Translational Neuroscience of Anorexia Nervosa

A genetic and environmental interplay underlying behavioural hyperactivity in mice

DISSERTATION

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TRANSLATIONAL NEUROSCIENCE OF ANOREXIA NERVOSA

A GENETIC AND ENVIRONMENTAL INTERPLAY UNDERLYING BEHAVIOURAL HYPERACTIVITY IN MICE

Translationeel neurowetenschappelijk onderzoek naar anorexia nervosa
een samenspel van genetische en omgevingsfactoren in de regulatie van gedragshyperactiviteit bij muizen

(met een samenvatting in het Nederlands)

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Për prindërit e mi, motrën dhe vëllain

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Chapter 1

General introduction and outline of the thesis

1.1 Anorexia nervosa

One of the first known descriptions of anorexia nervosa, written from Richard Morton in 1689[1] is as follows:

Mr. Duke's daughter in St. Mary Axe, in the year 1684, and the Eighteenth Year of her Age, in the month of July fell into a total suppression of her Monthly Courses from a multitude of Cares and Passions of her Mind, but without any Symptom of the Green-Sickness following upon it. From which time her Appetite began to abate, and her Digestion to be bad; her Flesh also began to be flaccid and loose, and her looks pale, with other Symptoms usual in an Universal Consumption of the Habit of the Body, and by the extream and memorable cold Weather which happened the Winter following, this Consumption did seem to be not a little improved; for that she was wont by her studying at Night, and continual poring upon Books, to expose her self both Day and Night to the injuries of the Air, which was at that time extreamly cold, not without some manifest Prejudice to the System of her Nerves. The Spring following by the Prescription of some Emperick, she took a Vomit, and after that I know not what Steel Medicines, but without any Advantage. So from that time loathing all sorts of Medicaments, she wholly neglected the care of her self for two full Years, till at last being brought to the last degree of a Marasmus, or Consumption, and thereupon subject to frequent Fainting Fits, she apply'd her self to me for Advice.

I do not remember that I did ever in all my Practice see one, that was conversant with the Living so much wasted with the greatest degree of a Consumption, (like a Skeleton only clad with skin) yet there was no Fever, but on the contrary a coldness of the whole Body; no Cough, or difficulty of Breathing, nor an appearance of any other distemper of the Lungs, or of any other Entrail: No loosness, or any other sign of a Colliquation, or Preternatural expence of the Nutritious Juices. Only her Appetite was diminished, and her Digestion uneasie, with Fainting Fits, which did frequently return upon her. Which Symptoms I did endeavour to relieve by the outward application of Aromatick Bags made to the Region of the Stomack, and by Stomack-Plaisters, as also by the internal use of bitter Medicines, Chalybeates, and Juleps made of Chephalick and Antihysterick Waters, sufficiently impregnated with Spirit of Salt Armoniack, and Tincture of Castor, and other things of that Nature. Upon the use of which she seemed to be much better, but being quickly tired with Medicines, she beg'd that the whole Affair might be committed again to Nature, whereupon consuming every day more and more, she was after three Months taken with a Fainting Fit and dyed.!

The term anorexia nervosa was used later around the late nineteenth century from Gull and Lasegue[2,3] to describe the wasting of body tissue for which no organic cause (like tuberculosis at the time) could be found. The illness is now recognized as psychiatric disorder and diagnostic criteria can be found in the "Diagnostic and Statistical Manual of Mental Disorders, 4th edition, text revised" (DSM IV-TR) of the American Psychiatric Association (APA) and the International Classification of Diseases (ICD) - 10 Classification of Mental and Behavioral Disorders defined by World Health Organization, Geneva.
Anorexia Nervosa (AN) is a severe mental illness which runs a chronic, relapsing course and is associated with high disability and mortality rates[4,5,6] with etiology still unknown and available treatment being only partially successful.[7,8,9] Prevalence of the disorder is different between females and males with rates of 0.9% and 0.3% respectively.[10] The hallmark of the disease is keeping a low body weight, less than 85% of what is expected. Even at this weight, there is an intense fear of gaining weight. There are two subtypes of anorexia nervosa: restricting and binge - eating/purging type. These and the other diagnostic criteria of AN based on the DSM IV-TR and ICD-10 are shown in table 1.1.[11,12]
Though not in the diagnosis criteria, excessive physical activity has also been described as a prominent feature of the disorder (seen in 40-80% of the individuals)[13,14,15,16,17] and is also recognized as such from the majority (≈85%) of the clinical specialists.[18] It plays a significant role in the development and maintenance of the disorder even when the patients are in an emaciated state. High activity levels are also known to have an impact on recovery rate[19,20,21,22] and outcome of the disorder.[13,23]
Excessive exercise does not simply imply an increase in the amount of activity expressed, but the motive (e.g. weight control, aim to improve appearance) and feelings (e.g. intense guilt when exercise is missed, annoyance when exercise is interrupted) associated with exercise, and giving it an obligatory nature are also important.[24,25,26,27] Excessive activity/exercise has also been associated with several aspects of the disorder such as anxiety, low mood,perfectionism, obsessions and compulsions.[28,29,17,30]

1.2 Genetics and epigenetics

The etiology of anorexia nervosa is complex (unknown), with risks involving environmental, temperamental, developmental and genetic factors.[31,32,33,34,35,36,37] Twin and family studies have shown a genetic component of the disorder with heritability estimates in a range between 0.22-0.56.[38,39]
Several genes involved in weight regulation, eating behavior, appetite neuropsychological profiles, mood, neurodevelopment and stress responsiveness have been associated with anorexia nervosa.[40,41,42,43,44] Some of the studies had initially shown positive results, but could not be replicated in a larger sample. Recent genome wide association studies conducted in AN have identified single nucleotide polymorphisms (SNP) associated with genetic risk of

TABLE 1.1 – *Anorexia nervosa diagnostic criteria*

DSM IV-TR Diagnostic Criteria for Anorexia Nervosa

- Refusal to maintain body weight at or above a minimally normal weight for age and height: Weight loss leading to maintenance of body weight <85% of that expected or failure to make expected weight gain during period of growth, leading to body weight less than 85% of that expected.
- Intense fear of gaining weight or becoming fat, even though under weight.
- Disturbance in the way one's body weight or shape are experienced, undue influence of body weight or shape on self evaluation, or denial of the seriousness of the current low body weight.
- In postmenarchal females, amenorrhea (i.e. the absence of at least three consecutive menstrual cycles). A woman is considered to have amenorrhea if her periods occurring only following hormone (e.g. estrogen) administration.

Specific types

- Anorexia Nervosa - Restricting type: During the current episode of anorexia nervosa, the person has not regularly engaged in binge - eating or purging behavior (i.e. self-induced vomiting or the misuse of laxatives, diuretics, or enemas).
- Anorexia Nervosa - Binge-eating/purging type: During the current episode of anorexia nervosa, the person has regularly engaged in binge-eating or purging behavior (self-induced vomiting or the misuse of laxatives, diuretics, or enemas).

ICD-10 Criteria for Anorexia Nervosa (F50.0)

A. Weight loss, or in children a lack of weight gain, leading to a body weight of at least 15

B. The weight loss is self-induced by avoidance of "fattening foods".

C. A self-perception of being too fat, with an intrusive dread of fatness, which leads to a self-imposed low weight threshold.

D. A widespread endocrine disorder involving the hypothalamic-pituitary-gonadal axis, manifest in the female as amenorrhoea, and in the male as a loss of sexual interest and potency (an apparent exception is the persistence of vaginal bleeds in anorexic women who are on replacement hormonal therapy, most commonly taken as a contraceptive pill).

E. Does not meet criteria A and B of Bulimia nervosa.

anorexia nervosa.[45,46] *(SNP = variation in a certain nucleotide between individuals.)* However, no SNP has passed the statistical significance threshold when corrected for multiple comparisons. It is highly likely that a large number of genes, each of small effect, contribute to the risk[47] and that genes and environmental factors also influence the etiology via epigenetic modifications.[31] Epigenetics refers to heritable changes in gene function that occur without alterations in DNA sequence. There are several ways via which these changes can occur e.g. DNA methylation, histone modifications. DNA methylation (most commonly studied modification) is the addition of a methyl group on the cytosine-phosphate-guanine (CpG) nucleotides on the pyrimidine ring of cytosine residues.[48,49] Epigenetic modifications are likely to contribute to the etiology of numerous non-malignant complex disease phenotypes including psychiatric disorders.[50,51,52,53] There have also been a couple of studies interesting for eating disorders e.g. DNA methylation studies in relation to weight[54,55] or stress.[56] There have also been few studies in anorexia nervosa.[57,58,59,60]

1.3 Treatment

Treatment of anorexia nervosa is aimed at normalizing body weight and eating behaviors. Based on the degree of illness, patients can be treated in outpatient, partially residential (day patient), or inpatient settings. Treatment consists of behavioral treatment and pharmacological treatment, or a combination of the two is also applied, but only with partial success.[7,8,9] There are several types of behavioral treatments such as cognitive behavioral therapy; interpersonal psychotherapy; cognitive analytical therapy; family-based therapy (Maudsley).[9] Pharmacotherapy involves many agents such as antidepressants (e.g.tricyclics - clomipramine and selective serotonin reuptake inhibitors - fluoxetine), antipsychotics (e.g. typical - haloperidol and atypical - olanzapine), antihistaminics and other pharmacological compounds (e.g. zinc, lithium). Recent guidelines on pharmacological treatment of eating disorders, show that controlled studies have not shown full evidence for any of the pharmacological treatments.[61] However some pharmacological agents, currently with limited evidence, are promising such as Olanzapine and zinc supplementation. Yet, there can not be a successful treatment without a better understanding of the neurobiology of the disorders.

1.4 Animal studies

Animal studies can permit the systematic study of the underlying neurobiology, environment, genetics and gene by environment interactions. Further, animal studies can accelerate our understanding of critical phases of illness development as the reproduction and lifetime of, e.g. rodents, is much shorter than of humans. As animal studies take place under controlled genetic and environmental conditions, the dissection of complex phenotypes is facilitated.

In the classical view, animal models should fulfill face, predictive and construct validity criteria for the disorder. However, anorexia nervosa is (i) complex (ii) of unknown etiology, (iii) diagnostic crossover occurs within eating disorders and (iv) treatment is only partly successful. All these factors make it difficult to design one animal model that will fulfill the validity criteria for this disorder. This is a challenge faced by other psychiatric disorders as well.[62,63] As a result, there are several animal models addressing different aspects of anorexia nervosa, instead of a single model.[64,65,66] These models are divided in two major categories: genetic and environmental.
In the genetic category, the phenotype resultant from a genetic modification to a gene, e.g. spontaneous mutations or generation of gene knockout mouse, is examined. The observed phenotype can be directly linked to the candidate gene. Studied examples include dopamine deficient mice,[67] M3 muscarinic receptor deficient mice[68] and other genes involved in regulation of food intake. The most studied gene within the genetic models is the autosomal recessive anx mutation in rodents where extreme decreased food intake leading to death, within 20-30 days after birth, is observed.[69,70,71,72,73]
In the environmental category, as the name suggests, the environment is manipulated either by dietary restriction, exposure to stress and behavioral hyperactivity models. Within this category, activity based anorexia model (ABA) combines two aspects: dietary restriction and excessive physical activity levels. This model can be also used in combination with genetic models, that is, we can test knockout mice of candidate genes in this model to understand the contribution of that gene under these conditions.
In the ABA model, animals have unlimited voluntary access to a running wheel throughout the experiment, while food is ad libitum available for a limited period at the same time during several consecutive days.[74,75] Under these conditions, a paradoxical physical hyperactivity resulting in body weight loss is observed in certain rat and mouse strains.[76,77,78] This phenotype is similar to the excessive physical activity observed in people with AN while they are emaciated providing some face validity to the model.

In this thesis, classical inbred, consomic and candidate gene knockout mice, each with their advantages, are used to understand the relationship between genetic and environmental background and pathophysiological processes of self-starvation. Mice have been used in research to study human disease for over a century now, and this can be attributed to the similarities in physiology, behavior and genetics to humans.

1.4.1 Classical Inbred

The classical inbred strains were initially bred as fancy mice before being introduced to the laboratory,[79] figure 1.1. Because of the continuous inbreeding, their genomes are homozygous and they share unique variation patterns attributed to mixing of diverse but limited founding populations.[80]

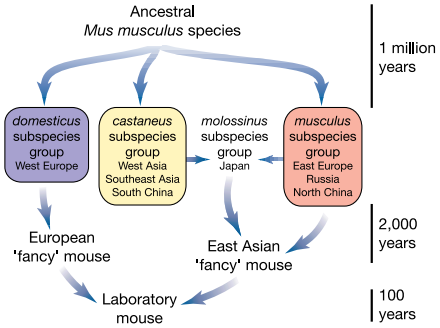

FIGURE 1.1 – *Origin of the laboratory mouse. Both European and Asian 'fancy' mice contribute to the genetic background of the laboratory mouse. Reprinted by permission from Macmillian Publishers Ltd: Nature,*[79] *Copyright 2002.*

1.4.2 Chromosome substitution strains

Mouse genetic reference populations have been generated to facilitate the genetic mapping of complex traits. For example, a panel of mouse chromosome substitution strains has been derived from C57BL/6J and A/J inbred mouse strains (C57BL/6J-Chr#$^{A/J}$/NaJ[81]). In this CS strain panel, one of the A/J (donor) chromosomes substitutes the corresponding C57BL/6J (host) chromosome after at least 10 generations of selective breeding, figure 1.2. After each generation, the mice are genotyped and only the mice with a high number of markers for the specified chromosome are used for the next generation. Nineteen autosomes, two sex chromosomes and one mitochondrial chromosome, comprise the complete CS strain panel. The generation of this panel (and others) facilitates the mapping of genetic loci for complex traits (QTLs) that involve small contributions from a variety of genetic loci. The genetic background variance is reduced which leads to each QTL explaining more of the phenotypic variance and also to a decreased significance level.[82,83] Observed phenotypical differences between a substituted chromosome and the host strain, suggest that there is at least one underlying QTL in that chromosome contributing to the phenotype.[84] This QTL can be fine mapped by first crossing the CS strain to the host strain which will result in an F1. Intercrossing of the F1 animals will result in unique recombinations of the substituted chromosome in the F_2 progeny.
The CS strain panel is a powerful tool in identifying loci affecting behavioral processes and has been successful in identifying several QTLs for complex traits.[85,86,87]

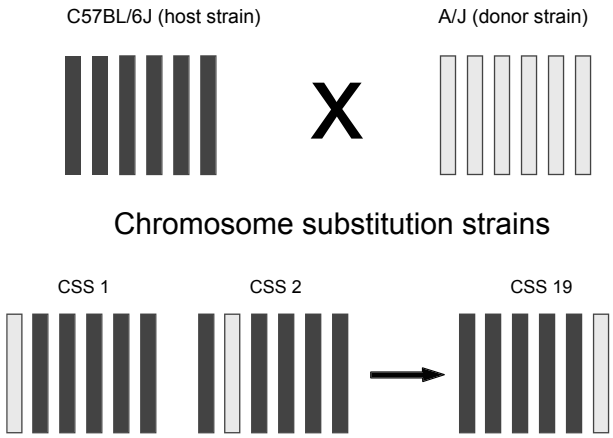

FIGURE 1.2 – *Chromosome substitution strains (CSS) where one chromosome of the host strain is fully substituted with the corresponding chromosome from the donor strain after at least 10 generations of selective breeding.*

1.4.3 Knockout mice

In the generation of the knockout mice for a single gene, an essential part of that gene is replaced with a selectable marker. This results in loss of the gene function associated with phenotypic (physical of biochemical) changes in the mouse. These changes help characterize the function of the gene, and the possible role of that gene in diseases. Considering that mice share many genes with humans, the data collected from animals studies helps to understand also the function of the human homologue gene. Today, double or even triple gene knockout mice can be generated to better understand the relationship between these gene′s functions. The number of genes being knocked out is increasing steadily.

1.5 Aim and outline of this thesis

This thesis aims at unraveling genetic and environmental factors affecting (i) hyperactivity behavior and (ii) susceptibility to the ABA model.

To unravel genetic factors for hyperactivity, a mouse genetic panel was exposed to the ABA model. Mouse chromosome substitution (CS) panel, generated from C7BL/6J and A/J mice, was behaviorally screened in the ABA model, to identify chromosomes carrying QTL(s) related to hyperactivity, chapter 2. This analysis would reveal three chromosomes carrying QTL(s) related to hyperactivity.

Following up on these results, to fine map the location of the QTL(s), contributing to the observed behavioral hyperactivity, an F_2 progeny was generated, genotyped with microsatellite markers and behaviorally screened in the ABA model, chapter 3.

Continuing with the results of chapter 2, CS strain 4 mice, apart from being hyperactive, were also found to be lower in body weight than the C57BL/6J control and highly susceptible to the ABA model. Interestingly, there was no body weight difference on day of birth between these two strains, but it became present on postnatal day (PND) 14. The body weight of CSS4 pups at PND 14 reached the C57BL/6J levels by cross - fostering with C57BL/6J mother and likewise, C57BL/6J pups had body weight similar to CSS4 pups when fostered by CSS4 mother. To further investigate how the change in early environment (fostering) would impact (i) adult body weight and (ii) ABA susceptibility, we carried out series of experiments with CSS4 and C57BL/6J inbred strains, chapter 4.

To identify susceptibility factors to develop anorexia nervosa, a set of eleven inbred mice, which are part of Mouse Phenome Database (MPD) priority strains, Tier 1 (http://phenome.jax.org/db/q?rtn=strains/searchandreqpanel=MPD) were behaviorally screened in the ABA model, chapter 5. Their response to daily scheduled food restriction and the relationship between their baseline phenotypes and their ABA susceptibility was investigated.

NPY infusion in scheduled food restricted mice had a significant effect on behavioral hyperactivity levels.[88] Since NPY can act through a set of five G-protein coupled receptors, to investigate the path via which NPY exerts its effect on different processes we compared behavioral responses of NPY Y1 and Y2 receptor knockout mice and their wild-type littermate controls in the model. The results are described in chapter 6.

Finally, in chapter 7, to directly probe gene by environment interactions in relation to anorexia nervosa, we examined quantitative DNA methylation in the promoter region of four well established candidate genes that have been

proposed to have a role in the development of anorexia nervosa.

Chapter 8 provides a summary and overall discussion of the thesis with a subsequent future perspective in view of epigenetics in eating disorders research presented in chapter 9.

Chapter 2

Chromosomal mapping of excessive physical activity in mice in response to a restricted feeding schedule

Çiğdem Gelegen, Eneda Pjetri, Iain C. Campbell, David A. Collier, Hugo Oppelaar, Martien J.H. Kas

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Abstract

Excessive physical activity plays an important role in the progression of anorexia nervosa (AN) by accelerating weight loss during dietary restriction. To search for mechanisms underlying this trait, a panel of mouse chromosome substitution strains derived from C57BL/6J and A/J strains was exposed to a scheduled feeding paradigm and to voluntary running wheel (RW) access. Here, we showed that A/J chromosomes 4, 12 and 13 contribute to the development of a disrupted RW activity in response to daily restricted feeding. This pattern is characterized by intense RW activity during the habitual rest phase and leads to accelerated body weight loss. Regions on mouse chromosomes 4, 12 and 13 display homology with regions on human chromosomes linked with anxiety and obsessionality in AN cohorts. Therefore, our data open new roads for interspecies genetic studies of AN and for unraveling novel mechanisms and potential effective treatment strategies for these neurobehavioral traits.

2.1 Introduction

Anorexia nervosa (AN) and bulimia nervosa (BN) are psychiatric disorders characterized by serious disturbances in eating behaviors and estimated prevalence rates for these disorders fall between 0.5–1% (AN) and 1–3% (BN).[89] In addition to severe restriction in food intake, excessive physical activity is observed in the majority of patients with AN[90,14,91,17] and although not part of the diagnostic criteria, it plays a significant role in disease progression by exacerbating the weight loss that results from dietary restriction.[92,93,94] Treatment for AN is unsatisfactory: there are no effective pharmaceutical treatments and the mortality for AN is one of the highest of any psychiatric disorder.[6,95] It is therefore important to develop models of the disease to better understand basic etiology and facilitate the development of new treatments to improve long term prognosis. Despite its high prevalence, factors underlying the development and maintenance of excessive activity in eating disorders remain to be clarified. Twin studies performed across several countries to determine the contribution of genes and unique environmental factors to variation in physical activity levels showed a heritability of 23–68% in males and 31–72% in females.[96,97,98,99,100,101] In these studies, in addition to genetic background, non-shared environmental factors were shown to explain a considerable amount of variation in physical activity levels in both males (29–59%) and females (30–52%). In their study with 3344 male twin pairs, Lauderdale et al. showed strong genetic effects for intense exercise (i.e. running, swimming, cycling) and a modest effect for moderate physical activities (i.e. walking, stair climbing) with 48–58% and 12–40% estimated heritability for intense exercise and moderate physical activity

respectively.[99] In patients with AN, excessive exercising may be a behavior with high reinforcing value in that they may engage in it to improve mood, reduce anxiety and relieve an anhedonic state, which are features of the illness.[102,90,103,104,94,105,106,29,107,17] Obsessive-compulsive disorder (OCD) in particular has been shown to be associated with the development of excessive exercise in eating disorders.[108,92,28,17,109] Indeed, the term excessive may be inappropriate and compulsive exercise has been suggested as an alternative.[110] Since it is an important indicator of a poor prognosis,[20] understanding the neurobiological mechanisms that could explain its links with other co-morbid traits such as obsessionality, anxiety and anhedonia is important.

As in the case of other psychiatric disorders, the etiology of AN is likely to involve a complex interaction between psychosocial and genetic risk factors. Although the disease has an estimated heritability of approximately 60%,[111] little is known about the contribution of specific genetic factors to the development and maintenance of the disease. Twin and family studies demonstrated the heritability of the disease,[38,112,113,114] suggesting genetic contribution to the development and maintenance of disease-related neurobehavioral traits. Rodent models are increasingly used in the search for genetic mechanisms underlying the development of traits characteristics of psychiatric disorders.[115,116,117,118] Chromosome Substitution Strains (CSS) provide a powerful strategy for the identification and mapping of polygenic traits.[81,84] In a CS strain, a full length chromosome from one inbred strain (donor) is substituted in the genetic background of the host strain, thus partitioning the genome into single chromosome substitutions on a uniform genetic background. The phenotype of interest is measured in each CS and the background strain. Any phenotypic divergence between a given strain in the panel and the background strain indicates the presence of a locus or loci on the substituting chromosome affecting the trait. Once a CSS is determined to carry a locus for a specific trait, fine mapping of the candidate genes affecting the trait is carried out with crosses between the CSS and the host parental strain or with a panel of congenic strains derived from the CSS.[81,84]

The progenitor strains of the CS panel used in this study, C57BL/6J (the host) and A/J (the donor) exhibit different physiological and behavioral characteristics.[119,120,77,121,122,123,87,124,83,125,126,127,128] When they have unlimited access to a running wheel, these two progenitor strains respond differently to a scheduled feeding paradigm, in that, during a restricted feeding schedule C57BL/6J mice reduce their running wheel activity (RWA). In contrast, A/J mice gradually increase their RWA in response to scheduled feeding.[77] Furthermore, during daily scheduled feeding, RWA levels in the first 2 h of the dark phase (when food is available), decrease over time from baseline to day 4 of food restriction in C57BL/6J, whereas it remains similar during this period in A/J mice. Based on these differences between the two parental lines, we screened the CS panel for the development of excessive physical activity under restricted feeding conditions together with unlimited access to a running wheel. By identifying substitution strains displaying disturbed locomotor activity in response to food restric-

tion, we aimed to determine mouse chromosomes harboring genetic pathways underlying this complex trait. Once the chromosomes are determined, further fine genetic mapping strategies will delineate the pathways underlying its development. We chose to investigate the development of excessive physical activity in response to restricted food access because of its important contribution to the acceleration and/or maintenance of body weight loss, similar to AN. We showed that strains showing accelerated body weight loss also displayed disturbed locomotor activity during scheduled food access.

2.2 Materials and methods

2.2.1 Animals

Initial breeding pairs for CSSs and their progenitors A/J and C57BL/6J were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Generation of the mouse CSS panel has been described previously.[81,84] Each strain in the panel has a chromosomepair substituted from the A/J strain on to a host C57BL/6J background. The complete CS panel is constituted from 21 strains (19 autosomes and 2 sex chromosomes). The panel nomenclature is C57BL/6J-Chr#$^{A/J}$/NaJ, where # refers to the substituted chromosome. For simplicity, however, the strains are referred to as CSS# in this report. All mice were bred in the Rudolf Magnus Institute of Neuroscience animal facility and were 3–5 months old at the start of the experiment. Following weaning at 3–4 weeks, female and male mice were separately housed in cages (2–4 animals per cage; Macrolon® Type II; # 1284 L) in a room maintained on a 12 h dark/12 h light cycle (lights on at 2am), with an ambient temperature of 22.0 ± 2°C. They were given unrestricted access to food and water. In total, 321 female mice were tested in the experimental procedure that lasted 11 days; C57BL/6J strain (n=42), A/J strain (n=19) and of the 19 tested CS strains (n=271; on average 14 mice per CS-strain). Mice from CSS-15 and CSS-16 were not tested due to low availability. The Animal Ethical Committee of Utrecht University approved all experiments.

