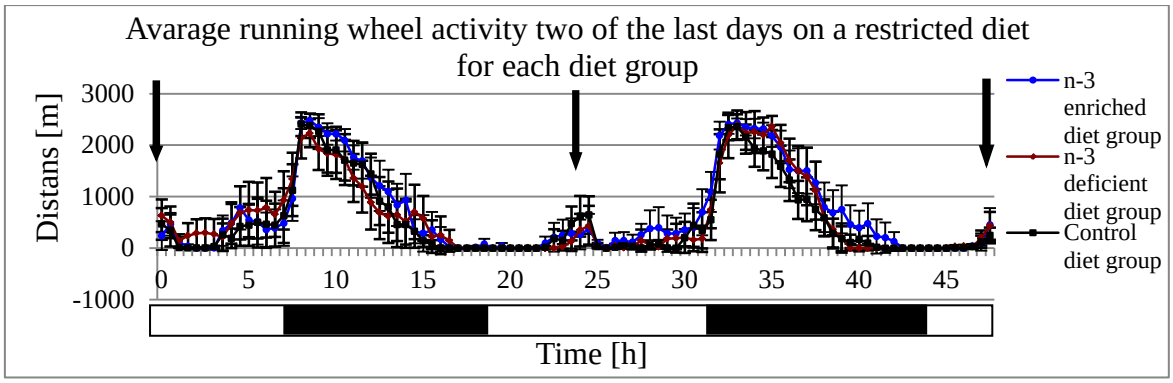

Figure 9: This is also the RWA in the restriction week but only showing two days in the end of the restriction period. The activity is measured in chunks of 30 minutes. The white and black blocks represent day time and night time respectively. The black arrows point at the FAA just before feeding. The error bars are the standard error of the mean (SEM). For the n-3 deficient group n=10, for the n-3 enriched diet group n=11 and for the control group n=11.

4.2 The *anx* -/- experiment

There was no difference in BW or FI between the groups of mice during the habituation period. During the first week of food restriction no difference in FI or BW could be found between the groups (fig. 10). During this period, two of the heterozygotes were removed from the study due to severe body weight loss. Data from these individuals are not included.

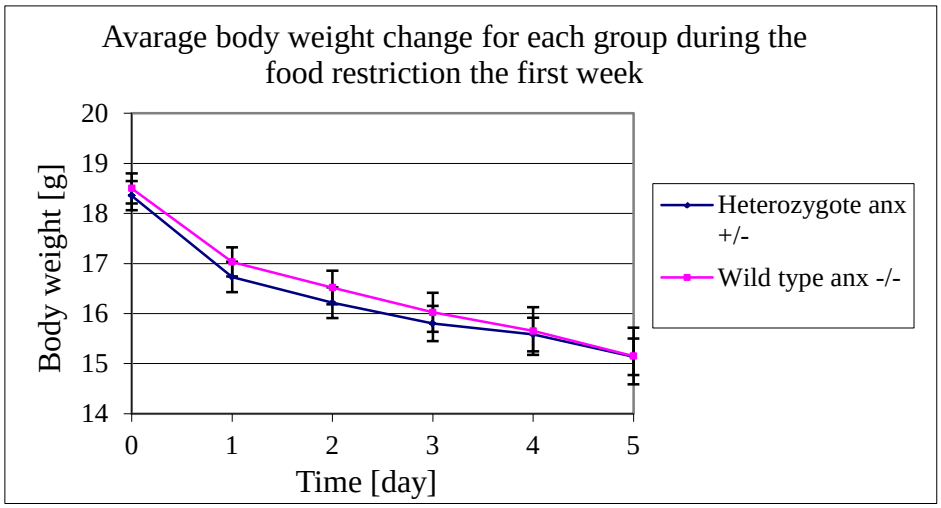

Figure 10: The graph shows the body weight change for the two groups of *anx* mice in the first week of food restriction. The error bars are the standard error of the mean (SEM). For the heterozygote *anx* +/- mice (n=16) and for the homozygote *anx* -/- mice (n=11).

The only significant difference between the groups was found on the last day, when the heterozygote mice showed a significant higher FAA, figure 11. The same result was found in the next week of restriction with the exception that this time all animals survived.