2.2.2 Experimental procedure

The scheduled feeding paradigm used in this study was previously described.[76,77] Briefly, following the adaptation of mice to running wheel cages (wheel circumference: 43.96 cm) for one week, mice were maintained in the same cages for an additional week. This period is termed “Baseline Conditions”. Under baseline conditions, mice had unrestricted access to food,water and the running wheel. Body weight and food intake were measured daily just before the beginning of the dark phase. Individual wheel running revolutions were continuously registered using Cage Registration Software (Department of Biomedical Engineering, UMC Utrecht, The Netherlands). Following baseline, mice were placed on a restricted feeding schedule for five consecutive days (2 h of daily access to food) and this period is defined as “Restriction Conditions”. During the re-

striction period, the food was given during the first two hours of the dark phase (the habitual activity phase of this nocturnal species). Body weight and food intake were measured before and after food access and running wheel revolutions were registered continuously. To quantify disturbed rhythms of RWA, we calculated the amount of RWA during the hours that C57BL/6J mice (the genetic control strain) has its habitual resting phase during the daily scheduled feeding paradigm (Zeitgeber Time ZT 0 (2 am)–ZT 7 (9 am)).

2.2.3 Statistical analysis

All group data are expressed as mean ± SEM unless otherwise indicated. Differences in body weight, food intake and physical activity were assessed by a general linear model (GLM) repeated measures procedure,using a between subject factor (STRAIN) and within subject factor (DAYS). Significance levels (p=0.05) were corrected using Dunnett′s approach to account for the comparison of multiple substitution strains with a single control background (p=0.004).[129] Differences in baseline body weights were assessed by one-way ANOVA. When a significant difference (p=0.004) between strains was observed, the analysis was followed by Bonferroni post hoc analysis. Differences in the percentage of mice remaining in the experiment between the control and affected strains (based on the 20% body weight loss criterion) were analyzed using Kaplan-Meier method with log rank analysis and the difference considered significant at $p<0.05$. Data were analyzed using SPSS 14.0 for Windows.

2.3 Results

2.3.1 Chromosomal mapping of the "disorganized wheel running pattern"

19 CS strains (n=14 mice on average per strain) together with their progenitor strains (A/J and C57BL/6J) were exposed to the scheduled feeding paradigm. Total daily dark phase RWA (2 pm-2 am) was significantly lower in A/J than in C57BL/6J at baseline (4602 ±620 vs 18,933 ±781 revolutions/day) and during restricted food access (5404 ±777 vs 19,159 ±692 revolutions/day on restriction day 1; 7429 ±796 vs 13,120 ±1057 revolutions/day on restriction day 4) ($F_{strain}(1, 43)=83.47$, p=0.0001) (Fig. 2.1b and f). Consistent with our previous results, C57BL/6J mice significantly reduced dark phase RWA in response to the scheduled feeding paradigm, while A/J mice exhibited a significant increase in the dark phase RWA during the scheduled feeding period.[77] Three CS strains, CSS 4, 12 and 13 displayed a disorganized wheel running pattern in response to the restricted feeding paradigm. This pattern is characterized by intense RWA during the light phase hours of the dark/light cycle, where C57BL/6J mice are normally resting. Fig. 2.1 shows case example plots of individual mice from the control (Fig. 2.1a) and from two of the affected strains (CSS12 (Fig. 2.1g), CSS13 (Fig. 2.1i)) and average daily RWA patterns in the three corresponding strains

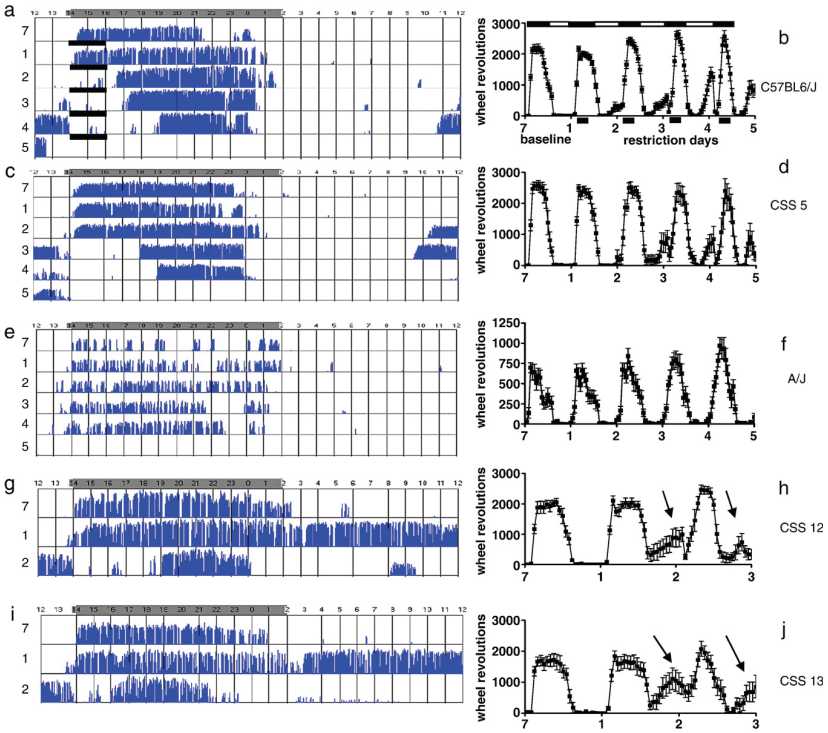

FIGURE 2.1 – ***RWA patterns in mice exposed to restricted feeding paradigm.*** *Actogram plots from 5 individual mice from the following strains: (a) C57BL/6J, (c) CSS 5 (C57BL/6J-like phenotype), (e) A/J, (g) CSS 12 and (i) CSS13 and mean RWA patterns in five corresponding strains (b) C57BL/6J, (d) CSS 5, (f) A/J, (h) CSS 12 and (j) CSS13. Disrupted wheel running rhythms in the affected strains CSS 12 and 13 are not observed during baseline conditions, but are only induced during scheduled food access (indicated by arrows). The numbers on the left side of the panels a,c,e,g,i indicate days of the experimental setting with 7 being the last day of baseline and 1, 2, 3, 4 and 5 days of the scheduled feeding. The numbers above panels a,c,e,g,i indicate the hours of the day of the early removal of the affected strains (CSS 12 and 13) the scheduled feeding the actogram plots and daily RWA patterns for these strains are shown for the first two days of the scheduled feeding only. Black bars in panel (a) indicate the hours of food access. Black-white bars above panel (b) indicate the 12-h light/dark cycle; dark boxes below x-axis of panel (b) indicates the hours of scheduled access.*

(Fig. 2.1b, h, and j). Most of the mice from affected strains were removed from the experimental setting by the third day of the scheduled feeding because they reached the maximum body weight loss criterion (20% decline from baseline body weight).

During the baseline period the number of wheel revolutions between ZT 0

and ZT 7 was similar in CSS 12, CSS 13 and C57BL/6J mice (173.42±44.71; 222.80±112.07; 110.83±33.68 respectively; p>0.05). Because of the early removal of most of the affected mice from CSS 12 and 13 (due to accelerated body weight loss), disturbed RWA was evaluated only for the first two days of the scheduled feeding paradigm. Fig. 2.2a shows the average RWA between ZT 0 and ZT 7 during the first two days of scheduled feeding for all strains tested in the panel, togetherwith the progenitor lines. Fig. 2.2b shows average RWA between ZT 0 and ZT 7 at baseline and during the first two days of the scheduled feeding for the control and the three affected strains. On the first day there was an increase in RWA in both control and the affected strain CSS12 (F_{days} (1,52)=14.55; p=0.0001) but the increase was more pronounced in the CSS 12 strain ($F_{days*strain}$ (1,52)=10.14; p=0.002). The average RWA between ZT 0 and ZT 7 for the first two days of restriction was significantly higher in CSS12 strain compared to the control C57BL/6J mice (F_{strain} (1,52)=13.48; p=0.001) (Fig. 2.2a, b). Similarly, the increase in RWA between ZT 0 and ZT 7 on the first day of restriction was significantly stronger in CSS13 strain ($F_{days*strain}$ (1,50)=23.45; p=0.0001) and the average RWA during the first two days of restriction was significantly higher in CSS13 compared to C57BL/6J (F_{strain} (1,50)=27.55; p=0.0001) (Fig. 2.2a, b). CSS 4 also displayed disorganized RWA during the hours of the light phase where the control strain C57BL/6J was inactive. However the difference in the increase in RWA from the baseline to the food restriction period between CSS 4 and the control strain did not reach the corrected significance level ($F_{days*strain}$ (1,54)=4.70; p=0.035). The lack of significant difference could be due to a higher baseline RWA between ZT 0 and ZT 7 in the CSS 4 compared to the two other affected strains (CSS 12: 173.42±44.71; CSS 13: 222.80±112.07; CSS 4: 676.07±302.33 respectively). While the difference in the increase in RWA between ZT 0 and ZT 7 did not reach the corrected significance level, the average RWA for the first two days of the restriction period was significantly higher in the CSS 4 compared to the control strain (F_{strain} (1,54)=8.82; p=0.004) (Fig. 2.2a).

2.3.2 Changes in body weight and food intake across strains

During baseline conditions, body weights of the three affected strains CSS4, CSS 12 and CSS 13 were significantly lower than that of the control strain C57BL/6J (CSS4: 20.87±0.25; CSS12: 20.52±0.39; CSS13: 20.29±0.19; C57BL/6J: 22.53±0.28) (F_{strain} (3, 77)=10.71; p=0.0001). In addition to the affected strains, a number of other strains in the panel, such as CSS 2 and CSS 5, also exhibited baseline body weight patterns similar to those of the affected strains (Fig. 2.3a). However, CSS 2 and CSS 5 lacked the disrupted light phase RWA and accelerated body weight loss in response to the restricted feeding paradigm. Food intake corrected to body weight was similar between the control and affected strains both at baseline (CSS 4: 0.185±0.08; CSS 12: 0.227±0.006; CSS 13: 0.212±0.01; C57BL/6J: 0.208±0.004) and on the second day of food restriction (CSS 4: 0.073±0.004; CSS 12: 0.078±0.009; CSS 13: 0.078±0.005; C57BL/6J: 0.086±0.002).

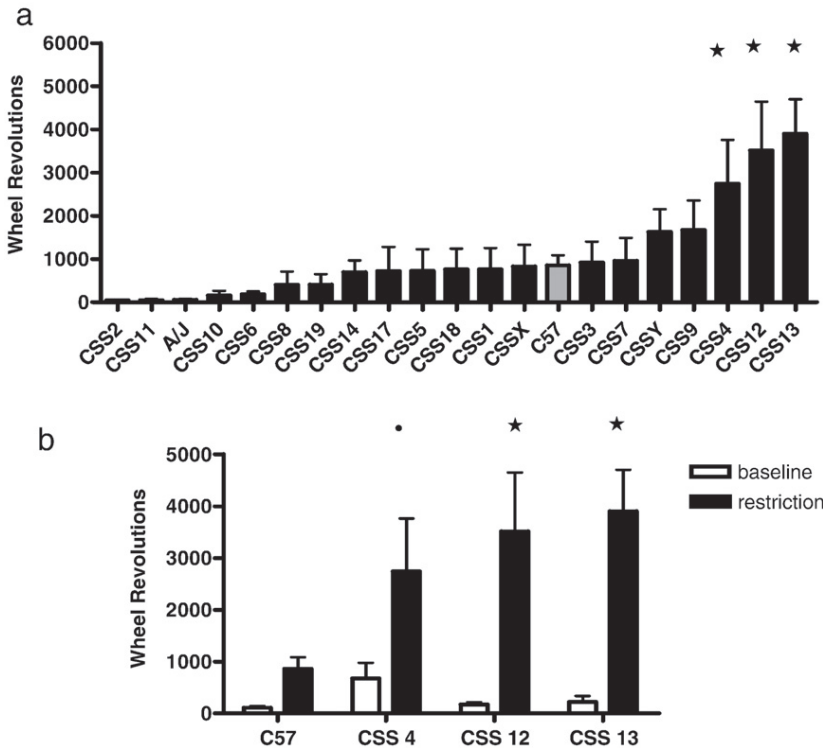

FIGURE 2.2 – ***ZT0-ZT7 RWA levels in CS strains and their progenitor lines.*** *Average RWA levels between ZT0 and ZT7 during the first two days of food restriction in all strains tested (a) and wheel activity levels between ZT0 and ZT7 at baseline and during the first two days of food restriction in the control and three affected strains (b). In response to scheduled feeding CSS4, CSS 12 and CSS 13 displayed ZT0-ZT7 RWA levels significantly higher than that of the control strain (indicated by ⋆). The increase in RWA from baseline to food restriction period reached the corrected significance level in CSS 12 and CSS 13 (indicated by ⋆); while CSS 4 displayed a similar increase, this did not reach the corrected significance level (indicated by •).*

Fig. 2.3b displays the body weight of each strain in the panel on the third day of scheduled feeding, as a percentage of the baseline body weight. Due to intense RWA during the first two days CSS 12 and CSS 13 strains displayed the lowest percentage body weight by the third day of the scheduled feeding (Fig. 2.2a and Fig. 2.3b), although their percentage body weight was not significantly different from that of the C57BL/6J. As can be seen from Fig. 2.4, by day three of food restriction a large number of mice from the CSS 12 and CSS 13

strains reached the body weight criterion (i.e. 80% of baseline body weight) and were removed from the experiment. As a consequence, the percentage of mice remaining for these strains in the scheduled feeding is significantly lower than in the control strain C57BL/6J (CSS 12: 58.33%; CSS 13: 50%; C57BL/6J: 87.04%) (Log Rank test: Chi Square=7.26; df=2; p=0.026) (Fig. 2.4). This shows that affected strains displaying disorganized rhythms during scheduled feeding have accelerated body weight loss in comparison to the genetic background control strain (C57BL/6J). The percentage body weight of the CSS 4 by the third day of food restriction period was similar to that of C57BL/6J (84.64%±1.10 and 84.98%±0.34 respectively) and 75.76% of the mice remained in the scheduled feeding following the removal of those reaching the body weight endpoint.

2.4 Discussion

In this study, a CS panel together with the progenitor strains A/J and C57BL/6J were screened for the development of excessive physical activity under the conditions of a scheduled feeding paradigm with unlimited access to a running wheel. Consistent with our previous results, C57BL/6J mice reduced their dark phase RWA levels in response to food restriction as opposed to A/J mice, which increased their dark phase RWA levels during times of low energy intake.[120,77] Three strains in the panel, CSS 4, CSS 12 and CSS 13 displayed disturbed RWA in response to the restricted feeding paradigm. This pattern is characterized by intense RWA during the light phase hours of the dark/light cycle, when C57BL/6J mice are normally resting, and accelerated body weight loss as a result. Overall, the data indicate that genetic loci on A/J chromosomes 4, 12, and 13 on a C57BL/6J genetic background contribute to the development of disorganized circadian locomotor activity levels and accelerated body weight loss following exposure to five days of scheduled feeding paradigm. This study provides a starting point for genetic fine mapping of loci affecting the development of disrupted locomotor activity in response to scheduled feeding paradigm in mice.
Body weights of the three affected strains CSS 4, CSS 12 and CSS 13 were significantly lower during the baseline period than that of the control strain C57BL/6J (see Results; Fig. 2.3a). The lower body weight in affected strains could therefore be associated with the expression of disorganized RWA during the hours of the light phase where the control strain C57BL/6J was inactive. However, as can be seen from Fig. 2.3a, a number of other strains in the panel, such as CSS 2 and CSS 5 had baseline body weights similar to that of the affected strains but did not display a disorganized wheel running pattern and accelerated body weight loss in response to the restricted feeding paradigm. This provides evidence that low body weight is not directly related to the development of disorganized rhythms, and genetic loci on A/J chromosomes 4, 12 and 13 regulate the development of disrupted locomotor activity in response to restricted feeding irrespective of the body weight patterns under ad libitum feeding conditions.
The three affected strains CSS 4, CSS 12 and CSS 13 are genetically identical to

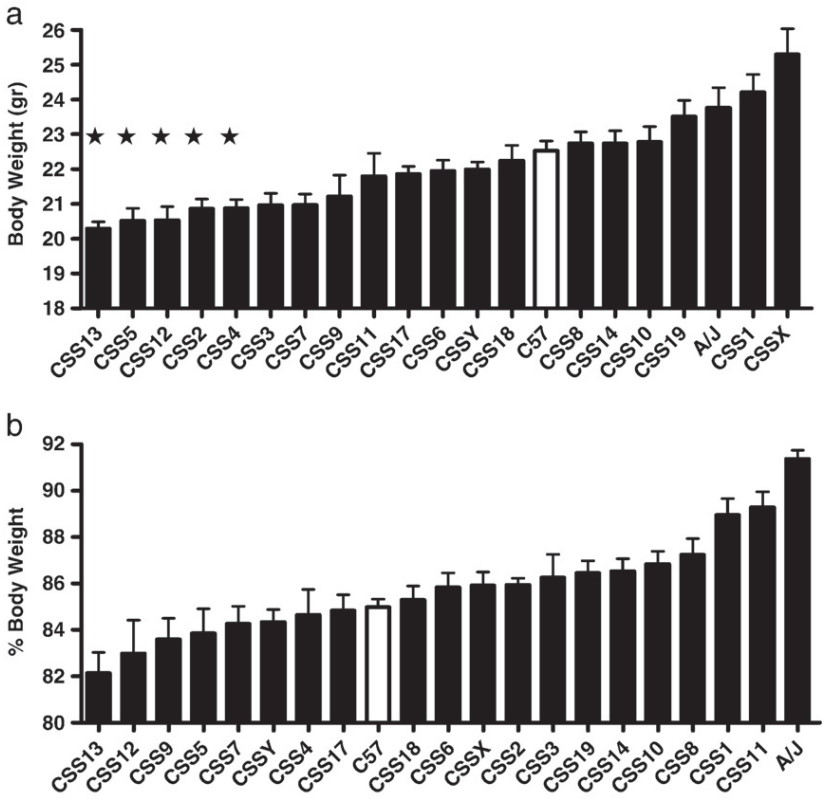

FIGURE 2.3 – ***Body weight during baseline and food restriction (as a % from baseline) in CS and their progenitor strains.*** *Average baseline body weight (a) and body weight on the third day of food restriction as a percentage of the baseline body weight (b) in all strains tested together with progenitor lines. Body weights are measured at the beginning of the dark phase (2 pm) during baseline and the food restriction period. During baseline conditions, body weights of the three affected and two other strains in the panel were significantly lower than that of the control strain (indicated by ⋆).*

C57BL/6J strain except for one entire chromosome (chromosome 4, 12, and 13) that is derived from the donor strain A/J. That these strains display disorganized RWA in response to scheduled feeding while the background strain lacks this phenotype suggests that at least one locus on A/J chromosomes 4, 12 and 13 contributes to the development of this disrupted locomotor activity. However, mice from the founder strain A/J did not display disorganized RWA when exposed to the same paradigm. This suggests the presence of transgressive segregation across the progeny, a phenomenon characterized by the emergence of

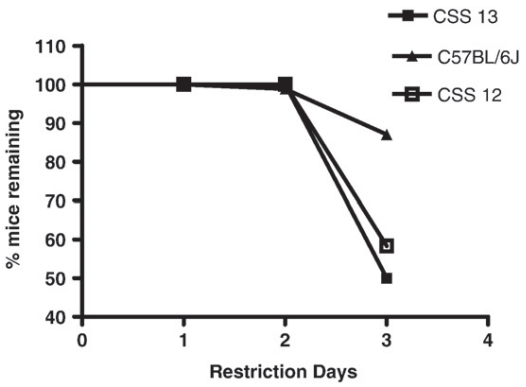

FIGURE 2.4 – ***Percentage of the mice from the control and affected strains that remained in the scheduled feeding.*** *Mice were removed from the experiment when they reached the 80% body weight criterion (relative to their baseline body weight level). 50% of mice from CSS 13 and 41.66% of mice from CSS 12 strains were removed from the scheduled feeding by the third day of food restriction period because they reached a body weight level that was 80% of their baseline body weight.*

new or extreme phenotypes in segregating hybrid populations when compared to parental strains.[130,131,132] Despite being more common in plants,[130,132] a number of animal studies have reported the emergence of transgressive phenotypes in hybrid populations such as recombinant inbred and CS strains of mice.[133,134,135,136,137] Transgressive segregation can be explained by the complementary actions of additive alleles that are amplifying and attenuating the trait and dispersed at different loci in the parental strains.[130,132] In such a case, there can be a combination of more increasing alleles among the offspring than found in either parent, leading to the emergence of transgressive phenotypes.[132] Epistatic interactions among different loci also provide an important genetic mechanism that might underlie the emergence of transgressive phenotypes in hybrid populations.[138,132,137,139] A number of studies with CS strains of mice have reported evidence of epistatic interactions among quantitative trait loci (QTLs).[140,141,142,137,84,83,143,139,144] In this study, we observed disorganized RWA in response to a scheduled feeding in three substitution strains and this pattern was absent in both parental lines. This suggests the presence of additive or epistatic interactions between loci on transferred A/J chromosomes and loci on the host genome in the affected strains.However, disrupted wheel running pattern in these strains are only induced during scheduled food access and are not observed during baseline conditions with ad libitum food intake, suggesting that these additive or epistatic interactions are dependent on environmental factors, such as limited food access. Although this pattern requires

an interaction between loci on the donor and host chromosomes, it may be possible to unravel the genetic pathways underlying its occurrence using QTL-mapping methodologies to allow the detection of epistatic QTLs that exert their effects on the traits through interactions with other genes.[145,146,147,148,149,150]
The disorganized wheel running pattern, which is characterized by an intense RWA during the light phase hours of the dark/light cycle, was apparent from the first restriction day in CSS 12 and CSS 13 strains, and from the second restriction day in the CSS 4 strain. The intense wheel activity in CSS 12 and CSS 13 strains on the first two days of the scheduled feeding paradigm led to the removal of the 40-50% of mice from the paradigm because they reached the 20% body weight loss criterion that we had incorporated in our study design. While the average RWA between ZT 0 and ZT 7 during the first two days of food restriction was significantly higher in CSS 4 compared to the control strain, only 25% of all mice tested from this strain were removed from the experimental setting on the third day of food restriction due to their early reaching of the body weight criterion. This could be due to the appearance of the disorganized running wheel pattern on the second day of the scheduled feeding, rather than the first day as was the case for the two other affected strains. The trait observed in affected strains has parallels in excessive exercising observed in AN. Several studies have shown excessive exercising in AN patients and this behavior was associated with anhedonia[90,103,106,107] or observed co-morbidities of AN such as depression and anxiety disorders[102,94,105,151,29] with OCD being significantly linked to the development of this phenotype in AN.[152,108,28,17]
The development of disorganized RWA in the affected strains following 1-2 days of scheduled food access (and the consequent removal of affected mice from the experimental setting) suggest that one or more loci on the transferred A/J chromosomes 4, 12 and 13 underlie the observed excessive weight loss in these strains during times of restricted food access. This is similar to the excessive weight loss observed in AN patients which is largely due to severe restriction in dietary intake combined with excessive exercising.[153,92,93,94] A region on human chromosome 1 (1p34.2) has been linked to the restricting subtype of AN (characterized by a severe limitation in food intake) and this region shows complete overlap with a long region of mouse chromosome 4 that was identified in our genetic screen.[154] Two suggestive linkages, one for obsessionality on chromosome 6 (6q21) and one for anxiety on chromosome 9 (9p21.3) have been found in a linkage analysis of AN[155] and both chromosomal regions show homology with mouse chromosome 4. In the same study, a significant linkage for body mass index (BMI) and a suggestive linkage for obsessionality have been found in BN and combined AN/BN cohorts on respectively human chromosomes 14 (14q21.1) and 7 (7p21.3) and both regions display homology with regions on mouse chromosome 12. Finally, a region on human chromosome 5 (5p15.3), displaying a suggestive linkage with BMI in a BN cohort, is syntenic with a region on mouse chromosome 13.[155] A linkage analysis of these regions with compulsive physical activity was not included in these studies, therefore it is not known if these regions are also linked with this phenotype.