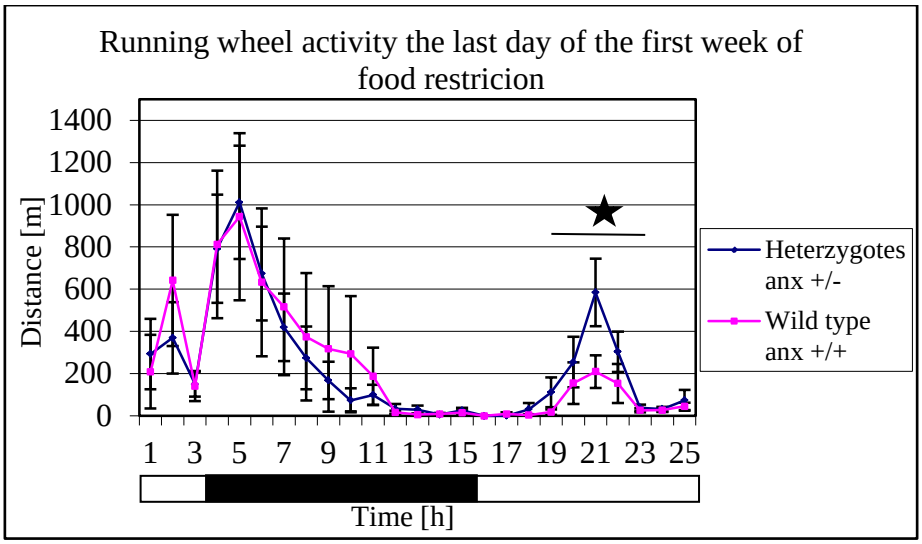

Figure 11: This is the last day of the first restriction period. The activity is measured in chunks of 60 minutes. The white and black blocks represent day time and night time respectively. The error bars are the standard error of the mean (SEM). It is a significant difference between heterozygote *anx* +/- mice (n=16) group and the homozygote *anx* -/- mice (n=11) group in FAA.

Between the restriction weeks there were two days of refeeding. When put on refeeding a small but significant difference in BW gain was found between the groups after 24 hours of refeeding, seen in figure 12, where the heterozygotes gained more in weight. However, this difference disappeared after 48 hours of refeeding.

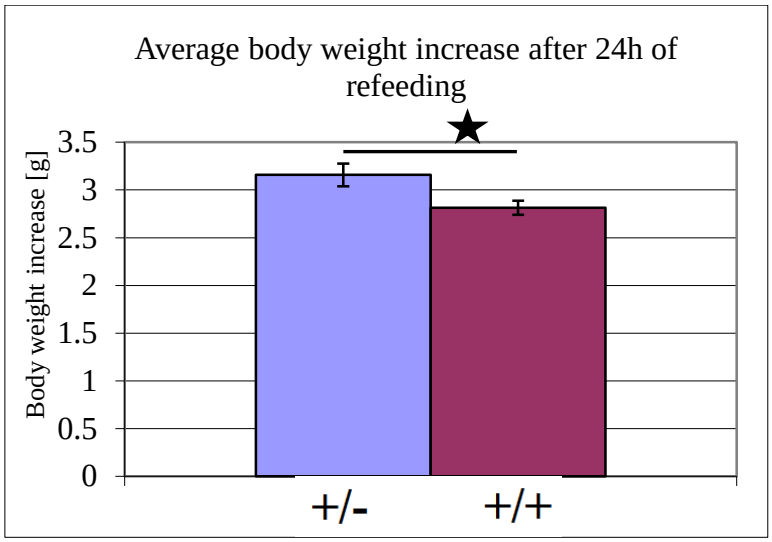

Figure 12: This graph shows the amount of the average weight gain the first 24hs of refeeding between the two groups of heterozygote *anx* +/- mice (n=16) and the homozygote *anx* -/- mice (n=11). The error bars are the standard error of the mean (SEM).

5. Discussion

In the experiments with different PUFA diets, no effect was found on BW, FI, RWA or body composition during the first six weeks of habituation or during the habituation to the separation and running wheel. During the week with restricted food (the ABA model) there was no difference in RWA or BW between the groups when the food was given in a fixed amount but unrestricted in time. However, when restricted food was given during a limited time, there was a difference in food intake between mice fed with a ω-3 enriched PUFA diet compared to mice fed on a control diet. Although despite this difference in food intake, no difference was found in activity/FAA, body weight or body weight change. These results are in agreement with the result from earlier experiments by another group (unpublished data).

When trying to cut the food pellets into pieces, it was found that different food items had different texture and some could be harder to chew than the other. For this reason the mice were put on refeeding once more. This time it was to test if it was the texture of the food that was the reason behind the difference in FI. All mice got the same novel diet (normal chow) where they showed no difference in intake. That means that it was likely due to the difference in texture of the food that influenced the difference in FI. Other less likely hypothetical reasons for this could be that it was differences in taste, visual appearance or olfactory differences. What was also detected when cutting the pellets into pieces was a strong fishy smell, clearly distinguished the ω-3 enriched PUFA diet from the other two diets. However, this would probably have resulted in different intake during the habituation.