However, these findings are relevant considering the likely contribution of anxiety and obsessionality levels to the development of excessive exercising in eating disorders.[90, 152, 108, 28, 94, 105, 29] These observed homologies at the genome level between human AN/BN and disturbed wheel running (with accelerated body weight loss) in mice provides a starting point for genetic fine mapping of loci affecting this trait. This will narrow the search for human candidate genes underlying the development of excessive physical activity during disease progress. Genes identified through fine mapping strategies can then be evaluated for their contribution to the development of excessive physical activity in human AN samples in population or family based association studies.
CS strains provide an effective and accelerated way for mapping QTLs affecting developmental, physiological and behavioral processes.[84] Since their generation, these strains have been used widely for the detection and localization of multigenic trait genes.[86, 156, 122, 123, 141, 84, 83, 128] Use of CS strains in mapping studies allows rapid identification of the chromosome(s) harboring genes that affect the trait of interest by phenotypic comparison of each inbred substitution strain with the background strain C57BL/6J. Once a strain in the panel is found to diverge from the host strain for the phenotype of interest, the underlying genetic factors can be mapped on the implicated chromosome by testing the progeny of mapping crosses between the selected substitution and the host strain.[81, 84] We showed that genetic loci on A/J chromosomes 4, 12, and 13, when present on a C57BL/6J genetic background, contribute to the development of disorganized circadian locomotor activity patterns and accelerated body weight loss during periods of negative energy balance, as observed in AN. Our data show that existing CS strains based on C57BL/6J and A/J strains are valuable tools for the chromosomal mapping of molecular determinants involved in the development of behavioral phenotypes associated with AN. Future studies involving high resolution mapping populations generated from these affected strains will help to understand the genetic mechanisms that integrate increased physical activity levels during food restriction with reward related pathways and conditions co-morbid to AN, such as mood and anxiety disorders. A better understanding of these genetic mechanisms will contribute to the development of effective novel therapeutic strategies for this disorder.

2.5 Acknowledgements

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Chapter 3

Heterosis QTL for behavioural hyperactivity on mouse chromosome 12

Eneda Pjetri, Hein A. van Lith, Ilse de Bruin, Hugo Oppelaar, Roger A. Adan, Berend Olivier and Martien J.H. Kas

Abstract

Excessive physical activity is considered an important feature of anorexia nervosa (AN) playing a significant role in the development, progression and maintenance of the disorder. Animal studies can contribute to understand the neurobiological factors that underlie the development of excessive physical activity. Similar to physical hyperactivity observed in AN patients, in activity based anorexia (ABA) rodent model, mice express paradoxical high voluntary wheel running activity levels when food restricted.

In previous studies, using a panel of mouse chromosome substitution strains, we have shown that different mouse chromosomes contribute to behavioural hyperactivity variability between different genetic mouse strains when exposed to the ABA model. In these studies, behavioural hyperactivity, in response to scheduled feeding, during the light phase hours (Zeitgeber Time 0 to 7) was mapped to A/J chromosome 12.[157] By means of phenotyping and genotyping an F_2 progeny derived from C57BL/6J and chromosome substitution strain 12 (n = 186 female mice), we genetically mapped the locus contributing to the hyperactivity phenotype on this mouse chromosome. Quantitative trait locus (QTL) analysis revealed a significant statistical association between the hyperactivity phenotype and the chromosomal segment ranging from 1 to 10 Mbp.; the QTL showed positive heterosis.

Further research, including fine mapping of the QTL, is needed to better characterize the peak and to provide a translational bridge to genetic findings in AN.

3.1 Introduction

Excessive exercise is considered a clinically relevant characteristic of anorexia nervosa (AN)[13,15,16] and is seen in 41 – 80% of people with AN.[14,17] This phenotype plays a significant role in the development, progression and maintenance of the disorder, as it is observed even when the patients are in an emaciated state. High physical activity levels are also known to have an impact on recovery rate[21,22] and outcome of the disorder.[13,23]

This behavioural feature of AN has been modelled in rodents in the activity based anorexia (ABA). In this model, rodents have unlimited voluntary access to a running wheel throughout the experiment, while food is ad libitum available for a limited period at the same time during several consecutive days.[74,75] There is a paradoxical physical hyperactivity observed under these conditions making it a core feature of this model.[76,77,78] Phenotypic screening of a mouse chromosome substitution (CS) panel, generated from C7BL/6J and A/J mice,

in the ABA model has shown potential quantitative trait loci (QTL) on mouse chromosomes 4, 12 and 13 that contribute to an increased behavioural activity, in response to scheduled feeding, in the light phase hours (Zeitgeber Time (ZT) 0 to 7).[157] In addition, these strains showed accelerated body weight loss under these conditions, being susceptible to the model.
In this CS strain panel, one of the A/J (donor) chromosomes substitutes the corresponding C57BL/6J (host) chromosome C57BL/6J-Chr#$^{A/J}$/NaJ.[81] Observed phenotypical differences between a substituted chromosome and the host strain, suggest that there is at least one underlying QTL in that chromosome contributing to the phenotype.[84] The CS strain panel is a powerful tool in identifying QTLs affecting complex traits, such as behavioural, neurological and physiological traits.[85,86,87]
To determine the location of the QTL, and identify possible candidate genes, contributing to the observed behavioural hyperactivity during ZT 0 to 7 on CS strain12, a female F_2 progeny was generated, genotyped and behaviourally screened in the ABA model.

3.2 Materials and methods

3.2.1 Animals and housing

Initial breeding pairs of C57BL/6J and C57BL/6J-Chr12$^{A/J}$/NaJ (CSS12) (JAX stock # 000664 and # 004390 respectively) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All mice used in behavioural experiments were bred in the Rudolf Magnus Institute of Neuroscience animal facility.
The housing facilities were maintained on a 12:12 h dark/light cycle with an ambient temperature of 21.0 ± 2°C and humidity of 55 ± 10%. During this period, the mice were given tap water and food ad libitum (Rat and Mouse Breeder and Grower Expanded-RM(E), Special Diet Services, Essex, UK). Following weaning at 28–30 days, female and male mice were separately housed in groups of 2 – 3 mice per cage (Macrolon®, Type II, Tecniplast, Milan, Italy). We used test - naïve, 3 – 4 months old female mice, because of the high prevalence of AN in females[6] and the observed phenotype was found in female mice.[157]
All animal experiments were approved by the Animal Experiments Committee of the University Medical Center Utrecht and Utrecht University, and were carried out in agreement with Dutch Laws (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

3.2.2 Activity based anorexia model

After an adaptation phase (7 days) where the mice had ad libitum access to food, water and voluntary access to a running wheel, they were exposed to a daily restricted feeding schedule (restriction phase) for 4 consecutive days. During this period, the food (5 pellets of about 1.2g each) was available only in the first two hours of the dark phase. Individual running wheel revolutions (RWR)

were continuously registered by a magnet activated counter (also during the adaptation phase) using Cage Registration Software (Department of Biomedical Engineering, University Medical Center Utrecht, The Netherlands).
During the restriction phase, body weight (BW) was measured daily before and after food administration. On the last day (day 4), BW was measured again at the end of the day and this measure was given the label 'day 5'.
The amount of RWR between ZT 0 to 7, corresponding to the resting hours of the host strain C57BL/6J, were measured on baseline and during the first two days of the restriction phase. The last two days of the adaptation phase were considered the baseline phase of the experiment. Quantification of the trait was calculated with the difference between restriction and baseline phase RWRs during ZT 0 to 7 (DZT).

3.2.3 Behavioural experiment

The chromosome substitution strain panel screening results of chromosome 12 were confirmed in a separate group of female C57BL/6J (n = 10) and CSS12 (n = 11) mice.

3.2.4 Generation of F_2 progeny

The F1 generation was derived by reciprocal mating of C57BL/6J and CSS12 mice. The F1 hybrids were intercrossed (brother × sister mating), producing 186 female F_2 progeny; two grandparent sets can be found on the F_2 progeny. These animals were behaviourally screened in the ABA model and genotyped with microsatellite markers.

3.2.5 DNA samples

Genomic DNA was isolated from ear punches. The mice were earmarked at weaning and the ear punches were stored at -20°C. Total genome DNA was isolated using standard DNA isolation procedures for tissue. Isolated DNA was re-suspended in distilled water at a concentration of ≈ 10 ng/µl. DNA samples were stored at 4°C.

3.2.6 PCR amplification of mouse chromosome 12 microsatellite loci

Seven microsatellites covering chromosome 12 and showing allelic difference between the C57BL/6J and A/J strains (of at least 8 base pairs), were selected from the mouse genome database (Mouse Genome Informatics Genes and Markers Query Form,
http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=markerQF) to generate the genetic map of chromosome 12.
Cox et al.[158] have constructed a revised genetic map of the mouse genome and

demonstrated that utilization of the revised genetic map improves QTL mapping. Therefore, marker positions (in cM) were taken from this map by using the 'mouse map converter' (http://cgd.jax.org/mousemapconverter/). The markers and their position are shown in figure 3.1.

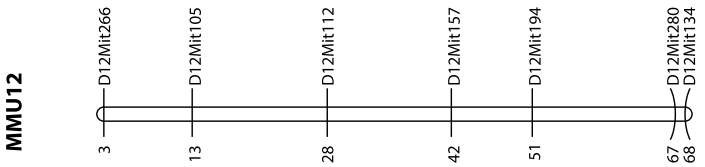


FIGURE 3.1 – *Mapping of the seven microsatellite markers in murine chromosome 12 (MMU12).*

Primers flanking these microsatellites (MIT mouse MapPair primers) were purchased from Sigma-Aldrich. Thermal cycling was performed in a DNA Engine (Applied Biosystems,Veriti® Thermal Cycler, CA, USA). For the following seven microsatellites: D12Mit266,D12Mit105, D12Mit112, D12Mit157, D12Mit194, D12Mit280, D12Mit134, the genomic DNA samples were amplified according to the supplied protocol accompanying the microsatellite primers using Taq polymerase (Bioline Biotaq). PCR products were analyzed by electrophoresis on 3% (w/v) agarose gels (Agarose MP, Roche, Germany) and visualized by ethidium bromide staining. From the gel, a digital image was created and alleles were assigned by sight by two different raters.

3.3 Statistical analysis

3.3.1 C57BL/6J and CSS12 behavioural data

One sample Kolmogorov-Smirnov test was used to check the Gaussian distribution and Levene′s test for the homogeneity of variances of the data. Data were parametric and two tailed, unpaired Student′s t-test was used for analysis between the C57BL/6J and CSS12 RWR between ZT 0 and ZT 7. Data are shown as means ± standard error of mean (SEM). The level of BW loss during the scheduled food restriction days was selected as a measure of susceptibility to the ABA. Based on the level of BW loss, a dichotomous variable for ABA susceptibility was generated, with each individual mouse assigned a value whether it was susceptible or not. If a mouse lost 15% or more of their baseline BW on any experimental day, that mouse was taken out of the experiment (based on the ethical humane endpoint criteria).
ABA susceptibility is a 'time to an event parameter' and therefore it was analyzed as survival data; the results were plotted as Kaplan-Meier curves. The Kaplan-Meier graphs were compared using the Mantel-Cox Log-Rank test.

3.3.2 Genetic Map

Segregation ratio of the genotypes of individual microsatellite markers (MSMs) was checked with the Chi-squared goodness-of-fit test. Even though the F1 mice were heterozygous for the MSMs, four of the markers showed significant segregation distortion from the expected ratio 1:2:1 (BB:BA:AA - homozygous C57BL/6J:heterozygous:homozygous A/J) (table 3.1). This significant difference from the expected ratio is called 'transmission ratio distortion' (TRD).[159,160] TRD event (two alleles of a heterozygote are transmitted unequally) happens in many organisms and several mechanisms have been involved in the process that occurs during fertilization.[161]

TABLE 3.1 – *Segregation ratio of individual microsatellite markers. Abbreviations: Df (Degrees of freedom), Sig. (Significance). Significant differences are indicated by (*).*

Marker	Chi-Square	Df	Sig.
D12Mit266	11.77	2	0.003*
D12Mit105	17.23	2	0.000*
D12Mit112	7.946	2	0.019*
D12Mit134	7.099	2	0.029*
D12Mit157	0.836	2	0.669
D12Mit194	1.121	2	0.581
D12Mit280	4.957	2	0.087

Recent data suggest an overdominance TRD QTL present on chromosome 12,[162] which may explain the TRD observed in our sample. In general, segregation distortion of markers will not have a great impact on the estimation of QTL position and effect.[163]

3.3.3 Quantitative Trait Loci (QTL) analysis of DZT

R/qtl software package[164] was used in the analysis of the QTL location. The DZT was not normally distributed and the non-parametric mapping approach was applied.[165] Results were expressed as LOD scores. Permutation tests (random shuffling of genotypes with phenotypes, 10000 permuations) were done to assess the statistical significance of a QTL; the 5% significance level was chosen. The percentage of total phenotypic variance explained by the QTL was calculated as $100\left(1-10^{-\frac{2}{n}\mathrm{LOD}}\right)$, where n is the number of individuals.

3.3.4 Co-segregation analysis of the F_2 progeny

After grouping by genotype for the DNA marker at the peak of the QTL, trait comparison of the F_2 animals was performed. If a DNA marker and the trait of interest are segregating independently, the values of the trait will be equally distributed among the homozygote and heterozygote genotypes. The Kolmogorov-

Smirnov one-sample test was used to check normality and Levene′s test for the homogeneity of variances of these data. The DZT data within genotype groups for the marker at the peak were not normally distributed.
Furthermore, the variances of these DZT data were not equal. Therefore Kruskal Wallis test was used for analysis. Post hoc analysis between the genotype groups was done with Wilcoxon-Mann-Whitney test. Significance was set at $p < 0.017$ after being adjusted for multiple testing with Dunn-Šidác correction $\alpha = 1 - (1 - 0.05)^{\frac{1}{n}}$, n = number of genotypes (3).[166] The mode of inheritance was based on the degree of dominance which was calculated as the ratio of dominance to additive effect (|d/a|).
Dominance was calculated as the DZT difference between heterozygote group and the average of two homozygote groups. Additive effect was calculated as half the DZT difference between two homozygote groups. Dominance and additive effects were also calculated with the ranks, as the DZT is not normally distributed. Based on the criteria by Stuber,[167] the QTL peak was additive when the ratio was < 0.2; partially-dominant 0.2 ≤ ratio < 0.8; dominant 0.8 ≤ ratio < 1.2 and over-dominant if ratio ≥ 1.2).

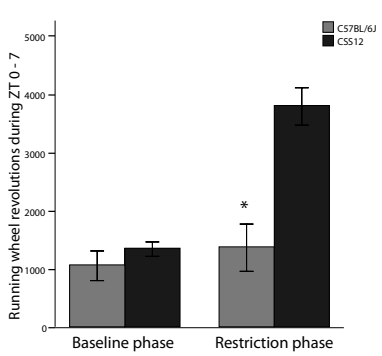
3.4 Results

3.4.1 C57BL/6J and CSS12 phenotypic data

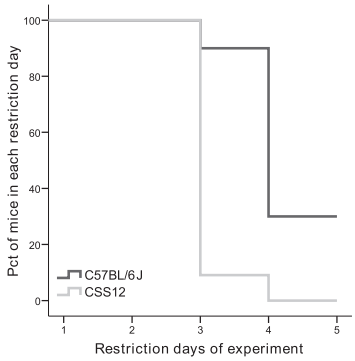
In a separate group of mice, we first confirmed the previous observations[157] that CSS12 mice become hyperactive during ZT 0 to 7 in the restriction phase, C57BL/6J: 1376 RWR (±406 SEM) and CSS12: 3680 RWR (±315 SEM), $t_{(19)}$ = -4.527, $p < 0.001$, figure 3.2(a)). This difference was not seen under baseline conditions when mice have food and water ad libitum, C57BL/6J: 1064RWR (±254 SEM) and CSS12: 1279RWR (±134 SEM), $t_{(19)}$ = -0.770, $p = 0.451$, figure 3.2(a)).
CSS12 mice showed also an accelerated body weight loss, with ≈ 90% of mice being taken out of the experiment on day 3, and were quite susceptible to the model when compared to the C57BL/6J mice (χ^2 = 13.02, df = 1, $p = 0.0003$, figure 3.2(b)).

3.4.2 QTL analysis of DZT in the CSS12-F_2 population

An F_2 progeny was generated by inter-crossing of CSS12 × C57BL/6J F1 hybrids, and the female F_2 progeny was both genotyped with microsatellite markers and behaviourally screened in the ABA model. The phenotype of the F_2 progeny showed a right shift towards an increased difference between restriction and baseline wheel running activity levels between ZT 0 to 7 (figure 3.3(a)).
QTL analysis revealed a significant association for this phenotype with the proximal region of chromosome 12; the highest LOD score was 2.14 (Figure 3.3(b)). The peak explained 5.16% of the DZT variance in the F_2 progeny. The exact location of the peak is, at this stage, difficult to define as only part of the peak is



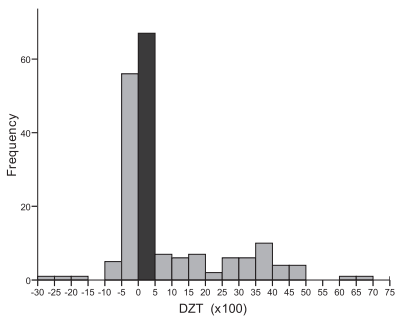
(a) *Zeitgeber Time (ZT) 0 to 7 running wheel revolutions levels during baseline and restriction phase. There is a significant difference between the strains during the restriction phase indicated by (*). Data are shown as mean ± SEM.*



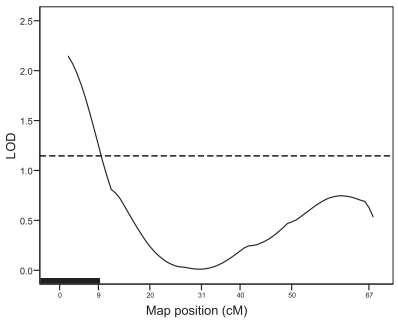
(b) *Percentage (Pct) of mice that completed the restriction days for C57BL/6J and CSS12 strain. CSS12 strain mice lost weight rapidly and ≈90% were taken out of the experiment on day 3.*

FIGURE 3.2 – *C57BL/6J and CSS12 phenotypic data.*

seen, however, if we were to take that LOD 2.14 is the actual height of the peak, QTL interval is located between 1 ≈ 10964203 bp (including the 1-LOD support interval for this LOD).



(a) *Histogram of the CSS12* F_2 *-progeny phenotype (the dark bar indicates the C56BL/6J average). A right shift in phenotype can be seen on the right.*



(b) *Proximal QTL peak with an LOD of 2.14; the supporting interval is shown by dashed line and the corresponding position with the dark bar on the x-axis.*

FIGURE 3.3 – *QTL mapping on chromosome 12.*

The microsatellite marker at the peak is D12Mit266. The co-segregation analysis revealed a difference in phenotype between the genotypes (Kruskal Wallis test, $\chi^2 = 9.6$, df = 2, p = 0.008). A (positive) heterosis effect was observed in the

phenotype with the heterozygous phenotype exceeding both of the parents (BA vs BB Z = -2.507, p = 0.012; BA vs AA Z = -2.351, p = 0.018); BB vs AA Z = -0.156, p = 0.881). The mean and the ranks of the phenotypes are shown in table 3.2.

TABLE 3.2 – *DZT phenotype of the D12Mit266 marker genotypes.*

Genotype	Mean DZT	Rank
BB	282.20	75.91
AA	513.78	79.13
BA	1063.65	103.10

The degree of dominance analysis revealed an overdominant effect of this heterosis peak with a ratio (|d/a|) of 5.75 when the analysis was carried out with the means. As the data were non parametric, the rank analysis revealed an even stronger overdominant effect with a ratio (|d/a|) of 15.92.
Thus, at the current stage, we have identified a heterosis peak located in the proximal region of chromosome 12 which explains 5.16% of the observed phenotypic variance in F_2 progeny.

3.5 Discussion

To indentify the QTL(s) on mouse chromosome 12 that contributed to the increased hyperactivity phenotype, as found in the initial screen with a mouse panel of chromosome substitution strains[157] and confirmed in a separate sample, we genotyped and behaviourally screened in the ABA model 186 female F_2 progeny. QTL analysis revealed a significant association with the proximal region (1 ≈ 10 Mbp) of chromosome 12 and points to positive heterosis.
The location of the QTL peak is of interest in the regulation of activity. Mapping of locomotor activity in another mouse genetic population has found a QTL peak also in the proximal region, 11 cM, of chromosome 12.[168] It is very interesting that these two peaks (from different genetic populations) are close to each other, suggesting an involvement of that region in the regulation of behavioural activity.
The phenotype of the heterozygous genotype (BA) exceeds both of the homozygous phenotypes (C57BL/6J and A/J). At the peak marker, BA genotype mice had a greater increase in activity during ZT 0 to 7 of the first two days of restriction phase, exceeding also the A/J genotype. There are several explanations for the heterosis effect, such as genetic, dominance, overdominance, epistasis or differences in gene expression,[169] and lately a role for epigenetic modifications has been hypothesized.[170] Our results support the overdominance hypothesis, since the mode of inheritance analysis revealed an overdominance effect.
Heterosis effect has also been observed in several traits in mice such as, body weight,[171] locomotor activity responses to ethanol[172] and relevant to our phenotype, it has been observed in F1 (male) lines that were selectively bred for high

voluntary running wheel activity.[173]
The first next step in the study is to further fine map the region by genotyping additional microsatellite markers and find the precise location of the peak, as currently we only see part of it. With these results we hope to find a genetic basis of the hyperactivity that occurs under scheduled food restriction, which is similar to the excessive exercise observed in people with AN, therefore increasing our understanding of this complex disorder.

Chapter 4

Maternal environment affects adult genetic susceptibility to activity based anorexia and epigenetic programming of neurodevelopmental genes in mice

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Abstract

Early life environment and genetic background interplay is thought to have a significant role in the neurodevelopmental trajectory of (mal-)adaptive behavior, in both humans and animals. By means of early life inter- and intra- strain cross-fostering experiments in mice, we show here that the genetic background susceptibility to activity based anorexia model (ABA) in adulthood can be reversed; ABA is a well-known rodent model for studying pathophysiological processes in anorexia nervosa. In these experiments, low C57BL/6J and high C57BL/6J-Chr $4^{A/J}$/NaJ (CSS4) ABA susceptible mouse inbred strains were used. Independent of the foster mother genetic background, fostered CSS4 mice were less susceptible to ABA when compared to CSS4 mice raised by their biological mother (same strain foster mother: χ^2 = 7.690, p = 0.006; different strain foster mother: χ^2 = 7.874, p = 0.005). In contrast, no cross-fostering effects were observed on ABA susceptibility in the low susceptible C57BL/6J strain. Global DNA methylation analysis revealed several differentially hypermethylated genome regions as a function of cross-fostering in CSS4 strain. These hypermethylated regions included genes, such as Cntnap2, that have been associated with neurodevelopmental disorders.
Thus, early life events may modulate genetic background susceptibility for adult maladaptive behavior via epigenetic modifications and provide a basis for studies towards further understanding of neurodevelopmental processes underlying adult behavior.

4.1 Introduction

Human studies have shown that the interaction between genetic and environmental factors contribute to adult expression of psychiatric phenotypes, such as anti-social behavior.[174] Recently, gene-environment interactions have been increasingly reported for a wide range of mental disorders such as schizophrenia, depression,[175] and eating disorders.[176,31,177,39] Anorexia nervosa (AN) is a complex eating disorder with highest prevalence in young adult females (age 15 – 19 years)[6,10] and is associated with the largest mortality rate among psychiatric disorders.[4,5] Various genetic, environmental and developmental risk factors are thought to contribute to this dramatic disease.[178,33,177,39,179,36,180] However, little is known about the developmental trajectory that preceeding this psychiatric disorder. Systematic longitudinal studies with controlled genetic and environmental background variation are necessary to identify and understand the mechanisms underlying the interplay between environment and genetic background susceptibility for maladaptive behavior. As it is very difficult

to control for these aspects in humans across different developmental stages, animal models are best suited for understanding the neurodevelopmental trajectory of adult maladaptive behavior.
The activity based anorexia (ABA) rodent model mimics the paradoxical expression of behavioral hyperactivity in periods of reduced food intake that is also observed in anorexia patients.[181,182] In this rodent model, animals have unlimited voluntary access to a running wheel throughout the experiment, while food is ad libitum available for a limited period at the same time during several consecutive days.[74,75] The physical hyperactivity observed during food restriction leads to accelerated body weight loss, reminiscent of the features of the disease.[77,78]
We have shown that ABA susceptibility is dependent on the genetic background[76] and further genetic mapping, using chromosome substitution (CS) strains of mice, pointed to chromosomes related to high ABA susceptibility.[157] In a CS strain panel, generated from C57BL/6J and A/J mice (C57BL/6J-Chr#$^{A/J}$/NaJ[81]), C57BL/6J-Chr4$^{A/J}$/NaJ (CSS4) mice apart from being hyperactive, had also found lower body weight than the C57BL/6J control and highly susceptible to the ABA model. Interestingly there was no body weight difference on the day of birth between these two strains which only became apparent on postnatal day (PND) 14. These findings indicate that maternal contributions during those early days of life is likely an important factor for their early life body weight levels.
In the present study, the relationship between maternal environment, body weight genetic background and epigenetic mechanisms was studied using high ABA (CSS4) and low ABA (C57BL/6J) susceptible mouse inbred strains.

4.2 Materials and methods

4.2.1 Animals and housing

All mice used in behavioral experiments were bred at the Rudolf Magnus Institute of Neuroscience animal facility. Initial breeding pairs of C57BL/6J (B6J; JAX stock # 000664) and C57BL/6J-Chr4$^{A/J}$/NaJ (CSS4; JAX stock # 004382) mice were obtained from The Jackson Laboratory, Bar Harbor, ME, USA.
The housing facilities were maintained on a 12:12 h dark/light cycle with an ambient temperature of 21.0 ± 2°C and humidity of 55 ± 10%. During this period, the mice were given tap water and food ad libitum (Rat and Mouse Breeder and Grower, Special Diet Services, Essex, England). Following weaning at 28 days, female and male mice were separately housed in groups of 2 – 3 mice per cage (Macrolon®, Tecniplast, Milan, Italy). We used test-naive, 3 – 4 months old female mice, because of the high prevalence of AN in females.[6,5]
All animal experiments were approved by the Animal Experimentation Committee of University Medical Center Utrecht and were carried out in agreement with the Dutch Law (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

4.2.2 Activity based anorexia model

The mice were placed in individual cages with voluntary access to running wheels with food and water ad libitum. After the mice were left to adapt to the conditions for 7 days, they were exposed to a daily restricted feeding schedule (restriction phase) for 4 consecutive days. During this period, the food (5 pellets of about 1.2g each with an energy intake of 3.31 kilocalorie (kcal) per gram) was available only in the first two hours of the dark phase.
Individual running wheel revolutions (RWR) were continuously registered by a magnet activated counter (also during the adaptation phase) using Cage Registration Software (Department of Biomedical Engineering, University Medical Center Utrecht, The Netherlands).
As baseline measures for body weight (BW) and food intake (FI), the average of last three days of the adaptation phase, measured just before the dark period, were used. Baseline wheel running activity (wheel revolutions) (WRA) levels were determined as the average WRA of the last two days of the adaptation phase. During the restriction phase, BW was measured daily before and after food administration.On the last day 4, BW was measured again at the end of the day and this measure was given the label 'day 5'.
The level of BW loss during the scheduled food restriction days was selected as a measure of susceptibility to the ABA. Based on the level of BW loss, a dichotomous variable for ABA susceptibility was generated, with each individual mouse assigned a value whether it was susceptible or not. If a mouse lost 15% or more of their baseline BW on any experimental day, the mouse was taken out of the experiment (based on the ethical humane endpoint criteria set prior to the onset of the experiments).

4.2.3 Cross fostering procedure

Breeding consisted of two multiparous females being put together with one male of the same strain. The females were separated in individual cages, with bedding and tissue from the mating cage, between 4 – 7 days before birth. After separation, pregnant mice were monitored daily in the morning and afternoon. The foster procedure took place within 12 – 24 hrs after birth. After taking the body weight, pups were put in the new foster nest, gently rubbed with it, and placed in the cage of the foster mother, either from the same genetic background strain (CSS4) or a different strain (B6J). During the fostering procedure, litters with similar number of pups were cross-fostered.
The procedure of weighing and fostering the pups lasted on average 2 – 3 min and the investigator wore gloves during the procedure; the gloves were changed between litters. From these procedures, 3 groups per strain were formed: pups raised by their biological mothers (BM), pups raised by the same strain foster mothers (SsFM) and pups raised by the different strain foster mothers (DsFM). Each litter was considered one experimental unit,however, because of the difficulties encountered with breeding of the CSS4 strain (see below in the section

of maternal behavior), two pups per litter were used for behavioral testing.

4.2.4 Maternal behavior toward biological pups

To have an indication of the time the dams spent with the litter, we observed the dams in their home cages with a top unit containing the PhenoTyper system (Noldus Information Technology, Wageningen, The Netherlands) which was connected to EthoVision® video tracking system (Noldus Information Technology, Wageningen, The Netherlands). This system can monitor the mouse behavior without any interference from the investigator for several consecutive days. Details on the home cages and top unit can be found in the paper by Kas et.al.[122]
Six B6J and five CSS4 dams were placed in these home cages after being separated from the mating cage. When the pups were born, the time the dam spent with the litter was monitored for the first 3 days (the sensitive period for bonding) through the recordings of the top unit. It was observed that the first three days are the most important in bonding with the pups. The nest position was monitored daily by the investigator two times per day. The place of the nest was corrected when necessary in the analysis. During this period the cumulative food intake of the dam was measured for a period of 15 days after the litter was born, which is the period when pups are fully dependent on the mothers′s food supply.[183]

4.2.5 Ultrasonic pup vocalization procedure

The pups were 10 days old at the time of testing. B6J (n = 5 females) and CSS4 (n = 4 females) pups coming from different litters were used to assess pups vocalization, with the protocol similar to Groenink et al.[184] Pups were separated from their mother 30 min before the test and were placed on a warm mat at 37°C.
Apparatus: Pup vocalization was measured for duration of 3 min on a 21°C plate. The temperature of the plates was maintained through a water system. The pup was placed on the middle of the plate and a plexiglass cylinder (diameter of 19cm). Calls were recorded using Ultra Sound Advice S-25 bat detector placed 14cm above the plate, on the lid of the cylinder. The detector is part of an automated system which transmits the sound to an audio filter converter connected to a computer with analysis software (Ultravox 2.0®, Noldus Information Technology, Wageningen, The Netherlands).

4.2.6 Body weight

Body weight was measured for each of the pups at PND 0 and 14. The gender of the mouse pup can be determined by the presence of a pigmentation spot on the perineum of the male mouse between the genital papilla and the anus.[185] However, on PND 0 it was difficult to identify the gender of the CSS4 mice as

the fur coat is light brown/gray and the pigment spots are not visible,[185] that is why all pups are included in the early life body weight analysis. At PND 14 the pups are still dependent on the mother for feeding so we included all the pups independent of gender in this analysis (at this stage we were able to differentiate between the gender and analysis revealed that there were no differences in the body weight between the male and female pups in any of the groups (data not shown).

4.2.7 Global DNA Methylation

DNA methylation levels in CSS4 strain BM and fostered groups (n = 2 per experimental group) were examined. DNA was isolated from right hippocampal tissue, dissected from thawed fresh frozen brains, at PND 35. Genomic DNA was extracted and purified by standard procedures.
Methylation analysis was carried out using Methyl Binding Domain (MBD)-based Genomewide Methylation by the Nxt-DX company, Gent, Belgium.

Quality Control
The purified genomic DNA (A260/A280 ratio of 1.8 – 2.0) was quantified by Quant-iT^{TM} PicoGreen (Invitrogen, UK).
After fragmentation a second quality control was done on Agilent 2100 HS DNA chip. Concentration was determined with smear analysis on the Agilent 2100 and the samples were checked for degradation.

Fragmentation and methyl binding domain capture
Fragmentation of methylated DNA was performed on Covaris S2 with the following settings: duty cycle 10%, intensity 5, 200 cycles per burst during 190 sec, to obtain fragments with an average length of 200 bp. The power mode was frequency sweeping,temperature 6 – 8°C, water level 12. Maximum 3 µg was loaded in 130 µl TE in a microtube with AFA intensifier (Covaris, Woburn, Massachusetts, USA). For samples with less input DNA (down to 500 ng), DNA was diluted in 1:5 TE. DNA with an input of 3 µg was then analyzed on the Agilent 2100 (Agilent Technologies, Santa Clara, California, USA). DNA with an input lower than 3 µg was concentrated in a rotary evaporator to 25 µl and the fragment distribution was checked on a high sensitivity DNA chip. Methylated DNA was captured using the MethylCap kit (Diagenode AF-100-0048, Belgium). The concentrations of the fragmented and captured DNA were determined on a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) with the Quant-iT^{TM} Picogreen® dsDNA assay kit (Invitrogen P7589, Merelbeke, Belgium) on 480/520 nm.

Library preparation, Amplification and Sequencing
This is a modification of the 'multiplexed paired end ChIP protocol' (Illumina, San Diego, California, USA). DNA Sample Prep Master Mix Set 1 (NEB E6040) in combination with the Multiplexing Sample Preparation Oligo Kit (96 samples,

Illumina PE-400-1001) was used. For the library preparation, 250 ng of fragmented DNA were used. Library preparation was performed on Apollo 324 with PrepX-DNA Library Kit 24 samples per kit (400007) or PrepX-32-DNA Library Kit 96 samples per kit (400021) according to the kit′s protocol.
Library amplification was performed according to multiplexed paired end ChIP protocol, including the indexes from Multiplexing Sample Preparation Oligo Kit. When necessary, smaller fragments were removed on 2% agarose gel (Low Range Ultra agarose Biorad 161-3107), with a 1 Kb Plus ladder (Invitrogen 10787-018), which was run at 120V for 2 hours. A fragment of 300 bp ±50 bp was excised and eluted on a Qiagen Gel Extraction Kit column (Qiagen28704), then eluted in 23 µl EB.
From this elution, 1 µl was put on an Agilent 2100 HS DNA chip to determine the concentration with smear analysis. Following the determination of the concentrations, the samples were diluted to a final concentration of 15nM. qPCR was performed on the (1:500 diluted) samples, with PhiX index3 as standard, results are shown in table 4.1.
All samples were adjusted to 10 nM and were pool-indexed per lane. The paired end (PE) flow cell was prepared according to the Cluster Station User Guide (Manual). The samples were sequenced in Illumina Hi-Seq 2000.
The average size of the fragments in the MBD library was 300-400 bp (with 90 bp of adapter ligation).

TABLE 4.1 – *qPCR measurements: Abbreviations: BM (Biological mother), SsFM (Same strain foster mother), and DsFM (Different strain foster mother), '1' (sample 1), '2' (sample 2).*

Samples	MID	Average c(t)	Log concentration	Input concentration qPCR	pM	nM (diluted library)
BM1	1	6.5	1.730582	53.78	26888	26.9
BM2	7	6.5	1.726304	53.25	26624	26.6
SsFM1	8	6.6	1.696356	49.70	24850	24.8
SsFM2	9	6.7	1.677816	47.62	23811	23.8
DsFM1	1	7.0	1.590824	38.98	19489	19.5
DsFM2	1	7.0	1.585120	38.47	19235	19.2

BaseCalling
The FASTQ sequence reads, forward and reverse, were generated using the Illumina Casava pipeline version 1.8.0.

Sequencing Quality Control
Initial quality assessment was based on data passing the Illumina Chastity filter. Subsequently, reads containing adaptors and/or Phix control signal were removed. The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.10.0.

Mapping
The paired end 51 bp sequence reads were mapped using bowtie (v0.12.7) software.[186] The bowtie parameters were set to 0 mismatches in the seed (first 28 nucleotides). Only unique paired reads were retained and both fragments must be located within 400 bp of each other on the mouse reference genome build NCBI37/mm9. An overview is shown in table 4.2.
The mapped paired ends reads allow giving every nucleotide in the genome a coverage value. Multiple paired reads with the exact same location in one sample are discarded, as these are most likely amplified from the same sequence. A histogram of the number of CpGs in the mapped PE sequences per sample, showing also the quality of the enrichment is shown below in figure 4.1. Methylated regions were annotated with the genes in their surroundings,

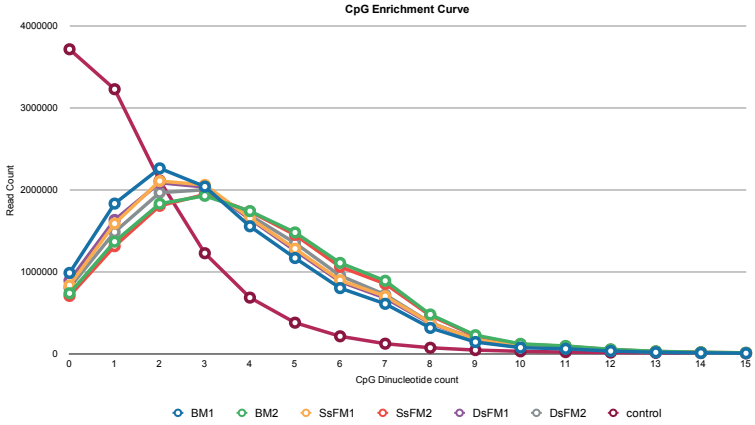

FIGURE 4.1 – *Amount of paired-end mapped reads versus the number of CpG's in the sequence for each sample. Abbreviations: BM (Biological mother), SsFM (Same strain foster mother), and DsFM (Different strain foster mother), '1' (sample 1), '2' (sample 2).*

and categorized as Promoter (-2000 to 500 relative to TSS), Exon, intronic or intergenic. Ensembl Mus musculus v64 was used to annotate the regions.

4.2.8 Statistical analysis

For all the experiments, the one sample Kolmogorov-Smirnov test was used to check the Gaussian distribution and Levene's test was used for homogeneity of variances. When data were not normally distributed, non-parametric statistical tests were used.
Maternal bonding per strain was assessed based on the number of litters still

TABLE 4.2 – *Overview of total reads and mapped reads per sample. Abbreviations: BM (Biological mother), SsFM (Same strain foster mother), and DsFM (Different strain foster mother), '1' (sample 1), '2' (sample 2).*

Samples	Lane	Index	Total number of reads	Paired end reads	Mapping percentage	Mapped paired end reads
BM1	7	1	51462234	25731117	59.44	15294576
BM2	7	7	52468854	26234427	53.85	14127239
SsFM1	7	8	52117700	26058850	57.57	15002080
SsFM2	8	9	45636902	22818451	52.76	12039015
DsFM1	8	10	69043548	34521774	60.36	20837343
DsFM2	8	11	58003184	29001592	56.72	16449703

being present at PND 3 as a percentage of the number of litters at PND 0.
ABA susceptibility was analyzed using Kaplan-Meier survival analysis (KMSA) with Mantel-Cox Log-Rank test. The data are shown in Kaplan-Meier graphs separated by strain.
Litter differences were analyzed using the Mann Whitney U test. The data are presented as mean ± standard error of mean (SEM). Significance was set at $p < 0.05$.
Home cage: The percentage of time that the dams spent in the nest zone in the first three days (day 1 (D1) is the day the nest was born) was used to measure the time the dams spent with their nests, after bonding had occurred. The data were parametric and independent samples T- test was used for analysis between the two strains for each of the days. The data are presented as mean ± SEM. Significance was set at $p < 0.05$.
Cumulative food intake: The data parametric and the independent samples T- test and Pearson′s correlations test were used for analysis between the two strains.
The difference in the number of pups was analyzed using Mann Whitney U test. The data are presented as mean ± SEM. Significance was set at $p < 0.05$.
Ultrasonic pup vocalization: in this experiment the number of calls were analyzed. The data were not normally distributed and the Mann Whitney U test was used to analyze the number of calls. The data are shown as median with min and max values.

Methylation analysis
To acquire quantitative results we studied the methylation status of all Methylation Cores (MC) — a set of genomic regions that are potentially and putatively independently from each other, methylated. MCs located in the promoter, exon and intron regions of the genome with at least 2 mapped reads in all samples combined were studied. Results were calculated with R statistical software package baySeq.[187] This package calculates the likelihoods with which a MC sup-

ports a predefined set of sample groupings. In this analysis, two models of interest were defined: equal methylation (EM) and differential methylation (DM) between the two groups. Every model gets a likelihood value and a false discovery rate (FDR) was used to rank the regions.
Analysis revealed differentially methylated gene regions in both fostered groups when compared to CSS4 BM group. The gene regions were either hypermethylated (the levels were higher) or hypomethylated (the levels are lower) relative to the BM group. A region was considered to be significantly differently methylated if (i) methylation change were 5 fold or higher, in all samples within a group, and (i) the FDR was < 0.5 or 0.05, based on the baySeq package threshold analysis. FDR is corrected for multiple testing and is used in the ranking of the values.

4.3 Results

4.3.1 C57BL/6J and CSS4 strains ABA susceptibility

Previous research in our laboratory has shown that adult CSS4 strain mice have a lower body weight and disturbed running wheel activity levels under scheduled food restriction.[157] This strain was also significantly more susceptible to the model, with all the mice being taken out of the experiment due to body weight criteria. In contrast, only 20% of B6J mice were taken out of the experiment based on this susceptibility criteria (χ^2 = 10.552, p = 0.001; figure 4.2).

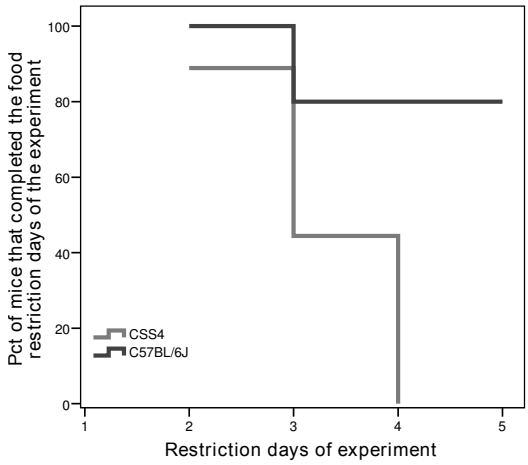

FIGURE 4.2 – *Percentage (Pct) of mice that completed the restriction days for CSS4 strain and C57BL/6J strain. There was a significant difference between the strains.*

4.3.2 Early body weight

While body weight was significant different in adulthood between the high (CSS4) and the low (B6J) ABA susceptible strains, there was no significant difference in body weight of pups (male and female) at PND0 (B6J 1.33g (± 0.01 SEM) and CSS4 1.34g (± 0.01 SEM); $t_{(413)} = 1.016$, p = 0.310) while at PND14 B6J pups were significantly heavier than CSS4 pups (7.06g (± 0.10 SEM) vs 6.48g (± 0.12 SEM); $t_{(144)} = -3.818$, p = 0.0002; figure 4.3(a)).
The number of pups per litter was not significantly different at PND0 (B6J $n = 7$ (± 0.7 SEM) and CSS4 $n = 6$ (± 0.4 SEM); $t_{(32)} = 0.708$, p = 0.484), but there was a significant difference at PND14, where the number of pups in CSS4 litter was significantly decreased (B6J $n = 6$ (± 0.6 SEM) and CSS4 $n = 4$ (± 0.4 SEM); $t_{(26)} = 2.978$, p = 0.006; figure 4.3(b)). Thus, despite a decrease in litter size, CSS4 pups showed a significant lower BW of the pups at PND14.

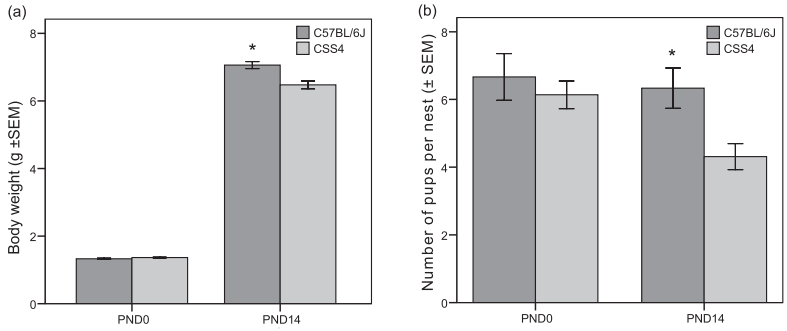

FIGURE 4.3 – ***C57BL/6J and CSS4 strain body weight and number of pups.*** *Body weight of the pups (a) and the number of pups per nest (b) in postnatal day (PND) 0 and PND14. Significant differences are denoted by (*).*

4.3.3 Early environment: dam and pup behavior

Maternal bonding, necessary for the initiation and maintenance of maternal behaviors, was highest in B6J dams with 95% of litters born still being alive at PND3 versus 39.6% in CSS4 dams. CSS4 mice were actively scattering the pups around the nest, while the B6J dams were keeping them together in the nest (figure 4.4(a)). However, when bonding had occurred, home cage recordings showed that CSS4 dams spent more time in the nest zone than B6J dams during these first 3 days (D1: B6J 70.30% (± 1.60 SEM) and CSS4 78.72% (± 1.12 SEM), $t_{(9)} = -4.145$, p = 0.003; D2: B6J 67.16% (± 1.20 SEM) and CSS4 74.92% (± 1.66 SEM), $t_{(9)} = -3.871$, p = 0.004; D3: B6J 63.39% (± 1.90 SEM) and CSS4 72.19% (± 2.73 SEM), $t_{(9)} = -2.718$, p = 0.024; figure 4.4(b)).

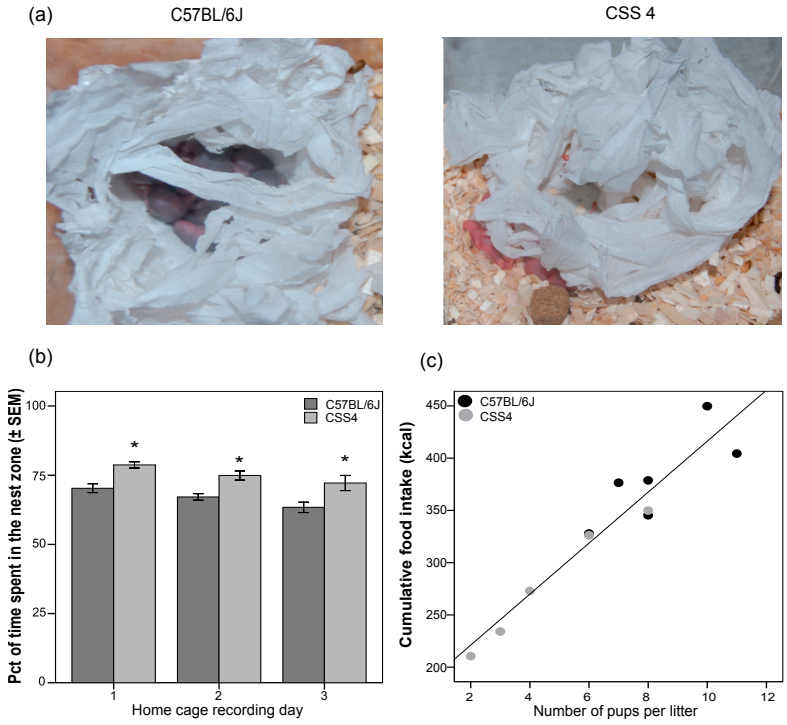

FIGURE 4.4 – ***Early environment: dam behavior.*** *Nest and litter composition of the C57BL/6J and CSS4 dams indicated more scattering of pups in CSS4 nests when compared to C57BL/6J nests (a). The percentage (Pct) of time the dams spent in the nest zone in the first 3 days; CSS4 dams spent more time than C57BL/6J dams (b). Significant correlation between the number of pups per litter (x-axis) and the cumulative food intake for 15 consecutive days of the dams from both strains (y-axis). For illustrative purposes, C57BL/6J dams are shown in black and CSS4 dams are shown in gray (c). Significant differences are indicated by (*).*

During the home cage observation, cumulative food intake of the dams was also measured. B6J dams ate more than CSS4 dams (B6J 380.41 kcal (± 17.69 SEM) and CSS4 278.72 kcal (± 7.99 SEM), $t_{(9)} = 3.295$, $p = 0.009$), however, there was a significant difference in the number of pups between the strains (B6J $n = 8_{(6-11)}$ and CSS4 $n = 4_{(2-8)}$ (median$_{(\text{min}-\text{max})}$), Mann-Whitney U Z = -2.124, $p = 0.043$). When corrected for the number of pups per litter, there was still a significant difference between the strains, but it were the CSS4 dams that were eating more per pup than B6J dams (B6J 46.78 kcal (± 2.76 SEM) and CSS4 69.93 kcal (± 10.60 SEM); $t_{(9)} = -2.306$, $p = 0.047$).
Independent of the dam background, a significant correlation between the cu-

mulative food intake and the pup number per litter was observed (Pearson′s r = 0.952, p < 0.0001; figure 4.4(c)). It is difficult to comment on the quality of time the dams spent with their nest via home cage recordings, however, these data clearly indicated that after the bonding occurred, the dams are attached to the pups and took care of them; food intake was significantly correlated to the number of pups in a litter (figure 4.4(c)).

Pup behavior was examined by measuring the number of calls at PND 10. Pup calls provide an important external stimulus to maintain the maternal behavior of the dams.[183] B6J pups were calling significantly more than CSS4 pups (B6J: $11_{(9-18)}$ and CSS4: $3_{(0-5)}$, $\text{median}_{(\text{min}-\text{max})}$); Z = -2.491, p = 0.016; figure 4.5(a)), suggesting that a low number of calls could be a reason why bonding is so low in the CSS4 strain.