Consequently, in the ABA-model, there was no significant effect of different PUFA diets when introduced in this way. It might had been possible to find differences between the diet if the amount of the ω-3 enriched PUFAs had been higher, if the variation in the ω-6/ω-3 ratios between the diets had been larger or if all the diets have had the same texture.

In the study by Avraham, they used the same kind of ω-3 enriched diet and control diet as in this study. They also had a restricted diet but did not use any running wheels. They found a better cognition and survival rate among animals fed with a ω-3 PUFA enriched diet [65]. The result from this experiment compared to the study of Avraham cannot be directly compared as this study was more focused on the physiological aspects of AN like BW change, FI and RWA, No mouse in this study was removed during the experiment, so nothing can be said about the survival rate and the change in cognition was not considered at all.

The diets were first given after the mice were three weeks old and this continued six weeks totally during the habituation. This should have been enough to make a difference in the cell membrane composition. PUFAs have many times been found to be important for the development of the brain. Around 40% of the PUFAs in the brain consist of DHA, which makes it extra important for the brain. This effect has been seen for children with ADHD [62]. To get an even better effect, the diets could have been introduced earlier, shortly before the gestation.

The promising effect of n-3 PUFA and EPA in particular, could not be replicated in this rodent model [53]. As humans and rodents are not used to eat the same kind of food, the effect of PUFA might not be the same. Mice are opportunists and eats mainly plant foods but also worms, insects and other dead mice. Humans, on the other side, are omnivores and are more used to have meat in their diets. This can have a profound

effect on how humans metabolize food and LCPUFAs. The positive effect seen in human studies is probably not possible to repeat in mice.

From the refeeding of the *anx* +/- and wild type mice it was seen a significant higher weight gain for the *anx* +/- mice after 24hs of refeeding. This was only an initial effect of the refeeding as there was no effect after 48hs of refeeding. The expectation was that the wild types would do best in the refeeding but here it was the *anx* +/- that had a slightly better recovery. This difference is hard to explain and it is likely to be biased data. It is probably better to draw conclusions on that matter first after this trial has been repeated.

In the experiments investigating the importance of the *anx* locus on the susceptibility to develop AN-like behaviors, it was found that heterozygote *anx* +/- mice had a higher FAA at the end of the food restricted period compared to wild type littermates. Even though the heterozygote mice have not been studied extensively to date no obvious phenotype relating to energy homeostasis (or other) has been identified in these mice (unpublished data).

Hence, the *anx* locus (or gene) has generally been considered recessive. However, all investigations of the heterozygote mice have so far been performed under normal environmental conditions. Our results show for the first time that the heterozygote mice, when challenged metabolically, seem to be more sensitive to develop starvation-induced activity changes indicating that this locus/gene might be of importance for the initiation and/or the progression of the disease.

A difference in FAA would normally lead to a difference in body weight. Though, in this experiment no such differences in body weight was found. It is very likely though that the time period during which the *anx* +/- mice display an increase in the FAA levels is too short to contribute to any difference in body weight. If the experiment would have been prolonged, such findings in body weight would have been likely.

The human homolog to the *Ndufaf1*gene (that is belived to be the cause of the anx phenotype) from the anx mouse is not seem to have the same phenotype in humans as in mice. In humans it is implicated in hypertrophic cardiomyopathy [66]. Although the homolog does not seem to induce *anorexia* by a mitochondrial dysfunction in important brain areas for food, the gene in the mouse can still be interesting. That is if another human gene is found that causes a mitochondrial dysfunction resulting in similar phenotype to the one found in the *anx* -/- mouse.

The cause of a reduced intake in AN is hard to explain. One could believe that it is different from patient to patient. One early theory in the history of AN was that AN patients had lost their hunger [16], but the indication is that most of them are hungry [67] but are able to withstand it. There are many different theories of how this is possible.

It has been suggested that as the activity level goes up in AN-patients, the increased activity makes the body release opioids, helping the anorexic to withstand from eating. These opioids are believed to be as addictive as external opioids. This could make the anorectics get stuck to the disease. This is the auto-addiction opioid theory. AN patients are generally more vulnerable to get addicted to various things, which indicates they are also more sensitive to opioids [68].

Moreover, there is a theory that states that AN patients get a reward or a kick from being able to maintain their restricted diet (refrain from eating). This kick is the same kind of reward coupled to the mesolimbic dopamine-system and very common to the type of reward the one can get from exercise as well as drugs of abuse (which interestingly has a high comorbidity in AN) [37].