Interestingly, body weight of the pups was different after intra-strain fostering: B6J pups raised by CSS4 foster mother had a similar body weight as the CSS4 pups raised by their biological mother (B6J-DsFM 6.26g (± 0.23 SEM) and CSS4 BM 6.48g (± 0.12 SEM)). Likewise, CSS4 pups raised by a B6J foster mother had a similar body weight then B6J pups raised by their biological mother (CSS4-DsFM 7.01g (± 0.18 SEM) and B6J-BM 7.06g (± 0.10 SEM); figure 4.5(b)).

These results show that body weight at this early life stage is influenced by maternal care. Both type of pups reduced calling for the mother, as well as the reversal in body weight loss by a different strain mother, indicate that both CSS4 pups and mothers contribute to the reduced maternal bonding.

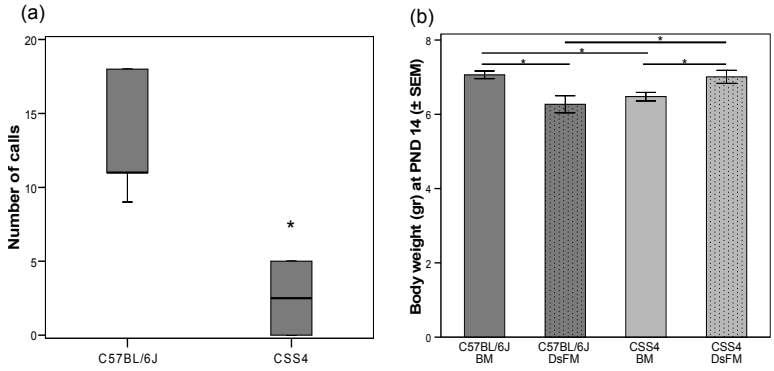

FIGURE 4.5 – ***Early environment: pup behavior and body weight.*** *Total number of calls at postnatal day (PND) 10; CSS4 pups call less than C57BL/6J pups (a). Body weight at PND14 of 4 groups: pups that were raised by biological mothers and pups that were raised by different strain foster mothers (b). Abbreviations: BM (Biological mother), SsFM (Same strain foster mother), and DsFM (Different strain foster mother). Significant differences are indicated by (*).*

4.3.4 Adult body weight

To study adult body weight and taking into account control groups for the effects of fostering itself, inter-strain fostered groups (pups raised by the same strain foster mother) were added, resulting in a total of six groups.
Body weight at adulthood (≈ 3 months old) was influenced by the genetic background of the pup ($F(1,49) = 26.42$, $p < 0.001$) and by environmental factors (dam) ($F(2,49) = 6.03$, $p = 0.005$). DsFM groups were significantly different from BM groups (Bonferroni post hoc analysis $p = 0.009$), but there was no difference from SsFM (Bonferroni post hoc analysis $p = 0.15$). SsFM groups were not different from BM groups (Bonferroni post hoc analysis $p = 0.69$) (figure 4.6).

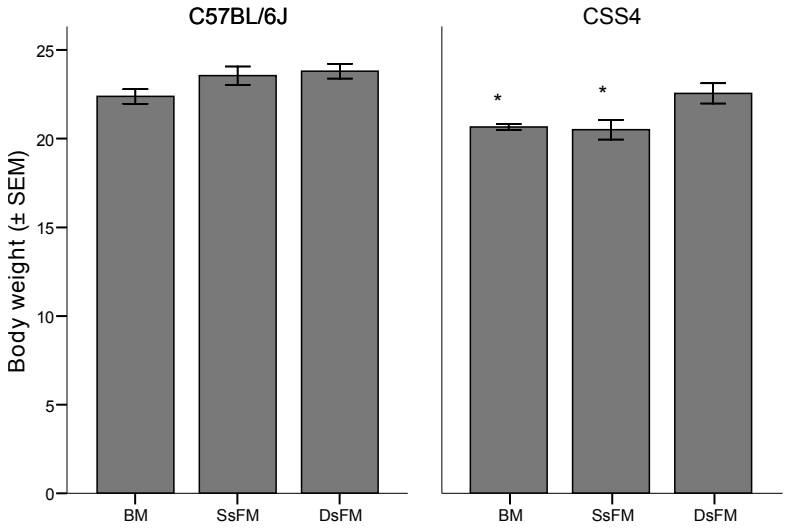

FIGURE 4.6 – ***Effects of fostering on adult body weight within each strain.*** *Significant differences between the foster and biological mothers are show with (*). Abbreviations: BM (Biological mother), SsFM (Same strain foster mother), and DsFM (Different strain foster mother).*

Within the strains, fostering did not have an effect on the B6J strain ($F(2,26) = 2.631$, $p = 0.091$). However, CSS4 strain groups showed differences in body weight ($F(2,23) = 5.615$, $p = 0.010$), with the DsFM group having a significantly higher body weight than BM and SsFM (DsFM 22.54g (± 0.56 SEM), BM 20.64g (± 0.16 SEM), Bonferroni post hoc analysis $p = 0.036$, SsFM 20.49g (± 0.56 SEM), Bonferroni post hoc analysis $p = 0.018$). The SsFM group was not significantly different from the BM group (Bonferroni post hoc analysis $p > 0.05$) (figure 4.6).

4.3.5 ABA susceptibility

To investigate the effects of fostering and body weight on the susceptibility to the ABA model, the six groups were subjected to the ABA model at adulthood to assess their ABA susceptibility.
Within the B6J strain, as with body weight, fostering did not have an effect on their low ABA susceptibility (BM vs SsFM: χ^2 = 1.154, p = 0.283, BM vs DsFM: χ^2 = 0.013, p = 0.908, SsFM vs DsFM: χ^2 = 0.840, p = 0.359) (figure 4.7(C57BL/6J)). However, following cross-fostering, the level of ABA susceptibility was signific-

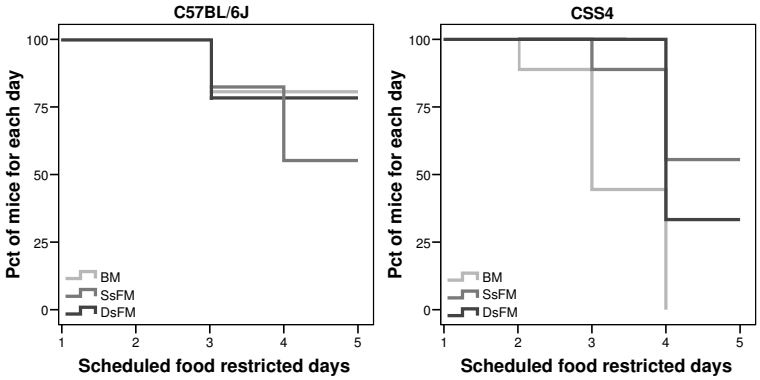

FIGURE 4.7 – *Percentage (Pct) of mice that completed the restriction days for C57BL/6J and CSS4 strain based on the 15% body weight loss criteria. Abbreviations: BM (Biological mother), SsFM (Same strain foster mother), and DsFM (Different strain foster mother).*

antly affected in the otherwise high ABA susceptible CSS4 strain. While the CSS4 mice raised by their BM were fully susceptible, the fostered groups were not. Interestingly, this also accounted for the CSS4 mice raised by the same strain foster mother (BM vs SsFM: χ^2 = 7.690, p = 0.006, BM vs DsFM: χ^2 = 7.874, p = 0.005). The fostered groups were also not significantly different from each other (χ^2 = 0.394, p = 0.930) (figure 4.7(CSS4)).
From these results, it became clear that body weight at PND 0 and at adulthood did not play a significant role in ABA susceptibility. Within the CSS4 strain, the SsFM group had an adult body weight similar to the BM group and yet, their susceptibility rate was lower. Further, low body weight at PND 14 did not play a role either, as CSS4 SsFM mice had even a lower body weight than BM groups (BM 6.48g (± 0.12 SEM) and SsFM 5.88g (± 0.13 SEM), $t_{(125)}$ = 3.420, p = 0.0008; figure 4.8(a)). The number of pups per litter at PND 14, corrected for the number of pups at PND 0, was not affected by fostering, as there was no difference between the two groups (BM *n* = 78.6% (± 10.24 SEM) and SsFM *n* = 78.0% (± 11.05 SEM), $t_{(11)}$= 0.37, p = 0.971; figure 4.8(b)), indicating that the impairment

of maternal bonding in CSS4 was also not directly related to ABA susceptibility in this strain.

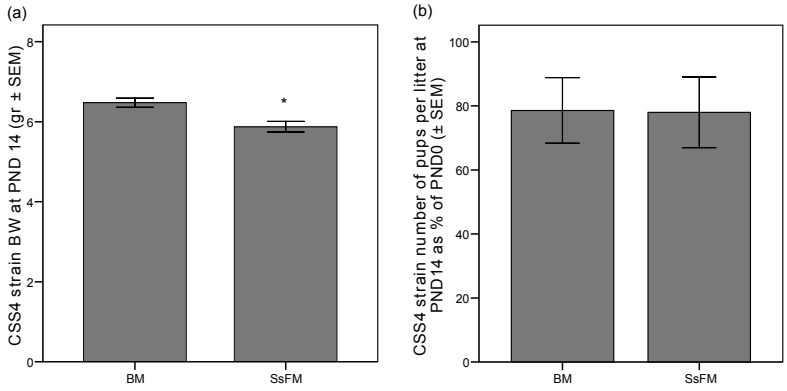

FIGURE 4.8 – ***CSS4 strain differences between biological mother (BM) and same strain foster mother (SsFM).*** *Body weight of CSS4 pups at postnatal day (PND) 14 (a) and the number of pups per litter at PND14 as percentage of PND0 (b). Significant differences are denoted by (*).*

4.3.6 Global DNA methylation analysis

Based on our current results, the impact of cross-fostering on the high ABA susceptibility in CSS4 can not be simply explained by their fixed genetic background. Therefore, we hypothesized that dynamic epigenetic modification may have occurred as a consequence of changes in maternal environment during early life. To investigate whether fostering was associated with epigenetic modifications, global DNA methylation analysis was examined in DNA extracted from hippocampal tissue in cross-fostered and non-cross-fostered mice a week after weaning the pups from their mother (PND 35).
Global DNA methylation analysis revealed hyper- and hypomethylated regions compared to the BM group. However, after the significance criteria were applied, only hypermethylated regions were significantly differently methylated in both the same strain and the different strain foster mothers group when compared to biological mothers group (table 4.3).

Table 4.3: List of genes that were hypermethylated in the two fostered groups versus the BM group. For each gene, the region modified and the fold methylation in both groups is given. Abbreviations: Chr (chromosome); begin (beginning chromosomal position of the region on mm9); end (ending chromosomal position of the region on mm9); location = Intronic, Exonic or Promoter, based on the mouse reference genome; INT (Intronic); Prom (Promoter); BM (fold methylation in biological mother group vs the corresponding foster group); FM (foster group fold methylation); FDR (false discovery rate).

Gene ID	group	chr	beg	end	location	BM	FM	FDR
Armc9	SsFM	1	88093825	88094157	INT	1	5.5	0.481
	DsFM	1	88093825	88094157	INT	1	7	0.032
Spag16	SsFM	1	70485822	70486061	INT	0.5	5	0.473
	DsFM	1	70485822	70486061	INT	0.5	7.5	0.021
Stau2	SsFM	1	16264006	16264151	INT	1	5.5	0.469
	DsFM	1	16244325	16244606	INT	0.5	6.5	0.021
Ush2a	SsFM	1	190581946	190582247	INT	1	6.5	0.451
	DsFM	1	190084586	190084684	PROM	0.5	6	0.021
AL845174.1	SsFM	2	150543400	150543571	PROM	1	5.5	0.474
	DsFM	2	150543400	150543571	PROM	1	7	0.028
Nup35	SsFM	2	80486930	80487187	INT	1	5.5	0.469
	DsFM	2	80488317	80488580	INT	1	8.5	0.022
Dnase2b	SsFM	3	146172975	146173117	INT	0.5	5.5	0.46
	DsFM	3	146099965	146100424	INT	1	7	0.028
Nbn	SsFM	4	15907431	15907688	INT	1.5	8	0.398
	DsFM	4	15903654	15903836	INT	0.5	5.5	0.023
Fggy	SsFM	4	95423383	95423656	INT	1	5.5	0.491
	DsFM	4	95546200	95546480	INT	1	6.5	0.036
Bach2	SsFM	4	32603763	32604136	Exon2	1	6	0.465
	DsFM	4	32462624	32462851	INT	1	6	0.044
Tmem132d	SsFM	5	128900583	128900801	INT	1.5	7	0.453
	SsFM	5	128549354	128549538	INT	1	6	0.458
	SsFM	5	128355215	128355415	INT	1	5.5	0.468
	DsFM	5	128633677	128633834	INT	0.5	6.5	0.021
Arhgap24	SsFM	5	103214042	103214271	INT	1	5.5	0.491
	DsFM	5	103204713	103205273	INT	1	6.5	0.04
9530036O11Rik	SsFM	5	29140287	29140512	INT	1.5	7.5	0.445
	DsFM	5	29140287	29140512	INT	1.5	9	0.04
	DsFM	5	29215427	29215600	INT	1	6.5	0.036
Casd1	SsFM	6	4567030	4567289	INT	1.5	9.5	0.229
	DsFM	6	4577161	4577362	INT	0.5	5	0.025
Eif4e3	SsFM	6	99602551	99603076	INT	1.5	7	0.461
	DsFM	6	99426156	99426626	INT	1	7	0.032
Cntnap2	SsFM	6	45232210	45232436	INT	0	5	0.23
	DsFM	6	47188927	47189241	INT	0.5	6	0.022
	DsFM	6	46063928	46064087	INT	0	5.5	0.009

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Table 4.3 – Continued

Gene ID	group	chr	beg	end	location	BM	FM	FDR
Sox5	DsFM	6	143892374	143892721	Exon3	1.5	11.5	0.023
	DsFM	6	144213536	144213928	INT	1	7.5	0.024
	DsFM	6	144455382	144455604	INT	1	7	0.032
	DsFM	6	143852971	143853224	INT	0.5	5	0.025
	SsFM	6	144213536	144213928	INT	1	6.5	0.461
Cacna1c	SsFM	6	118722984	118723453	INT	1.5	7	0.453
	DsFM	6	118579534	118579723	INT	1	6.5	0.036
	DsFM	6	118823471	118823628	INT	1	6	0.045
	DsFM	6	118989344	118989705	INT	0.5	6	0.022
Odz4	SsFM	7	103817478	103817780	INT	1	5.5	0.481
	DsFM	7	103817478	103817780	INT	1	8.5	0.022
	DsFM	7	103335084	103335169	INT	0	5.5	0.009
Sox6	SsFM	7	123171622	123171995	INT	1.5	7.5	0.44
	DsFM	7	122703163	122703394	INT	0.5	5.5	0.023
	DsFM	7	122975739	122975896	INT	0.5	5.5	0.023
Gm10155	SsFM	7	133444501	133444887	PROM	1	5.5	0.473
	DsFM	7	133443296	133443464	PROM	0.5	6.5	0.021
Tll1	SsFM	8	66668885	66669236	INT	0.5	5	0.474
	DsFM	8	66668885	66669236	INT	0.5	7	0.021
Myo16	SsFM	8	10272264	10272836	PROM	1	6	0.469
	DsFM	8	10272264	10272836	PROM	1	6.5	0.036
Fam155a	SsFM	8	9673069	9673309	INT	1	5.5	0.469
	DsFM	8	9618734	9619179	INT	1	7.5	0.025
AC114005.5	SsFM	8	123543664	123543883	INT	1	7	0.451
	DsFM	8	123543664	123543883	INT	1	6.5	0.035
	DsFM	8	124378460	124378727	INT	1	8	0.024
Ntm	SsFM	9	29731140	29731243	INT	1	6	0.474
	DsFM	9	28863029	28863242	INT	1	6	0.045
Grik4	SsFM	9	42476525	42476971	INT	2	8.5	0.402
	DsFM	9	42582058	42582134	INT	0.5	5.5	0.023
Sik2	SsFM	9	50799185	50799385	INT	1	5.5	0.473
	DsFM	9	50799185	50799385	INT	1	7	0.029
Helb	SsFM	10	119544559	119544848	INT	1	8.5	0.35
	DsFM	10	119544559	119544848	INT	1	8	0.024
Gcc2	SsFM	10	57737612	57737819	Exon1	0.5	6	0.45
	DsFM	10	57767439	57767688	Exon2	1	6.5	0.036
Ipmk	SsFM	10	70821811	70822029	INT	1	5.5	0.492
	DsFM	10	70816160	70816356	INT	0.5	7	0.021
Stat5b	SsFM	11	100653677	100653897	INT	0.5	6	0.452
	DsFM	11	100653677	100653897	INT	0.5	9.5	0.021
Odz2	SsFM	11	36726900	36727179	INT	1	5.5	0.473
	DsFM	11	36726900	36727179	INT	1	6.5	0.04

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Table 4.3 – Continued

Gene ID	group	chr	beg	end	location	BM	FM	FDR
Fam179b	SsFM	12	66084309	66084543	Exon6	1	7	0.45
	DsFM	12	66084309	66084543	Exon6	1	6.5	0.035
	DsFM	12	66104439	66104637	INT	1	6.5	0.035
Ap3b1	SsFM	13	95238928	95239319	INT	1.5	7	0.461
	DsFM	13	95238928	95239319	INT	1.5	10.5	0.024
Arl15	SsFM	13	114628652	114628941	INT	1.5	7	0.46
	DsFM	13	114628652	114628941	INT	1.5	9	0.042
Rreb1	SsFM	13	37883963	37884152	INT	1.5	7	0.453
	DsFM	13	37969448	37969673	INT	0.5	6.5	0.021
Mast4	DsFM	13	103561131	103561412	Exon1	1	6.5	0.036
	SsFM	13	103617016	103617271	INT	1	7.5	0.395
Erc2	SsFM	14	28437590	28437869	INT	2	9.5	0.271
	DsFM	14	29282230	29282429	INT	1	6	0.043
Dzip1	SsFM	14	119296068	119296403	INT	2.5	10.5	0.245
	DsFM	14	119291203	119291460	INT	0.5	6	0.021
Rarb	SsFM	14	17596069	17596373	INT	0.5	5.5	0.461
	DsFM	14	17502444	17502717	INT	0.5	6	0.022
Ptprg	SsFM	14	12740553	12740812	INT	1	5.5	0.482
	DsFM	14	12991841	12992106	INT	1	6.5	0.035
	DsFM	14	12456458	12456758	INT	1	6	0.045
	DsFM	14	12875590	12875813	INT	1	6	0.045
Tdh	SsFM	14	64118855	64119145	Exon1	1	5.5	0.482
	DsFM	14	64116691	64116975	INT	1	7.5	0.026
Ctnnd2	SsFM	15	30454683	30454938	INT	1	5.5	0.482
	DsFM	15	30411359	30411596	INT	0.5	6.5	0.021
	DsFM	15	30898288	30898483	INT	0.5	5.5	0.023
Fndc1	SsFM	17	7976677	7976849	INT	1	6.5	0.457
	DsFM	17	7971868	7971982	INT	1	6	0.045
Loxhd1	SsFM	18	77522876	77523273	INT	1	5.5	0.492
	DsFM	18	77522876	77523273	INT	1	9.5	0.021
Nrg2	SsFM	18	36306999	36307209	INT	0.5	6	0.457
	DsFM	18	36318936	36319064	INT	0.5	6.5	0.021
Mkx	SsFM	18	6964324	6964690	INT	1.5	9	0.37
	DsFM	18	6964324	6964690	INT	1.5	10.5	0.028
Sorcs3	SsFM	19	48524162	48524342	INT	0.5	5.5	0.462
	DsFM	19	48621780	48621792	INT	0.5	5.5	0.023
	DsFM	19	48337968	48338047	INT	0.5	5.5	0.023
Trpm3	SsFM	19	23059591	23059978	INT	1.5	7	0.458
	DsFM	19	22459787	22459858	INT	0.5	6	0.022
	DsFM	19	22359199	22359362	INT	0.5	5.5	0.023

4.4 Discussion

This study shows that early life environmental changes lead to long-lasting adaptive behaviors in adulthood dependent on genetic background. We observed behavioral effects related to fostering on the CSS4 strain but not on the C57BL/6J strain. Our data suggests that the long-lasting effects on adaptive behavior are modulated by DNA hypermethylation in regions throughout the genome. Interestingly, those regions involve previously identified candidate genes across the neuro-psychiatric spectrum, such as contactin associated protein-like 2 (Cntnap2) gene;[188] α-1C subunit of the L-type voltage-gated calcium channel (Cacna1c) gene;[189] catenin delta 2 (Ctnnd2) gene;[190] and family with sequence similarity 155, member A (Fam155a), a gene recently associated with anorexia nervosa.[46]

Impact of fostering on ABA susceptibility

ABA susceptibility of CSS4 mice raised by foster mothers was significantly reduced compared to the mice raised by their biological mothers. The change in ABA susceptibility could not be explained by the genetic background of the fostering mothers as CSS4 pups raised by a foster CSS4 and B6J mother both showed reduced ABA susceptibility. In addition, the altered ABA susceptibility could not be explained by body weight levels in adulthood, as the mice raised by B6J foster mothers had a higher body weight, while the mice raised by CSS4 foster mothers were not different from the biological mothers group. Body weight at PND 14 could not have played a role either, as body weight was higher, (mice raised by B6J foster mothers) or lower, (mice raised by CSS4 foster mothers) compared to biological mothers group. These observations indicate that reduced body weight levels observed in CSS4 mice at adulthood and at PND 14 did not relate to ABA susceptibility.

One possible factor affecting the ABA susceptibility is the connection between the mothers and pups during the pre-weaning period. When we first consider the maternal environment, in which the pups are raised by their biological mothers, there are differences between B6J and CSS4 strain. The most striking difference between the two strains is the bonding with the litter: CSS4 mothers had a weak bonding with the litters. It is notable that, after bonding had occurred, CSS4 mothers stayed longer with the nest compared to the B6J mothers, though it is difficult to comment on the quality of time spent (in terms of nursing and licking). There were differences in pup behavior between the strains as well. CSS4 pups were calling significantly less than the B6J pups. Callings of the pups are important in keeping the mother engaged with pup care.[183] These suggest that the connection between biological mother and pup in CSS4 strain is different compared to the B6J strain and this may impact their adult ABA susceptibility.

Though we did not observe the behaviors of the foster mothers, a literature search[191,192] does not suggest significant differences in the behavior of the foster mothers (either same or different strain) towards the pups. Hence, even

though CSS4 mice should have received similar care with the same strain foster mothers as with biological mothers, we observed beneficial effects on ABA susceptibility similar to the group raised by B6J foster mother. These results suggest that the connection between the CSS4 mice and foster mothers is somewhat different from biological mothers with positive influence in adult behavior.
Another factor could be the stress occurring with the fostering process. However, literature data on the effects of early life stress such as maternal separation at an early age in rats has led to discordant results with either increased[193] or decreased[194] vulnerability in the ABA model (separation protocol was similar in both studies). Further, early weaned rats were more vulnerable to ABA.[195] Possibly it is not just the initial stress associated with fostering, but the whole mother-pup bonding and their relationship that has an effect on the adult mice behavior, via epigenetic modifications.
In order to identify mechanisms that may explain the change in ABA susceptibility in cross-fostered CSS4 mice, a genome wide DNA methylation study was performed. Our analysis in hippocampal extracted DNA, showed various hypermethylated regions throughout the genome between the fostered groups and biological mother group in the brain of CSS4 mice. Several genes were hypermethylated and some of these have been previously associated with psychiatric disorders, including anorexia nervosa.
For example, it was interesting to find Fam155a gene in the list of hypermethylated regions as the human FAM155a gene has been found to have a single nucleotide polymorphism marker that was most significantly associated with AN in a genome-wide association study.[46] Not much is known about the function of this gene, but the fact that it has come up in two different studies (and approaches) related to anorexia nervosa, makes it a potentially interesting candidate gene for this disorder. Another hypermethylated gene identified was Cntnap2 gene, In humans, CNTNAP2 gene has been recently implicated with several aspects of the autism spectrum disorders.[188] Notably, anorexia nervosa has been linked with autism spectrum disorders via shared similarities in social cognitive features.[196,197,198,199]
The epigenetic modifications observed in regions of several neurodevelopmental genes, suggests that early life events related to brain structures are already shaping the individual susceptibility to later life events.

Impact of fostering on body weight
In addition to the effects on ABA susceptibility, we also observed significant time dependent effects on body weight that were partially dependent also on genetic background. Body weight differences were not present on PND 0 but became apparent at PND 14. At this age, when the pups are fully dependable on their mothers, cross-fostering to different strain mothers had similar effects on both pup strains. Namely, genetic background of the mothers determined the body weight of these pups at PND14. However, a difference between the strains was observed when the pups were raised by same strain foster mothers. While body weight of the B6J pups was similar to the biological mothers group (data

not shown), CSS4 pups were low in body weight.
At adulthood, genetic background prevailed in B6J mice, with the mice in all groups having a similar body weight. In CSS4 strain, we observe an interplay of the genetic background and environmental effects where, mice with high body weight at PND14 (B6J foster mother group), are still keeping up the difference in weight compared to biological group while the CSS4 mice with low body weight (CSS4 foster mother group) now have caught up in weight with the biological group. These results show how the interplay between genetic background and environment is dynamic and has different impacts in different stages of life, and support the literature that body weight regulation is quite complex.[55, 200, 201]

Together, our findings highlight the importance of genes and environment interplay within the trajectory of anorexia nervosa and other developmental psychiatric disorders. They introduce the notion that early life changes (e.g., maternal environment) can modulate adult susceptibility to mal-adaptive behavior via epigenetic modifications of genes related to neurodevelopmental processes.

4.5 Acknowledgments

We thank Jan Brakkee, Marina de Wit, Mark Broekhoven and Inge Wolterink-Donselaar for their technical assistance.

Chapter 5

Identification of predictive factors for activity based anorexia susceptibility using eleven mouse inbred strains

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Manuscript submitted

Abstract

Animal studies are very useful in detection of early disease indicators and in unravelling the pathophysiological processes underlying core psychiatric disorder phenotypes. Early indicators are critical for preventive and efficient treatment of progressive psychiatric disorders like anorexia nervosa (AN). Comparable to physical hyperactivity observed in AN patients, in the activity based anorexia (ABA) rodent model, mice express paradoxical high voluntary wheel running activity (WRA) levels when food restricted. Eleven inbred mouse strains were exposed to the ABA model in the search of identifying susceptibility predictors. Body weight, food intake and WRA levels of each individual mouse were measured. Mouse strains with high WRA levels during food restriction exhibited accelerated body weight loss. Linear mixed models for repeated measures analysis showed that baseline WRA levels preceding the scheduled food restriction phase strongly predicted ABA susceptibility (Beta = -0.0158 ($\pm$0.003 SE), $P < 0.0001$) compared to other baseline parameters. These results suggest that physical activity levels play an important role in ABA susceptibility in mouse inbred strains with a natural variation in genetic background. These findings support previous retrospective studies on physical activity levels in AN patients and indicate that pre-morbid physical activity levels could reflect an early indicator for disease severity.

5.1 Introduction

Eating disorders are severe psychiatric illnesses with high morbidity and mortality. Particularly anorexia nervosa (AN) has the highest mortality rate among psychiatric disorders.[4,6] AN affects young females and has an incidence rate of around 0.9%.[10] Hallmark of the illness is the refusal to maintain a normal body weight.[11] Various risk factors have been identified and genetics is found to play a role in the development of AN.[202,203,34,39,36,37] However, little is known about aetiology or predictive factors of this disease. Identifying predictive factors of specific AN phenotypes, such as physical hyperactivity,[204,16] could improve the treatment approach and increase treatment efficacy.
Physical activity levels are important in AN; they play a role in the onset and maintenance of illness[90,205,15] and have an influence on the recovery rate.[19,21,22] High activity levels may precede dieting,[14] suggesting that premorbid physical activity may be a predictor of illness course during times of reduced food intake. During the illness, regardless of low body weight, an increase in physical activity is observed.[206,16] The effects of limited food intake and hyperactivity under these conditions can be modelled in animals.[74,75]

Animal studies take place under controlled genetic and environmental conditions, minimizing their complex interaction effects on phenotypic heterogeneity. They may provide novel insights in pre-morbid factors that can affect and/or predict the course of the disorder. This is a challenge in human studies, as currently the research on these factors is determined either via cross-sectional or retrospective studies. Longitudinal prospective studies are very sparse (e.g. Nicholls & Viner[207]) and face the difficulty of low incidence rate. Activity based anorexia (ABA) is an animal model of pathophysiological processes of AN where the combined effects of daily scheduled limited food availability and voluntary running wheel activity mimic the physical hyperactivity behaviour observed in AN patients while their food intake is severely reduced. In this model, animals have unlimited voluntary access to a running wheel throughout the experiment, while food is ad libitum available for a limited period at the same time during several consecutive days.[74,75] The resulting physical hyperactivity under this conditions is a core feature of this model[77,78] and has clinical relevance.[108,94] Previous studies performed in our laboratory mapped the hyperactivity behaviour in chromosome substitution (CS) strain mice and has identified CS strains becoming hyperactive or being susceptible to this model.[157] Following these results, we continued our research focusing on the question of what are the factors that could influence and/or predict susceptibility to the model. By using this animal model, we investigated the response of 11 different inbred mice strains to daily scheduled food restriction and studied the relationship between their baseline phenotypes and their ABA susceptibility.

5.2 Materials and methods

5.2.1 Animals and housing

Initial breeding pairs were obtained from The Jackson Laboratory, Bar Harbor, ME, USA,and the mice used in the experiment were bred at the Rudolf Magnus Institute of Neuroscience animal facility. The following strains were tested: A/J (n = 8) (JAX stock# 000646), AKR/J (AKR n = 8) (JAX stock# 000648), BALB/cByJ (BALB n = 7) (JAX stock# 001026), C3H/HeJ (C3H n = 12) (JAX stock# 000659), C57BL/6J (C57BL n = 10) (JAX stock# 000664), CAST/EiJ (CAST n = 8) (JAX stock# 000928), DBA/2J (DBA n = 7) (JAX stock# 000671), FVB/NJ (FVB n = 8) (JAX stock# 001800), KK/HlJ (KK n = 10) (JAX stock# 002106), NZW/LacJ (NZW n =10) (JAX stock# 001058) and WSB/EiJ (WSB n =10) (JAX stock# 001145). The selected strains are part of Mouse Phenome Database (MPD) priority strains, Tier 1 (http://phenome.jax.org/db/q?rtn=strains/search&reqpanel=MPD).
Following weaning at 3 – 4 weeks, female and male mice were separately housed in groups (2 – 5 mice per cage) in cages (Macrolon®, Tecniplast, Milan, Italy). The housing facilities were maintained on a 12:12 h dark/light cycle with an ambient temperature of 21.0 ± 2°C and humidity of 55 ± 10%. During this period, the mice were given water and food ad libitum (Rat and Mouse Breeder and Grower, Special Diet Services, Essex, England). For this study we used test-

naive 3 – 4 months old female mice, because of the high prevalence of AN in females.[6] All animal experiments were approved by the Animal Experimentation Committee of University Medical Centre Utrecht and were carried out in agreement with Dutch Laws (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

5.2.2 Experimental procedure

The experimental procedures are the same as previously described.[208] Briefly, after an adaptation phase (7 days in individual cages) where the mice had ad libitum access to food, water and running wheel, they were exposed to a daily restricted feeding schedule (restriction phase) for 4 consecutive days. During this period, food (5 pellets of about 1.2g each with an energy intake of 3.31 kilocalorie (kcal) per gram) was available only in the first two hours of the dark phase.
As baseline measures for body weight and food intake, the average of last three days of the adaptation phase, measured just before the dark period, were used. Baseline wheel running activity (wheel revolutions) (WRA) levels were determined as the average WRA of the last two days of the adaptation phase.
During the restriction phase, body weight (BW) was measured daily before and after food administration. On the last day (day 4), BW was measured again at the end of the day and this measure was given the label 'day 5'. WRA levels were measured daily during the restriction phase.
The level of body weight loss during the scheduled food restriction days was selected as a measure of susceptibility to the ABA. If a mouse lost 15% or more of their baseline BW on any experimental day, that mouse was taken out of the experiment (based on the ethical humane endpoint criteria) and was considered susceptible to ABA. Based on the level of body weight loss, a second dichotomous variable for ABA susceptibility was generated, with each individual mouse assigned a value whether it was susceptible or not. In this way, a percentage of mice within each strain that was susceptible to activity based anorexia model (ABA) i.e. that could not maintain the body weight above 85% of their baseline body weight, was determined.

5.2.3 Statistical analysis

The data were expressed as means with standard error of the mean (SEM) unless otherwise specified. One sample Kolmogorov-Smirnov test was used to check the Gaussian distribution and Levene′s test was used for the homogeneity of variances.
To assess the effects of the baseline parameters (body weight, food intake, activity and food intake per body weight) as predictive factors, linear mixed models for repeated measures analysis, which takes into account the longitudinal data and within-mouse correlations, were used. To analyze the susceptibility to the model, body weight loss during the restriction phase was used as the dependent

variable. Animal′s identification number was entered as random effects, while in the fixed effects, the days of the experiment and each of the baseline factors were entered. To check whether there was a specific strain that contributed to the effect of these factors, the analysis was repeated with each of the strains being taken out of the analysis in turns.
Two-sided P values of < 0.05 were considered statistically significant. The effects were expressed as standardized beta (Beta) with standard error (SE), denoting the change in dependent variable with one standard deviation change of the independent variable. Spearman′s coefficient of rank correlation was used for correlation analysis as the data were not normally distributed. Significance was set at $P < 0.05$. Statistical analysis was carried out using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA).

5.3 Results

5.3.1 Baseline parameters

There was a wide distribution of baseline body weight between strains. However, the majority of the strains had a baseline body weight (BW) between 20g and 26g. CAST and WSB mice, had the lowest BW with 13.72g (±0.09 SEM) and 16.27g (±0.24 SEM) respectively. On the other side of the baseline body weight spectrum are NZW and KK strains, with a BW of 32.99g (±0.71 SEM) and 38.94g (±0.92 SEM) respectively (figure 5.1(a)). Food intake (FI) among strains varied, with FVB strain having the highest baseline FI 21.60kcal (±0.77 SEM) followed closely by BALB strain 19.51kcal (±0.53 SEM; figure 5.1(b)). When food intake was corrected for body weight, CAST strain had the highest food intake per body weight (FI/BW) levels between the strains (1.22kcal ±0.06 SEM) followed by FVB strain (0.95kcal ±0.04 SEM; figure 5.1(c)). Baseline WRA levels ranged from approx. 750 to 35000 revolutions per day, corresponding to 0.33 and 15.58 kilometres (km) per day, respectively (figure 5.1(d)).

5.3.2 Predictive factors for ABA susceptibility

Nearly half of the strains were particularly susceptible in this model. Within these strains, less than 25% of the mice remained at a BW level above 85% of their baseline BW until the end of the experimental days (figure 5.2). Linear mixed models for repeated measures analysis revealed that among baseline factors (BW, FI and WRA), WRA levels had the strongest effect on ABA susceptibility (body weight loss), based on Beta estimate (Beta = - 0.0158 (±0.003 SE), $P < 0.0001$). BW was the second factor (Beta = 0.0118 (±0.003 SE), $P = 0.0005$) and FI was third (Beta = - 0.0102 (±0.004 SE), $P = 0.0057$). The derived baseline FI/BW had also a significant effect (Beta = - 0.0106 (±0.004 SE), $P = 0.0041$). The interaction of the derived FI/BW and baseline WRA levels (FBxW), had also a strong effect on ABA susceptibility, where the effect of WRA levels was increased with the effect of FI/BW (Beta = 0.0199 (±0.004 SE), $P < 0.0001$).

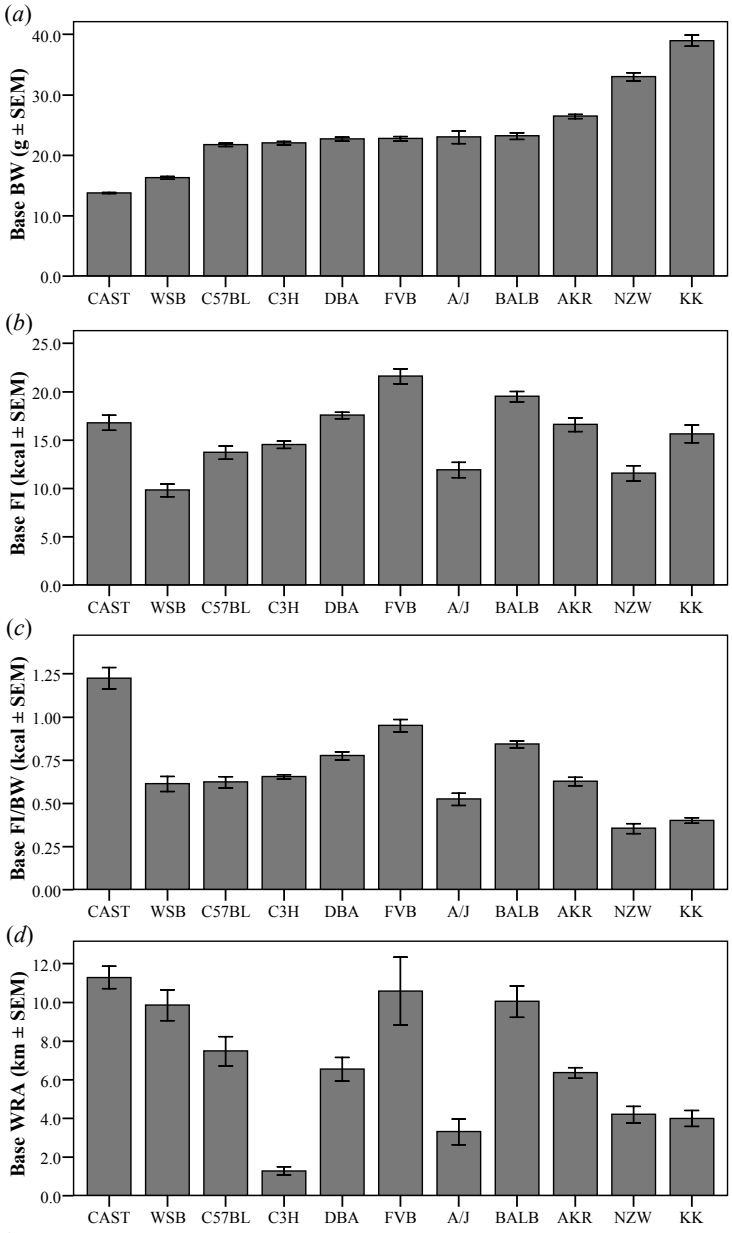

FIGURE 5.1 – ***Baseline parameters.*** *Baseline (Base) body weight (BW) (a), food intake (FI) (b), food intake corrected per body weight (FI/BW) (c) and wheel running activity (WRA) levels (d). The data are presented as mean ±SEM. The ranking for all graphs in this panel is based on baseline body weight.*

The high predictive value of baseline WRA levels for ABA susceptibility was not dependent on a specific genetic background, as was revealed by a repeated single strain exclusion analysis (data not shown). Based on this additional analysis, FI became an insignificant predictor when FVB strain was taken out (Beta = - 0.0053 (±0.004 SE), P = 0.164). Similarly, when NZW strain was taken out, FI/BW effect became insignificant (Beta = - 0.0066 (±0.004 SE), P = 0.0810). Further, the FB×W interaction became insignificant as predictor when CAST strain was taken out of the analysis (Beta = 0.0059 (±0.003 SE), P = 0.080), indicating that these specific strains dominated the effects of these measures.

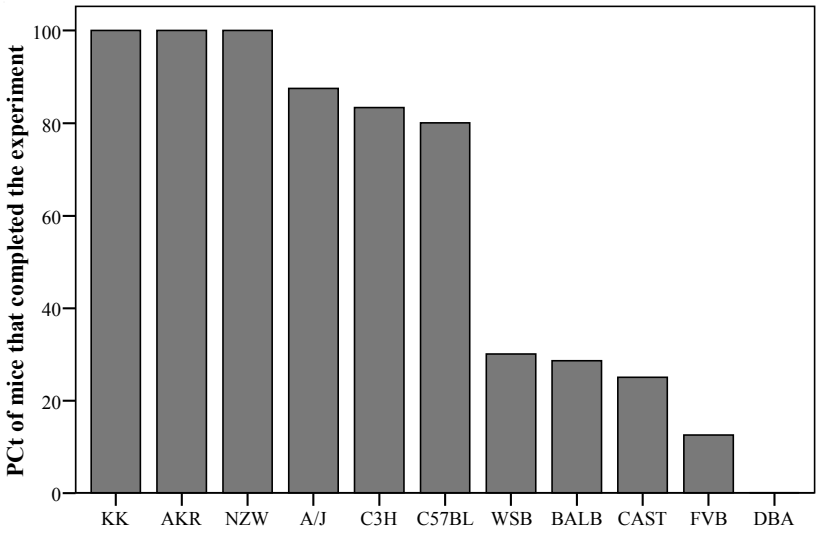

FIGURE 5.2 – *Percentage (PCT) of mice within each strain that was susceptible to activity based anorexia model (ABA) i.e. that could not maintain the body weight above 85% of their baseline body weight.*

5.3.3 Correlations

Based on all individual values, baseline BW was not correlated with baseline FI, but was weakly and negatively correlated to baseline WRA levels (r_s = - 0.380, P < 0.001; data not shown). Baseline FI was also weakly correlated to baseline WRA levels, but in a positive direction (r_s = 0.418, P < 0.001; data not shown). There was a positive correlation between baseline WRA levels and FI/BW (r_s = 0.609, P < 0.0001). Baseline WRA and restrictive days (R) 1, 2, 3 and 4 WRA levels were strongly correlated to each other (R1 (n = 98) (r_s = 0.885, P < 0.0001; R2 (n = 98) r_s = 0.767, P < 0.0001; R3 (n = 78) r_s = 0.768, P < 0.0001; R4 (n = 64) r_s = 0.593, P < 0.0001; figure 5.3 shows the correlation of baseline and restriction days 2 (R2)

WRA levels, where all the mice are still in the experiment). Restriction days WRA

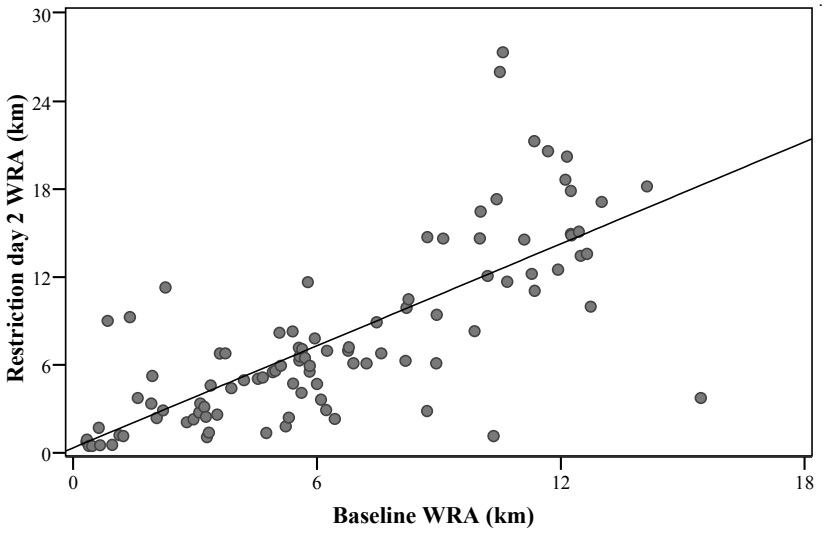

FIGURE 5.3 – *Significant correlation between baseline wheel running activity (WRA) levels (x-axis) and restriction day 2 WRA levels (y-axis) across individual mice (n = 98) from 11 different mouse inbred strains* ($r_s = 0.767$, $P < 0.0001$).

levels were also negatively correlated ABA susceptibility (dichotomous variable) (R1 ($n = 98$) ($r_s = -0.575$, P = <0.0001; R2 ($n = 98$) $r_s = -0.573$, P = <0.0001; R3 ($n = 78$) $r_s = -0.360$, P = 0.001; R4 ($n = 64$) $r_s = -0.365$, P = 0.003; overlay figure 5.4.

5.4 Discussion

In the present study, using eleven inbred mouse strains, we found that baseline WRA (physical activity) levels was the strongest factor to predict ABA susceptibility when compared to other baseline factors, such as baseline food intake and baseline body weight. These findings support the notion from retrospective studies in human AN indicating that physical hyperactivity levels may precede anorexia nervosa onset and can also predict the physical activity levels during AN illness.[205,14,209] These findings provide novel opportunities to study neurobiological mechanisms underlying this potential clinical relevant trait. Genetic studies may be a good starting point, as initial genetic factors for physical activity levels have been identified in animals[210,211,212,213,214] and in humans.[98,215] Genetic mapping studies in different genetic reference populations have found a peak on mouse chromosome 1 related to activity.[168,123] Furthermore, studies have shown that specific genes are coupled to specific components of physical hyperactivity levels during limited food access.[208] Understanding the neurobi-

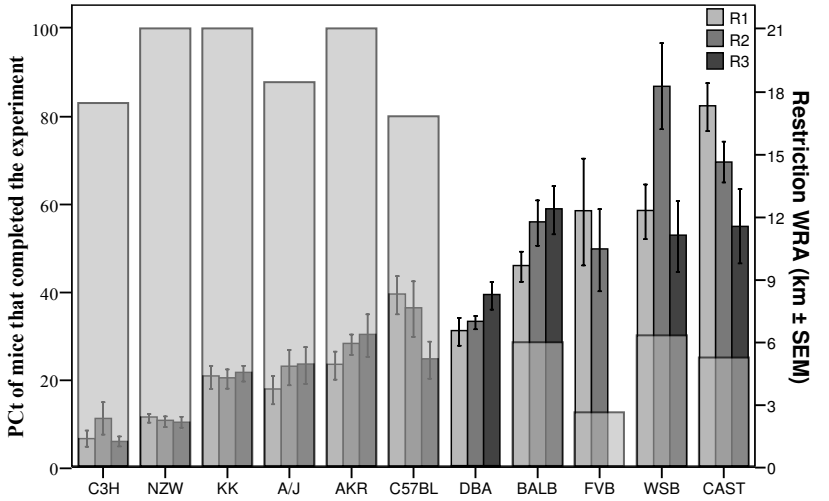

FIGURE 5.4 – *Overlay between percentage (PCT) of mice within each strain that completed the experiment, left y-axis (transparent gray bars), and restriction wheel running activity (WRA) levels on day 1 (R1), day 2 (R2) and day 3 (R3) right y-axis (ranking based on the right y-axis); in 11 different mouse inbred strains (x-axis). Please note that because on R3, more than 80% of FVB strain mice were taken out of the experiment, the data for the remaining mice on this day (n = 2) are not shown. Strains with overall high activity levels and strains that increased their activity during the restriction days (e.g. DBA), are susceptible to the model.*

ological contributions of these genes to these behavioural phenotypes may be a critical step forward in the development of aetiology - directed treatment.
In addition, the interaction between physical activity levels and food intake per body weight had initially a substantial effect on ABA susceptibility. However, further analysis revealed that this effect was mainly driven by the CAST mouse strain which had the highest food intake per body weight levels when compared to the other strains, and was also one of the strains with the highest baseline activity levels. Similarly, the effect of baseline food intake and highest food intake per body weight became non significant when FVB and NZW strains were taken out of the analysis respectively.
Interestingly, FVB strain had the highest FI levels among the inbred strains and was also one of the strains with high baseline activity levels, while NZW strain had the lowest FI/BW levels. These data suggests that individual mice with high baseline food intake per body weight and high innate physical activity levels have more difficulty to maintain body weight during scheduled food availability than mice that were less active or had lower food intake per body weight un-

der baseline conditions. Translating these mouse findings to human behaviour, they would suggest that patients with high pre-morbid activity levels and high energy requirements are prone to lose weight at a higher rate in the development of illness. Subsequently, with illness progression, the basal metabolic rate decreases and the amount of energy spent with any activity is less than that in normal controls.[216] Nevertheless, the physical activity levels do affect the rate of recovery[21] indicating the importance of this trait as a predictive factor as well as a factor affecting disease maintenance.

The other baseline factors, such as body weight and food intake, and their derivative food intake per body weight, also exhibited a significant predictor effect on ABA susceptibility. However, the magnitude of their predictive value was lower than that of baseline physical activity levels. A closer look at body composition data available in the existing literature showed that body fat percentage was similar to the body weight range. The strains with high body weight had also the highest fat percentage, while the rest of the strains had a similar percentage around 20%.[217] These data, even though it was not possible to directly statistically test their effect on ABA susceptibility, suggest that body composition effect could be similar to that of body weight with a low magnitude effect. Nonetheless, as pre-morbid BW levels have been previously correlated in AN with the BW at referral[218] and also at follow up[219] this may still be an important predictive factor to consider. Interestingly, the relationship between baseline body weight and ABA susceptibility was strong in some of the mouse inbred strains tested and not present in others (data not shown), suggesting that this could be independent of their body composition (they were different in body weight and fat percentages) and their levels of prediction may depend on other factors related to their genetic background.