One theory with the ω-3 enriched diet is that it can inhibit the inflammation/depression, which would make the patients feeling more hunger [67]. Another theory is that ω-3 supplementation will improve the cell signaling (and dopamine in particular) which would also improve the dopaminergic reward system making the AN patient eat more. Both these theories work on different aspects of AN but are both possible and can work in concert.

In the ABA model the reward consist probably most of the hyperactivity reward because the mice do not do any conscious dieting and during the habituation and there was no preference for any of the diets given. An increase in BW in the ABA model is expected to lead to a decreased activity that would help the mice increase their weight even more.

The mutation in the *anx* -/- mouse has been proposed affect the dopamine transmission and thus mainly influences the hedonic system [69]. If that also is the case for *anx* +/-, this together with the findings of an increased FAA in *anx* +/-, it would expect to lead to a reduced FI. This was also believed to happen if the *anx* experiment would have been prolonged.

It has been shown that a changed diet can improve the diseased with mitochondrial dysfunction [12]. A possible way to rescue the *anx* phenotype is with supplementation of LCPUFAs in their diet. Currently a project of this type is going on at KI.

6. Conclusion

It was not found any effect of ω-3 enriched diets or ω-3 deficient diets in the activity-based *anorexia* (ABA) model. It is advisable to do further research with other rodent models together with different polyunsaturated fatty acid (PUFA) diets that model for other AN features not here investigated. This could be models for cognition and emotion.

For the heterozygote *anx* +/- mouse a significant difference was found between the heterozygotes and the wild types in food anticipation activity (FAA) in the ABA model, although no difference in food intake (FI) or body weight (BW) was found. Therefore it can be claimed that heterozygotes are more vulnerable in ABA. With this information the *anx* +/- mice can be used to further study the *anx* locus and its relation to *anorexia*.

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Appendix A

Typical fatty acid content in the diets manufactured from Research Diets, Inc.

Ingredients (g):	**The control diet (Soybean oil)**	**The ω-3 enriched diet (ROPUFA 75EE)**	**The ω-3 deficient diet (Safflower oil)**
ROPUFA 75	0	40	0
Safflower oil, USP	0	0	50
Soybean oil	50	10	0
Total	**50**	**50**	**50**
Myristic acid (tetradecanoic acid, 14:0)	0	0.04	0
Palmitic acid (hexadecanoic acid, 16:0)	5.20	1.16	3.20
Palmitoleic acid (hexadecenoic acid,16:1n-7)	0	0.08	0
Margaric acid (heptadecanoic acid, 17:0)	0	0.04	0
Stearic acid (octadecanoic acid, 18:0)	1.90	1.34	1.15
Oleic acid (octadecenoic acid, 18:1n-9)	12.15	4.51	6.00
Linoleic acid (octadecadienoic acid, 18:2n-6)	26.75	5.59	39.20
Linolenic acid (octadecatrienoic acid, 18:3n-3/6)	3.90	1.02	0.07
Nervonic acid (cis-tetracosenoic acid, 24:1n-9)	0	0.16	0
Stearidonic acid (18:4n-3)	0	0.44	0
Arachidic acid (eicosanoic acid, 20:0)	0	0.20	0
Gadoleic acid (eicosenoic acid, 20:1n-9)	0	1.04	0
Eicosadienoic acid (20:2n:6)	0	0.16	0
Eicosatrienoic acid (20:3n-3)	0	0.20	0
Arachidonic acid (eicosatetraenoic acid, 20:4n-6)	0	0.88	0
Timnodonic acid (eicosapentaenoic acid (EPA), 20:5n-3)	0	16.92	0
Heneicosapentaenoic acid (21:5n-3)	0	0.84	0
Behenic acid (docosanoic acid, 22:0)	0	0.12	0
Erucic acid (docosenoic acid, 22:1n-9)	0	0.16	0
Clupanodonic acid (docosapentaenoic acid (DPA), 22:5n-3)	0	1.84	0
Docosahexaenoic acid (DHA) (22:6n-3)	0	9.08	0
Total	**49.90**	**45.82**	**49.62**
Saturated (g)	7.10	2.90	4.35
Monounsaturated (g)	12.15	5.95	6.00
Polyunsaturated (g)	30.65	36.13	39.27
Saturated (%)	14.23	6.33	8.77
Monounsaturated (%)	24.35	12.99	12.09
Polyunsaturated (%)	61.42	78.85	79.14
Linoleic acid (%)	53.61	12.20	79.01
Oleic acid (%)	24.35	9.84	12.09
α-linolenic acid (%)	7.82	2.23	0.13
Eicosapentaenoic acid , EPA (%)	0	36.93	0

Docosahexaenoic acid, DHA (%)	0	19.82	0
Total Omega-3 (%)	**7.82**	**63.95**	**0.13**

Appendix B

Ingredient declaration in grams for the diets manufactured from Research Diets, Inc.