Although fat percentages were not very different between the strains, circulating leptin levels could be different as leptin release is associated to the fat cell size. Therefore, we were also interested if strain differences in leptin signalling may be related to physical activity levels. While the leptin data for the eleven strains are available only after a fat diet for several weeks,[220] the strain ranking pattern for leptin signalling was very different from that of the strain ranking for physical activity levels in the present study. Other metabolic factors such as insulin levels (after fat diet) or glucose levels (baseline or after fat diet)[220] again did not show a specific pattern that could be correlated to ABA susceptibility. These data indicate that none of these metabolic factors was a common factor that could explain the ABA susceptibility.

Taken together, there is a general factor, physical activity levels, which can strongly predict the ABA susceptibility. The effect of physical activity levels on ABA susceptibility in individual strains is dependent on the genetic background. Other baseline factors, such as body weight and food intake are in general less strong predictors of ABA susceptibility. The effect of body weight and food intake on ABA susceptibility is sometimes strongly dominated by a single strain (e.g. CAST mouse strain effect on the interaction between physical activity levels and food intake per body weight and FVB mouse strain effect

on food intake). Literature search revealed no common metabolic factor that could contribute to body weight loss. While further studies are necessary to unravel the neurobiological mechanisms of these factors and their interrelatedness, these findings and the retrospective AN studies on physical activity levels suggest that pre-morbid physical activity levels of each patient could already be taken into consideration as a possible predictor of illness severity.

Chapter 6

NPY receptor subtype specification for behavioral adaptive strategies during limited food access

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Abstract

The neuropeptide Y (NPY) system in the brain regulates a wide variety of behavioral, metabolic and hormonal homeostatic processes required for energy balance control. During times of limited food availability, NPY promotes behavioral hyperactivity necessary to explore and prepare for novel food resources. As NPY can act via 5 different receptor subtypes, we investigated the path through which NPY affects different behavioral components relevant for adaptation to such conditions. We tested NPY Y1 and Y2 receptor knockout mice and their wild-type littermate controls in a daily scheduled limited food access paradigm with unlimited access to running wheel. Here we show that NPY Y1 receptor deficient mice lack the expression of appetitive behavior and that NPY Y2 receptors control the level of hyperactive behavior under these conditions. Thus, receptor specificity determines the differential expression of NPY-mediated behavioral adaptations to overcome a negative energy status.

6.1 Introduction

Neuropeptide Y (NPY) in the brain has received broad attention for its involvement in the regulation of energy balance, especially as a potent orexigenic factor.[221,222,223] In addition, NPY has been implicated in the regulation of different behavioral, metabolic and hormonal homeostasis processes, such as bone metabolism[224,225] and regulation of behavioral activity levels.[88] Brain NPY levels respond to sudden and chronic environmental situations, such as acute stressful events[226] and during daily scheduled food restriction,[227] however, the question remains how NPY exerts its variety of functions to establish compensatory responses to these environmental challenges.

During daily scheduled limited food availability, mammalian species express distinct forms of behavioral activity that each may serve different adaptations during times of low energy status. For instance, in addition to increased behavioral activity levels, there is a strong evolutionary conserved expression of motor activity just before the time of food availability. This so-called food anticipatory activity (FAA) to the expected upcoming food can be observed in a large variety of species, including insects, fish and primates.[228] As NPY can act through a set of five G-protein coupled receptors,[223] we tested whether NPY receptor types underlie the expression of these different behavioral components relevant for proper adaptation to limited food access.

To investigate the path via which NPY exerts its effect on these processes we compared behavioral responses of NPY Y1 and Y2 receptor knockout mice and their wild-type littermate controls in a daily scheduled limited food access paradigm with unlimited access to running wheel.

6.2 Materials and methods

6.2.1 Animals and housing

For this study, initial mouse breeding pairs were received from the Herzog Laboratory, The Garvan Institute of Medical Research, Australia. The NPY Y1 and Y2 germline receptor knockout mice were generated as described by Baldock et al.[229] Briefly, a targeting vector for the NPY Y1 and Y2 receptor gene has been designed that allows the production of germline NPY Y1 receptor knockout $\left(Y1^{-/-}\right)$ and NPY Y2 receptor knockout $\left(Y2^{-/-}\right)$ mice in a 129/SvJ strain. Positive embryonic stem cells from this strain were selected and injected into blastocysts from C57BL/6J. The $Y1^{-/-}$ mice were backcrossed to a C57BL/6J background for five generations and the $Y2^{-/-}$ mice for three generations. Both homozygous $Y1^{-/-}$ and $Y2^{-/-}$, as well as $Y1^{+/+}$ and $Y2^{+/+}$ littermates, were generated by crossing the respective heterozygous animals. Genotypes of the knockout and wild-type mice were determined by polymerase chain reaction analysis of DNA extracted from ear punch.
All mice used in the experiment were bred at the Rudolf Magnus Institute of Neuroscience animal facility and were 3 – 4 months old at the start of the experiment. In line with our previous studies,[77,157] test-naive female mice were used in the experiments; 10 $Y1^{-/-}$ and 10 $Y1^{+/+}$ littermates and 15 $Y2^{-/-}$ and 10 $Y2^{+/+}$ littermates. As the effects of the estrous cycle are much more subtle in mice than in rats[230] and previous studies found no relationship between the variation in estrous cycle and variation in mouse behavior,[231] we did not systematically monitored the different stages of estrous during this study. Following weaning at 3 – 4 weeks, female and male mice were separately housed in groups (2 – 5 mice) in cages (Macrolon® Type II, Tecniplast, Milan Italy) with sawdust bedding and 1 – 2 tissues per cage (Kleenex®, Kimberly-Clark B.V., Ede, The Netherlands). The housing facilities were maintained on a 12:12-h dark/light cycle with an ambient temperature of 21 ± 2°C and relative humidity of 55 ±10%. During this period, the mice were given water and food ad lib (Rat and Mouse Breeder and Grower diet CRM; Special Diet Services, Essex, UK). All the procedures described were approved by the Animal Experiment Committee of the Academic Biomedical Centre, Utrecht, The Netherlands.

6.2.2 Experimental procedure

During the experiment, all mice were individually housed in cages with voluntary access to a running wheel. The wheel is made of a metal grid with a circumference of 44.5 cm, a diameter of 14 cm and a width of 8.5 cm. The distance between the grid wires is approximately 1 cm. The mice were left to adapt to the conditions for 7 days before the experiment (with food, water and running wheel access ad lib). After this period, food was available ad lib only in the first 2 h of the dark phase for four consecutive days, which is the phase that the mice consume most of their food. The food was presented in pellets, five

pellets were introduced in the cage at the beginning of the dark phase. After 2 h, the food and each small part were collected and the cage was carefully checked that there were no food pieces left. We were not able to collect and weigh some food left over in the form of powder as these are generally very small amounts. The experiment ended on the fifth day before the dark phase (and food access). Body weight and food intake were measured daily before and after food administration. Baseline data of food intake and body weight were collected on experiment day 1, as the mice had food ad lib before the first 2 h of the limited food access episode. Individual running wheel revolutions (RWR) were continuously registered by a magnet activated counter (also during the adaptation phase) using Cage Registration Software version 5.5 (Department of Biomedical Engineering, University Medical Center Utrecht, The Netherlands). The average RWR of 2 days before the experiment was taken as the baseline RWR level. The activity level during the experiment was calculated as the average RWR of 2 days before the end of experiment or individual animals last day (based on the humane end-points). During the experiment we also calculated the FAA as the sum of the RWR during the 4 h prior to food intake as defined by Mistlberger.[228] This coincided with the last 4 h of the light phase, because food was given during the first 2 h of the dark phase. Total FAA was calculated for days 2, 3 and 4 of the experiment correcting for the corresponding total day activity.
At the end of the experiment (day 5), all mice were sacrificed within 3 h prior to the dark phase. We collected truncal blood from 8 $Y1^{-/-}$ and 8 $Y1^{+/+}$ and from 8 $Y2^{-/-}$ and 5 $Y2^{+/+}$ mice; and adrenal glands from 9 $Y1^{-/-}$ to 10 $Y1^{+/+}$ and from 8 $Y2^{-/-}$ to 5 $Y2^{+/+}$ mice. The weight of the right plus left adrenal glands was calculated for each mouse. The blood, collected in eppendorf tubes with 80 µM disodium ethylenediaminetetraacetate dihydrate and 1 mg aprotonin, was spun for 10 min at a relative centrifugal force of 1520 *g* at 4°C. The supernatant was collected and stored until the time of the assay at -20°C. Corticosterone levels in plasma were assayed using the protocol of the radioimmunoassay ^{125}I-labeled kit (MP Biomedicals, Orangeburg, NY, USA).

6.2.3 Statistical analysis

In this experiment, daily body weight levels just before the 2 h of limited food access, food intake, plasma corticosterone levels and the weight of the adrenal glands were compared between the gene knockout mice and their corresponding wild-type controls. We also assessed running wheel activity levels of the mice; the change in running wheel activity levels relative to baseline levels and the expression of FAA, as absolute values and relative to their total daily wheel running activity levels (TDA). The data were expressed as means with standard error of the mean unless otherwise specified.
One sample Kolmogorov-Smirnov test was used to check the Gaussian distribution and Levene′s test for the homogeneity of variances of the data. Two-tailed Student′s t test or Mann-Whitney U test, if the data were nonparametric, was

used for analysis between the knockout and wild-type mice. Pearson′s correlation test was used for the correlation analysis. Significance level was set at $P < 0.05$. The statistical analysis was carried out using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA).

6.3 Results

The results are shown separately for each receptor as the genetic background of the different receptor gene knockouts and corresponding wild-type controls were different (i.e. the NPY Y1 mice were backcrossed five times while the NPY Y2 mice were backcrossed three times to a C57BL/6J genetic background). Therefore, each knockout is compared with its own wild-type littermate and the wild-type mice of each receptor are not compared with each other.

6.3.1 NPY Y1 receptor

Body weight and food intake
During ad lib food conditions, $Y1^{-/-}$ mice were, on average, slightly heavier than their $Y1^{+/+}$ littermate controls ($median_{(min-max)}$, $Y1^{-/-}$: $22.8_{(19.5-29.3)}$g and $Y1^{+/+}$: $20.93_{(19.8-23.2)}$g) but not significant (Mann-Whitney U test, Z = -1.965, P = 0.052). This difference in body weight increased during the following 2 days of the scheduled feeding paradigm (Mann-Whitney U test, Z = -2.041, P = 0.043 and Student′s t test, $t_{(18)}$ = -2.390, P = 0.028, respectively) [Figure 6.1(a)]. One $Y1^{-/-}$ and four $Y1^{+/+}$ mice reached the humane end-point at day 3 of the experiment (indicating that they lost 15% body weight relative to their individual ad lib body weight) and were not included in further analysis. After these mice were taken out, the difference in body weight was no longer present. The difference in body weight of the $Y1^{-/-}$ and the $Y1^{+/+}$ mice was associated with differences in food intake. In general, the $Y1^{+/+}$ mice tended to consume more food than the $Y1^{-/-}$ mice, the cumulative (days 2, 3 and 4) food intake was significantly different (Student′s t test, $t_{(14)}$ = 2.862, P = 0.013; $Y1^{-/-}$: 3.8 ±0.2 g and $Y1^{+/+}$: 4.7 ±0.2 g). Food intake differences were statistically significant during the scheduled feeding paradigm on day 1 (Student′s t test, $t_{(18)}$ = 2.811, P = 0.01) and on day 4 (Student′s t test, $t_{(14)}$ = 2.793, P = 0.014) [Figure 6.1(b)].

Behavioral activity levels
General running wheel activity levels of the $Y1^{-/-}$ mice during the baseline (5903.35 ±1540.13 RWR or 2.63 ±0.69 km) and experiment (8952.35 ±2622.65 RWR or 3.98 ±1.17 km) were not different from the $Y1^{+/+}$ mice (baseline 8082.35 ±1738.04 RWR or 3.60 ±0.77 km and experiment 11 814.6 ±2493.41 RWR or 5.26 ±1.11 km) [Figures 6.1(c),(d), respectively]. Both showed a relative increase in wheel running activity levels during the restriction phase when compared with their baseline levels [$Y1^{-/-}$: 206.2 ±61.8% and $Y1^{+/+}$: 170.8 ±36.6%; Figure 6.1(e)].

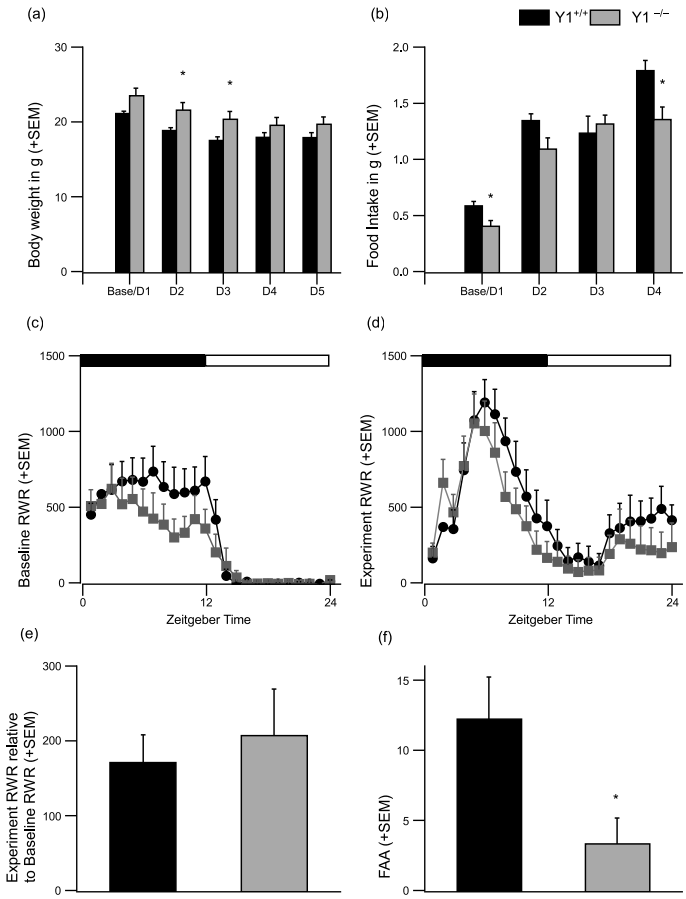

FIGURE 6.1 – ***NPY Y1 receptor results.*** *(a) Body weight of $Y1^{-/-}$ and $Y1^{+/+}$ mice on day 1/baseline P = 0.052, and on subsequent restriction day 2 (P = 0.043) and day 3 (P = 0.028). (b) Food intake during the 2 h of the first (baseline) through fourth day of the daily scheduled limited food access paradigm. In this paradigm, $Y1^{-/-}$ and the $Y1^{+/+}$ mice showed significant differences in food intake on day 1/baseline and on day 4. Removal of the four $Y1^{+/+}$ mice with accelerated body weight loss on day 4 likely explains the change in genotype effect in body weight and food intake on the subsequent days. (c) and (d) Hourly activity levels during the baseline and experiment (the average RWR of 2 days before the last experimental day), respectively. Zeitgeber time has been indicated on the x-axis. The corresponding dark and light cycle has been indicated with a dark and white bar on top of the graph, respectively. Note that the FAA shown in (d) represents FAA levels during 2 days before the end of the experiment. (e) Average activity levels of the experiment as percentage of the baseline levels. (f) Average FAA during the experiment. The data are presented as mean (± standard error of mean) and (*) indicates genotype effect.*

However, there was a significant difference in the total levels of FAA. $Y1^{-/-}$ mice showed less FAA when compared with their $Y1^{+/+}$ littermates ($Y1^{-/-}$: 3.4 ±1.8% and $Y1^{+/+}$: 12.2 ±3%), relative to their TDA (Student′s t test, $t_{(18)}$ = 2.529,P = 0.021) [Figure 6.1(f)]. The absolute values of the total FAA were also significantly different (Mann-Whitney U test, Z = -2.117, P = 0.035, data not shown).

Adrenal glands and corticosterone levels

The weight of the adrenal glands was corrected for body weight at the time of tissue collection. The $Y1^{+/+}$ mice had significantly larger adrenal glands than the $Y1^{-/-}$ mice ($Y1^{-/-}$: 0.36 ±0.03% and $Y1^{+/+}$: 0.47 ±0.04%; Student′s t test, $t_{(17)}$ = 2.373, P = 0.030) [Figure 6.2(a)]. The absolute weight was not correlated with plasma corticosterone levels (data not shown). The plasma corticosterone levels were not significantly different [$Y1^{-/-}$: 47.3 ±4.6 µg/dl and $Y1^{+/+}$: 37.5 ±3.6 µg/dl; Figure 6.2(b)].

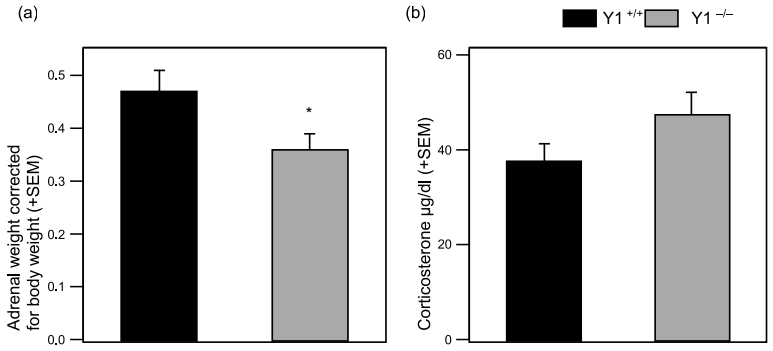

FIGURE 6.2 – ***The weight of the adrenal glands, corrected for body weight, graph (a), and the corticosterone levels, graph (b), for $Y1^{-/-}$ and $Y1^{+/+}$ mice.*** *The data are presented as mean (± standard error of mean) and (*) indicates a significant genotype effect.*

6.3.2 NPY Y2 receptor

Body weight and food intake

During the scheduled feeding paradigm $Y2^{-/-}$ mice and their $Y2^{+/+}$ littermates did not differ in body weight throughout the experiment [Figure 6.3(a)]. Consistent with these body weight similarities, $Y2^{-/-}$ and $Y2^{+/+}$ mice consumed similar levels of food during ad lib and experimental conditions [Figure 6.3(b)], the cumulative (days 2, 3 and 4) food intake was not significantly different ($Y2^{-/-}$: 4.7 ±0.1 g and $Y2^{+/+}$: 4.6 ±0.2 g, data not shown).

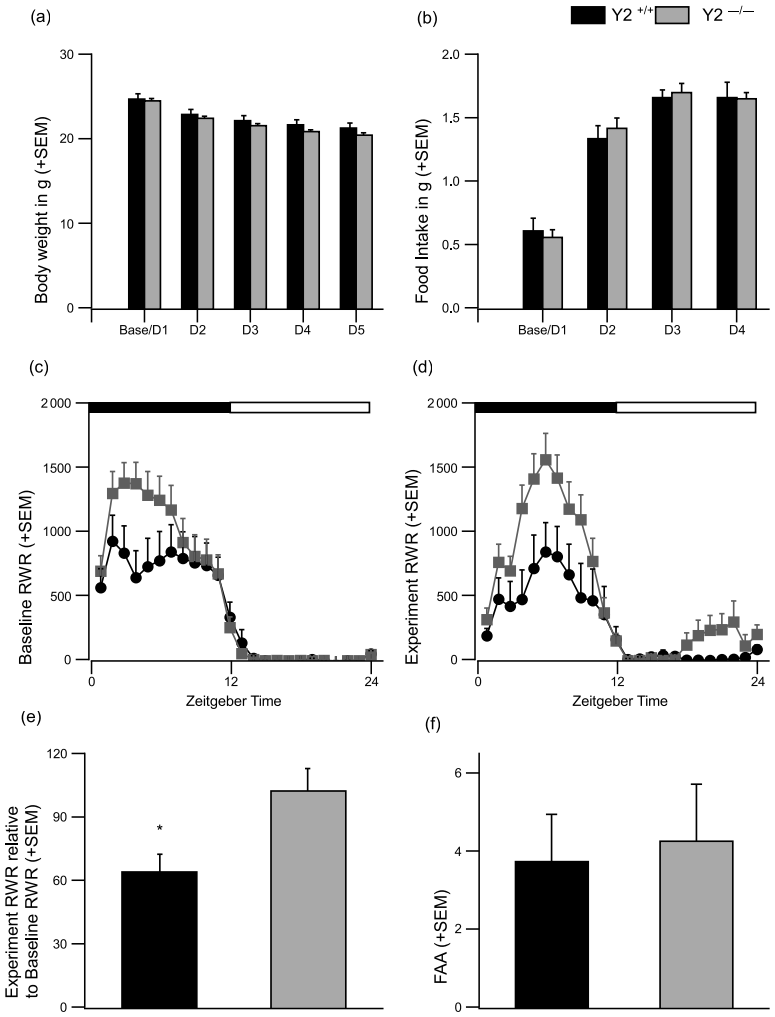

FIGURE 6.3 – ***NPY Y2 receptor results.*** *$Y2^{-/-}$ and the $Y2^{+/+}$ mice showed similar body weight levels (a), as well as food intake (b) during both the baseline and food restricted phase. (c) and (d) Hourly activity levels during the baseline and experiment (the average RWR of 2 days before the last experimental day), respectively. Zeitgeber time has been indicated on the x-axis. The corresponding dark and light cycle has been indicated with a dark and white bar on top of the graph, respectively. Note that the FAA shown in (d) represents FAA levels during 2 days before the end of the experiment. (e) Average daily running wheel activity levels during the restriction phase relative to baseline levels. (f) Average FAA during the experiment. The data are presented as mean (± standard error of mean) and (*) indicates a significant genotype effect.*

Behavioral activity levels

In contrast to the $Y1^{-/-}$ mice, $Y2^{-/-}$ mice showed a robust effect on the general running wheel activity levels during the scheduled feeding paradigm (baseline 11 990.5 ±1618.33 RWR or 5.34 ±0.72 km and experiment 12 359.3 ±1834.87 RWR or 5.50 ±0.82 km) [Figure 6.3(c),(d)] when compared with their wild-type controls (baseline 8822.95 ±2107.92 RWR or 3.93 ±0.94 km and experiment 6373.45 ±2274.2 RWR or 2.84 ±1.01 km). Under these conditions, the $Y2^{+/+}$ mice decreased and maintained their reduced wheel running activity levels, while the $Y2^{-/-}$ mice, after an initial decrease, significantly increased their wheel running activity levels ($Y2^{-/-}$: 101.7 ±10.4% and $Y2^{+/+}$: 63.9 ±8.3%; Student's t test, $t_{(23)}$ = -2.604, P = 0.016) [Figure 6.3(e)]. In contrast to $Y1^{-/-}$ mice, there was no difference between $Y2^{-/-}$ mice and their $Y2^{+/+}$ littermate controls in FAA levels, either as absolute values (data not shown) or relative to the TDA [$Y2^{-/-}$: 4.3 ±1.5% and $Y2^{+/+}$: 3.7 ±1.2%; Figure 6.3(f)]. Note that the FAA shown in Figure 6.3(d) shows FAA during 2 days prior to the end of the experiment. Absolute FAA levels of $Y2^{-/-}$ appear slightly higher than $Y2^{+/+}$, however, there is no significant difference in these FAA levels ($median_{(min-max)}$, $Y1^{+/+}$: $94.5_{(8-442.5)}$ RWR and $Y1^{-/-}$: $195.5_{(39-5114.5)}$ RWR; Mann-Whitney U test, Z = -1.609, P = 0.112).

Adrenal glands and corticosterone levels

$Y2^{-/-}$ mice and their $Y2^{+/+}$ littermates showed no significant differences for adrenal glands weight corrected for body weight [Figure 6.4(a)] nor for their corticosterone levels ($median_{(min-max)}$, $Y2^{-/-}$: $31.3_{(6-69)}$ µg/dl and $Y2^{+/+}$: $38.9_{(28-46)}$ µg/dl) [Figure 6.4(b)]. As in $Y1^{-/-}$ mice, there was no correlation between the absolute adrenal glands weight and the plasma corticosterone levels (data not shown).