	The control diet (Soybean oil)	**The ω-3 enriched diet (ROPUFA 75EE)**	**The ω-3 deficient diet (Safflower oil)**
Protein (g%)	14	14	14
Carbohydrate (g%)	72	72	72
Fat (g%)	5	5	5
Total (%)	100	100	100
Ingredient (g):			
Casein	140	140	140
L-Cystine	1.8	1.8	1.8
Corn Starch	473.192	473.192	473.19
Maltodextrin 10	125	125	125
Sucrose	100	100	100
Cellulose, BW200	50	50	50
Soybean Oil	50	10	0
ROPUFA-75EE	0	40	0
Safflower Oil	0	0	50
t-Butylhydroquinone	0.008	0.008	0.008
Mineral Mix S10022M	35	35	35
Vitamin Mix V10037	10	10	10
Choline Bitartrate	2.5	2.5	2.5
FD&C Yellow Dye #5	0.05	0	0
FD&C Red Dye #40	0	0.05	0
FD&C Blue Dye #1	0	0	0.05
Total	**987.55**	**987.55**	**987.55**

Appendix C

Declaration of ingredient in calories for the diets manufactured from Research Diets, Inc.

	The control diet (Soybean oil)	**The ω-3 enriched diet (ROPUFA 75EE)**	**The ω-3 deficient diet (Safflower oil)**
Protein (kcal%)	15	15	15
Carbohydrate (kcal%)	74	74	74
Fat (kcal%)	12	12	12
kcal/g	3.9	3.9	3.9
Ingredient (kcal)			
Casein	560	560	560
L-Cystine	7	7	7
Corn Starch	1893	1893	1893
Maltodextrin 10	500	500	500
Sucrose	400	400	400
Cellulose, BW200	0	0	0
Soybean Oil	450	90	0
ROPUFA-75EE	0	360	0
Safflower Oil	0	0	450
t-Butylhydroquinone	0	0	0
Mineral Mix S10022M	0	0	0
Vitamin Mix V10037	40	40	40
Choline Bitartrate	0	0	0
FD&C Yellow Dye #5	0	0	0
FD&C Red Dye #40	0	0	0
FD&C Blue Dye #1	0	0	0
Total	**3850**	**3850**	**3850**

Appendix D

The ROPUFA 75EE fatty acid content.

Saturated fatty acids	%
Myristic acid (tetradecanoic acid, 14:0)	0.1
Palmitic acid (hexadecanoic acid, 16:0)	0.3
Margaric acid (heptadecanoic acid, 17:0)	0.1
Stearic acid (octadecanoic acid, 18:0)	2.4
Arachidic acid (eicosanoic acid, 20:0)	0.5
Heneicosylic acid (heneicosanoic acid, 21:0)	0.1
Behenic acid (docosanoic acid, 22:0)	0.3
Total	3.8
Mono-unsaturated fatty acids	
Palmitoleic acid (hexadecenoic acid, 16:1n-7)	0.2
Heptadecenoic acid (17:1n-9)	0.1
Oleic acid (octadecenoic acid, 18:1)	3.7
Vaccenic acid (18:1n-7)	1.5
Gadoleic acid (eicosenoic acid, 20:1)	2.6
Erucic acid (docosenoic acid, 22:1)	0.4
Nervonic acid (cis-tetracosenoic acid, 24:1)	0.4
Total	8.9
Polyunsaturated fatty acids	
Linoleic acid (octadecadienoic acid, 18:2n-6)	0.6
γ-linolenic acid (octadecatrienoic acid, 18:3n-6)	0.3
α-linolenic acid (octadecatrienoic acid, 18:3n-3)	0.6
Stearidonic acid (18:4n-3)	1.1
Eicosadienoic acid (20:2n:6)	0.4
Dihomo-gamma-linolenic acid (DGLA) (20:3n-6)	0.5
Eicosatrienoic acid (20:3n-3)	0.4
Arachidonic acid (eicosatetraenoic acid, 20:4n-6)	2.2
Eicosatetreenoic acid (20:4n3)	2.0
Timnodonic acid (eicosapentaenoic acid (EPA), 20:5n-3)	42.3
Heneicosapentaenoic acid (21:5n-3)	2.1
Clupanodonic acid (docosapentaenoic acid (DPA), 22:5n-3)	4.6
Docosahexaenoic acid (DHA) (22:6n-3)	22.7
Total	79.8
Unassigned fatty acids	**7.5**