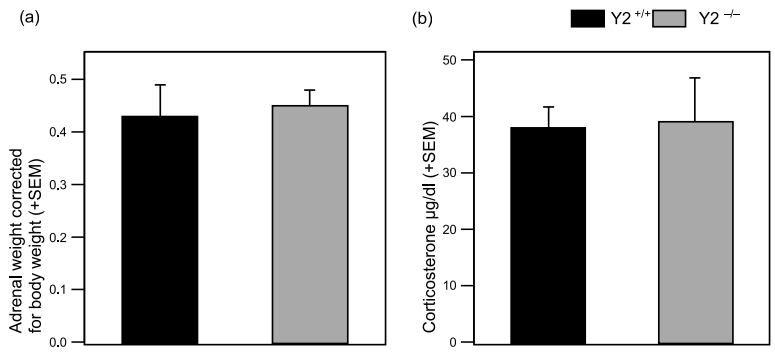

FIGURE 6.4 – ***The weight of the adrenal glands, corrected for body weight, graph (a), and the corticosterone levels, graph (b), for $Y2^{-/-}$ and $Y2^{+/+}$ mice.*** *The data are presented as mean (+ standard error of mean).*

6.4 Discussion

The data obtained show that NPY receptors have different roles in regulating behavioral adaptive strategies in mice during daily scheduled food restriction. Mice with a genetic deletion of Y1 receptors expressed significant lower levels of FAA when compared with their wild-type littermate controls. The reduced expression of this appetitive behavior is consistent with pharmacological studies in various rodent species showing that NPY Y1 receptor ligands affect the appetitive phase of food intake.[232,233,234] In contrast to $Y1^{-/-}$ mice, $Y2^{-/-}$ mice showed increased daily motor activity levels specifically during the daily scheduled feeding paradigm when compared with wild-type littermate controls. These data indicate that, during scheduled limited food access, NPY Y1 receptors promote appetitive behaviors, such as FAA, whereas NPY Y2 receptors are involved in regulating daily energy expenditure levels during a negative energy balance. On the second and third day of the scheduled food restriction paradigm, we observed that $Y1^{-/-}$ mice had a significantly higher body weight than their $Y1^{+/+}$ littermates. Furthermore, four $Y1^{+/+}$ mice needed to be taken out of the experiment before the intended end of the daily feeding schedule because of accelerated body weight loss. While we did not observe high body weights in NPY $Y1^{-/-}$ mice under baseline conditions,[235] our data suggest that the NPY Y1 receptor in mice affect body weight maintenance during limited food access. Alternatively, the reduced levels of FAA in NPY $Y1^{-/-}$ mice during limited food access may have contributed to the body weight changes, because the final hour of FAA coincided with the time of our daily body weight measurements.

The induction of behavioral hyperactivity levels during limited food access that was observed in $Y2^{-/-}$ mice resembles the behavioral response following NPY injections under similar food limiting conditions.[88] As NPY Y2 receptors have been considered auto receptors[236,237] that are located on NPY producing neurons,[238] the genetic deletion of the NPY Y2 may have resulted in behavioral hyperactivity via reduced inhibition on NPY release. Further studies are necessary to confirm this and to answer the question how NPY subsequently stimulate behavioral hyperactivity, however,our results support the notion that this is not via the NPY Y1 receptor. Alternatively, behavioral hyperactivity during limited food availability may be a compensatory behavior to deal with altered anxiety levels that NPY Y2 receptor deficient mice are known to express.[239,240] Indeed, a study in eating disorder patients indicated that anxiety symptoms and food restriction synergistically contribute to increased levels of physical activity in the acute phase of anorexia nervosa.[94] It would, therefore, be of interest to study the relationship between increased physical activity and anxiety levels, in the acute phase, in anorexia nervosa patients as a function of the occurrence of NPY and NPY Y2 receptor mutations in this human eating disorder population. In the past few years, several genes have been implicated in the regulation of FAA, Orexin receptor,[241,242] Mu Opioid receptors[243] and Ghrelin,[244,245] among others. A recent study in NPY knockout mice showed a delayed onset of FAA

when compared with controls.[246] Our data suggest that this NPY effect in the development of FAA may be modulated via the Y1 receptor.
Reduced FAA in $Y1^{-/-}$ mice was associated with reduced adrenal glands weight, but not with release of the corticosterone hormone, suggesting that FAA levels are not related to acute responses of the hypothalamus-pituitary-adrenal axis at this time of day.[247] Although several discussions are ongoing about the existence of a food entrainable oscillator that is driving these scheduled induced behavioral responses to limited food access,[248,249,250,251] none of gene knockout mouse strains that affect FAA showed a complete ablation of FAA during scheduled feeding. This suggests that various gene products can have a modulating effect on this anticipatory behavior rather than that of being necessary and required for the generation of FAA. Interestingly, some of these systems identified seem to functionally interact, providing some more ground for the underlying neurobiological mechanisms of FAA. For example, different studies have shown that the ghrelin-stimulating effects on foraging behavior and food intake can be blocked by a NPY Y1 receptor antagonist.[252,232] These findings indicate that more emphasis should be put toward the functional and neuroanatomical integration of neuropeptide systems that each are known to modulate FAA levels.
Taken together, it is expected that different receptors of NPY have different functions in behavioral processes regulating energy balance. Here we show that NPY Y1 receptors promote the expression of appetitive behavior prior to daily scheduled limited food access and that NPY Y2 receptors control behavioral hyperactivity levels under these conditions.

6.5 Acknowledgments

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Chapter 7

Quantitative promoter DNA methylation analysis of four candidate genes in Anorexia Nervosa: a Pilot Study

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Manuscript submitted

7.1 Introduction

Gene and environment interactions play a role in the development (and possibly maintenance) of complex psychiatric disorders including anorexia nervosa (AN).[253,177] The biological underpinnings of gene and environment interactions (G×E) may involve epigenetic processes,[31] and of relevance to eating disorders, epigenetic modifications have been linked to weight, fat cells and abnormal stress responses.[254,55] In this pilot study, we examined promoter region DNA methylation in four well established candidate genes that have been associated with AN in genetic studies. The leptin gene (LEP) is the first candidate, as leptin is involved in the regulation of fat stores and has been proposed as a diagnostic marker in AN.[255] The serotonin transporter gene (SERT/ SLC6A4) has been examined as SERT polymorphisms have been associated with G×E in people with AN.[177] The third gene is brain - derived neurotrophic factor (BDNF) which has been implicated in AN by some but not all studies.[256,257] Because of the complexity of the BDNF gene,[258] two exons (IV and VI) have been examined. Lastly, we sought to replicate the data showing that dopamine receptor D2 gene (DRD2) hypermethylation is present in people with AN.[60]

7.2 Materials and Methods

Participants, aged 16 – 60 years, were selected at random from the Maudsley eating disorder unit volunteer database, AN (n = 45). A group of matched healthy controls (HC) (n = 45) was recruited from several sites as described previously.[259] The study was approved by the Institute of Psychiatry ethics committee and the South London and Maudsley National Health Service Trust research ethics committee. All participants had given written informed consent after the procedures had been explained. DNA was extracted from venous blood using standard protocols. DNA methylation analysis was conducted as previously described.[260] Details on primers and the number of samples included in the analysis after the quality control check are provided in table 7.1. All samples were randomized and processed blind to sample identification. The average level of DNA methylation (% 5meC) for each genomic region was calculated by taking the mean of the multiple cytosine-phosphate-guanine (CpG) sites. Data are presented as mean ± standard error of mean (SEM), unless otherwise specified. Statistical analysis was carried out using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA). Two-tailed Student′s t-tests or Mann-Whitney U tests were used for analysis between the groups. Pearson′s or Spearman′s correlation tests were used for the correlation analyses. The results were corrected for multiple testing.

TABLE 7.1 – *Details of the primers used in methylation analysis and the number included in analysis after quality control. For Serotonin Transporter (SERT), Brain derived neurotrophic factor (BDNF) exon IV, BDNF exon VI and Dopamine Receptor (DRD2) genes, the cycling conditions in the bisulfite - PCR amplification were 45 cycles with an annealing temperature of 56°C; for Leptin gene (LEP), 50 cycles with an annealing temperature of 56.5°C was used.*

Gene	Genomic Region UCSC Human Genome Feb. 2009 (Grch37/Hg19) Assembly	CpG Units and sites	[a]Primer sequences Designed online on Sequenom EpiDesigner software (http://www.epidesigner.com/start3.html)	Total Number Included In Analysis	
				AN	HC
BDNF exon IV	chr11:27,722,495-27,723,494	11 units (13 sites)	F: TTGATTTTTTTAGAGTTTGTTTAATAGGA R: AACATAAAATCTCCCTACCTCTACC	29	37
BDNF exon VI	chr11:27,721,388-27,722,272	9 units (14 sites)	F: GATGGGGGAGAAAATTTTTTAAG R: AAAAACTTTAATAAAACACCCACC	35	42
DRD2	chr11:113,345,648-113,346,601	8 units (11 sites)	F: TTTTGGTTTTTGAGTTTTTAAAGGA R: CCACCAAAAAAACTATACCTCCTC	40	42
SERT	chr17:28562435-28562812	11 units (19 sites)	F: GGTTAGTTTTAGTTTTGGTTTTTGTT R: CAAAAATTCTTCAAAAACTCTTTAAC	30	41
LEP	chr7:127,881,054-127,881,410	14 units (21 sites)	F: TTTTTGGAGGGATATTAAGGATTT R: CTACCAAAAAAAACCAACAAAAAAA	38	38

[a]The standard Sequenom MassCLEAVE tags (F: aggaagagag, R:cagtaatacgactcactatagggagaaggct) were added to these primers.

7.3 Results

Participants did not differ in age but there was a significant difference in the current body mass index (BMI) between AN (19.5kg m^{-2} ± 0.44 SEM) and HC (22.5kg m^{-2} ± 0.43 SEM) participants (Student′s t-test, $t_{(81)}$ = -4.871, $p < 0.0001$). In the AN group, almost half of the participants had a BMI above 18kg m^{-2}, however, there was no significant difference between those above or below 18kg m^{-2} on any of the parameters.
DNA methylation levels in the promoter regions were not significantly different between the two groups for any of the genes (shown as % 5meC,
LEP (median$_{(min-max)}$): AN 23.3$_{(15.2-33.1)}$ and HC 24.5$_{(13.8-30.2)}$;
BDNF exon IV (median$_{(min-max)}$): AN 4.8$_{(2.1-10.4)}$ and HC 5.0$_{(2.5-12.9)}$;
BDNF exon VI (median$_{(min-max)}$): AN 7.0$_{(5.0-9.8)}$ and HC 7.4$_{(3.1-13.1)}$;
SERT: AN 4.3 ± 0.2 SEM and HC 4.6 ± 0.3 SEM and
DRD2: AN 9.5 ± 0.3 SEM and HC 9.7 ± 0.2 SEM).
None of the individual CpG site analysis showed significant differences between the groups. We also examined correlations between the current BMI and DNA methylation but none of the correlations passed the significance threshold after correcting for multiple testing.

7.4 Discussion

In this pilot study, we did not observe any significant differences in the levels of promoter DNA methylation between the groups for any of the candidate genes, nor did we find a significant correlation between BMI and DNA methylation for any of the genes or CpG sites. Given that the genes for LEP, SERT and BDNF have been implicated in AN, and for example, in fat regulation or in G×E associated with AN, but strong evidence is missing, it might have been expected that some differences would have been observed between the AN and HC groups. The lack of difference in LEP gene methylation levels between the HC and AN groups, while they have different BMIs, suggests that regulation of leptin levels is not associated with changes in promoter DNA methylation.
We did not replicate reports of significant hypermethylation of DRD2 in AN, probably because not all our AN participants were acutely ill and the BMI was relatively higher. However, we did not observe differences between those above or below BMI of 18kg m^{-2} (probably due to the low sample number), but also the methylation levels of our control group were much lower.
Our study has some limitations. Participants were randomly selected from a database and thus not all were ill at the time of testing: it is possible that factors related to illness status might affect results. Secondly, the AN group consisted of both binge and purging subtypes, and this might influence methylation status. Thirdly, DNA was from blood and there is tissue specificity in the epigenome; this is a major limitation of psychiatric studies performed on peripheral cell types. Lastly, we only looked at the methylation levels in a specific locus, the

promoter region.
Owing to these limitations, the present results do not rule out epigenetic changes at these loci. Replication in a sample where the limitations are reduced, e.g. controls for the current status of illness and AN subtype, is needed. Further, it is unclear how differences in methylation relate to gene expression, and incorporation of a three-way association design, between genotype, methylation and gene expression would offer more evidence in the biological mechanisms of AN.

Chapter 8

Summary and discussion

8.1 Anorexia nervosa and activity based anorexia mouse model

Excessive exercise or excessive physical activity, one of the core features of anorexia nervosa, plays an important role in the development, maintenance, and recovery rate of the disorder.[19,20,13,14,15,21,16,17,22] This feature can be modeled in mice using the activity based anorexia model. In this paradigm, mice show a paradoxical hyperactivity under scheduled food restriction conditions resulting in accelerated body weight loss. With this model we have studied the genetics, candidate gene effects and gene by environment interactions across different developmental stages of this behavioral phenotype.

8.2 Chromosome substitution strains and genetic fine mapping of hyperactivity

The screening of chromosome substitution strains, and the parental strains, revealed that under scheduled limited food access, three chromosomes: 4, 12 and 13, harbored loci leading to hyperactivity under these conditions. The observed hyperactivity was associated with disorganized rhythms as observed by behavioral activity occurring mostly during the light phase hours of the dark/light cycle (Zeitgeber time 0 to 7), when C57BL/6J mice are normally resting. This phenotype was apparent from the first restriction day in CSS12 and CSS13 strains, and from the second restriction day in the CSS4 strain and led to accelerated body weight loss.

Though initial body weight of these chromosome substitution strains was lower than C57BL/6J (the genetic background control strain), body weight level was not associated with an increase in hyperactivity as other strains such as CSS2 and CSS5 also had a low body weight but displayed no hyperactivity. Thus, the genetic loci on chromosomes 4, 12 and 13 regulate the development of disrupted locomotor activity in response to restricted feeding irrespective of the body weight patterns under ad libitum feeding conditions.

One interesting aspect of the observed hyperactivity, in these three chromosomal strains, is that it was not observed in either of the parental strains of the chromosome substitution strains (A/J and C57BL/6J) and became apparent under certain environmental conditions. This suggests the presence of transgressive segregation across the progeny, a phenomenon characterized by the emergence of new or extreme phenotypes in segregating hybrid populations when compared to parental strains.[130,131,132]

In order to identify the location of the locus (/loci) on chromosome 12, we generated a female F_2-progeny (n = 186) that was genotyped and behaviourally screened in the ABA model. A locus was found in the proximal region of chromosome 12 and points to positive heterosis, that is, the phenotype of the heterozygous genotype exceeds both of the homozygous phenotypes (C57BL/6J and A/J). The mode of inheritance analysis revealed an overdominance effect

of the phenotype.
The current location of the QTL peak is of interest in the regulation of activity levels, as this region has also been found in another mapping study for locomotor activity using a different mouse genetic population.[168] However, further genetic fine mapping of the region by genotyping additional microsatellite markers is necessary to specify the precise location of the peak for the QTL interval. Subsequently, a search for candidate genes within the QTL interval that contribute to the observed phenotype can be initiated. The observed heterosis effect suggests that this will not be an easy task.

8.3 Candidate genes

When a gene is knocked out, the observed phenotype can be directly linked to the candidate gene. In this thesis, we focused on knockout mice of the Neuropeptide Y (NPY) Y1 and Y2 receptor genes and their wild-type littermate controls, to investigate their role in the regulation of behavioral activity levels when exposed to the ABA model. These receptors were selected on the basis of the effect of NPY infusion on behavioral hyperactivity levels in scheduled food restricted mice.[88] The two receptors of NPY revealed specific functions in regulating behavioral adaptation to daily scheduled food restriction: Y2 receptors regulate the daily energy expenditure (daily running wheel activity levels) and Y1 receptors are involved in promoting appetitive behaviors, such as food anticipatory activity during the daily scheduled restriction phase.
These results suggest that it is not Y1 receptors via which NPY may exert its role in behavioral hyperactivity. Possibly Y2 receptors, considered auto receptors[236,237] and located on NPY producing neurons,[238] regulate NPY release, and loss of these receptors may have resulted in behavioral hyperactivity via reduced inhibition of NPY release. Further studies are necessary to confirm this role of Y2 receptors.

8.4 Gene and environmental interplay

The role of gene and environment interplay in neuropsychiatric disorders has become more prominent lately. In this thesis we carried out a series of experiments to investigate the effects of changing the maternal environment on body weight and ABA susceptibility.
The results on the CSS4 strain were quite exciting as the change in maternal environment, independent of the foster mother's genetic background, improved ABA susceptibility in highly susceptible adult CSS4 mice. There was no effect on ABA susceptibility following cross-fostering in the low susceptible C57BL/6J strain, suggesting that the effects of early life events on ABA susceptibility are also dependent on the genetic background of the mice.
The effects observed on body weight, on the other hand, were different: we observed an interaction between developmental stage and pup genetic back-

ground and maternal environment. When maternal x pup environment was isogenic (same genetic backgrounds), CSS4 mice were affected by fostering with a further decrease in body weight at postnatal day 14. CSS4 mice with a CSS4 mother, with and without cross-fostering, had similar body weights during adulthood, however, their body weight was still lower than in the C57BL/6J control strain. When maternal x pup environment was non-isogenic (different genetic backgrounds), body weight was determined by the genetic background of foster mothers at postnatal day 14. In C57BL/6J mice, at adulthood, genetic background prevailed, with mice in all groups having a similar body weight. However, the CSS4 strain still showed strong environmental influences: body weight was significantly higher in the foster mothers group. These results show a complex dynamic regulation of body weight throughout different life stages.
Gene and environmental interplay can influence biological processes via epigenetic modifications.[261,31,262] Therefore, we analysed DNA methylation in the CSS4 strain groups. Change in maternal environment was associated with significant differences in DNA methylation when compared to the mice raised by their own biological mothers. Several genes implicated in different psychiatric disorders showed hypermethylated regions. Importantly, there was also a gene, Fam155a, whose SNP in the human homologue has come up as one of the most significant in a genome wide association study in anorexia nervosa.[46] Of course, further research is necessary to find the causal relationship between the gene and environmental interplay, methylation and the resulting behavior. Nevertheless, these findings provide a new link between animal studies and human research and, further emphasizes the role of animal studies in understanding the neurobiology of anorexia nervosa and other psychiatric disorders.

8.5 Factors affecting ABA susceptibility

Chromosome substitution strain screening revealed that strains with increased hyperactivity during the restriction phase also showed accelerated body weight loss resulting in reaching the humane end point early (based on a 15% body weight loss criteria) and being taken out of the experiment.
As early intervention is better than treatment, we continued our research with the question: “What are the factors that influence and/or predict susceptibility to the model prior to the restriction phase?” To answer this question, instead of a hybrid panel, we chose eleven inbred mouse strains and screened them in the ABA model. As a starting point, this set of inbred strains was diverse in body weight, food intake and activity levels under baseline conditions.
We identified strains with different susceptibility levels (body weight loss levels) when exposed to the ABA model. Similar to the chromosome substitution strain panel results, high activity levels during the restriction phase were associated with increased body weight loss. The baseline factors: body weight, food intake and activity influenced the ABA susceptibility to a certain level. The available mouse inbred strain literature data on body fat, leptin, insulin and glucose did

not point to a strain correlation with ABA susceptibility.
Among baseline factors, it was the activity levels that highly predicted the susceptibility across all the strains. The interaction between physical activity levels and food intake per gram body weight had initially a substantial effect on ABA susceptibility but this effect was driven by one of the strains who had high levels for both of these factors. The effect of these factors is also dependent on the genetic background of the strains, where other baseline factors, or a combination of factors are more prominent than the effect of activity. These findings provide novel opportunities to further study the underlying neurobiological mechanisms of these factors and their interrelatedness.

8.6 Clinical implications

Excessive exercising is an important feature with significant influence throughout the trajectory of the illness: development, maintenance, recovery rate and outcome. In this thesis, to expand our understanding on the genetic basis of this feature, we made a start by screening different mouse panels in the ABA model; searching the role of candidate genes; studying the role of hyperactivity in ABA susceptibility. We also examined a gene and environmental interplay role in ABA susceptibility. Though further research is necessary to understand through which mechanisms hyperactivity and susceptibility behavior are affected, the current results provide a couple of starting points relevant to the clinical phenotype.
For example, it would be of interest to study the relationship between increased physical activity, NPY and NPY Y2 receptor mutations in acutely ill anorexia nervosa patients, as a recent study has found higher plasma NPY levels in people with bulimia nervosa whereas these levels were further increased as a result of exercising.[263] The NPY levels, in cerebrospinal fluid, were reduced in people with anorexia nervosa after some period of recovery[264] suggesting a relationship between illness status and circulating NPY levels.
Second, our findings suggest that high activity levels before scheduled limited food access are associated with increased susceptibility to the ABA model. These findings indicate that excessive activity levels preceding illness onset are an important feature to be taken into consideration. Our study design suggests that excessive exercise is more of a proximal risk factor associated with illness onset. Additionally, we found a genetic component for behavioral hyperactivity observed under scheduled food restriction (data from chromosome substitution strains), which may explain why excessive activity is not seen in all people with AN. We can speculate that close to illness onset, other factors such as increased desire to loose weight and increased concerns about body shape possibly play a role in triggering excessive exercise behavior depending on the genetic background.

8.7 Quantitative promoter DNA methylation of four anorexia nervosa candidate genes

In this pilot study we examined four candidate genes that have been proposed to alter the risk of developing an eating disorder. There was a difference in the levels of DNA methylation in the different genes, but we did not observe differences between anorexia nervosa and healthy control participants for any of the genes or for specific CpG sites, after correcting for multiple testing, probably because of the low number of patients.
The participants in the study were selected at random from the Maudsley eating disorder unit database, which carries some limitations with it. However, because of these limitations and the number of participants, we can not exclude epigenetic modifications of these candidate genes. The next step is carrying out a larger study including also the genotype and gene expression analysis. This combined design would increase our understanding of the genotype x epigenetic x phenotype effects in this disorder.

8.8 Concluding remark

The studies described in this thesis suggest that the trajectory of anorexia nervosa is likely defined by an ongoing gene and environment interplay starting at an early stage. Close to the onset of illness, the role of excessive exercise or excessive physical activity as a risk factor becomes prominent and it should also be considered as a factor of illness severity. These results warrant further studies to investigate what modifications of brain structures i.e. neuronal organizations or/and connections, have occurred with the epigenetic modifications (of neurodevelopmental genes) associated with fostering and how these changes affect susceptibility to adult mal-adaptive behavior.

Chapter 9

Future perspective in view of epigenetics in eating disorders

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Abstract

Purpose of review: Eating disorders are complex psychiatric disorders in which genes,environment, and gene-environment interactions (G×E) have a role. Such G×E may occur in adulthood or during development. They may also be modified by factors such as (mal)nutrition or stress and this may result in acute or long-term epigenetic modifications. This review discusses the potential for recent developments in epigenetics to address ongoing aetiological issues in eating disorders.

Recent findings: Epigenetic studies in eating disorders have focussed on the DNA methylation status of promoter regions of candidate genes: differences have been reported between people with eating disorders and healthy controls, and between subtypes of eating disorders. Animal studies related to eating disorders have focussed on understanding the acute and long-term effects of environmental manipulation on epigenetic changes and on the resultant phenotypes: these studies are promising, but they have also identified some of the complexity of epigenetic processing.

Summary: Because of the difficulties in obtaining brain samples, epigenetic studies in eating disorders (like in other psychiatric illnesses) have used peripheral tissues, usually blood: this raises various problems. It is likely, therefore, that in the immediate future,animal, rather than human studies will guide the progress in epigenetics studies of eating disorders and other psychiatric disorders.

9.1 Introduction

Eating disorders include anorexia nervosa, bulimia nervosa, binge eating disorders (BED), and eating disorder not otherwise specified (EDNOS).[31] They are severe psychiatric illnesses with high levels of psychological and physical comorbidity. Mortality is also raised, especially in anorexia nervosa, which has the highest mortality rate amongst psychiatric disorders.[4] Typically, eating disorders occur in young women at a (peri)pubertal age[265] and may persist for several years.[266,267] The majority of cases (50%) fall into the broad group of EDNOS.[268] Diagnostic crossover between different eating disorders diagnoses can occur over time, for example, symptom changes can occur such that a patient moves from anorexia nervosa to bulimia nervosa.[269,179] These various diagnostic issues are important for our understanding of the aetiology of eating disorders, but in addition, they have to be considered in genetic studies.[270,271] The combination of eating disorders and obesity[272,273] is an emerging problem, rising faster than either eating disorders or obesity alone.[274] However, the relationship between obesity and eating disorders (if any) is unclear, although in

some cases, being overweight during adolescence may be part of the trajectory into an eating disorder.[272] This issue of weight and eating disorders is important both clinically and academically because there is a central question about the extent to which eating disorders are about psychological variables such as perfectionism, cognitive rigidity, or anxiety and/or about set points for weight regulation, eating, or nutrition.[267] However, this is not an 'either' or 'or' issue, as weight loss can also give rise to symptoms of eating disorders and hence may contribute to the maintenance of illness (e.g. after bariatric surgery[275] or weight loss[276,277]).

Eating disorders are multifactorial diseases and there is an extensive literature on neurobiologically based aetiological models (e.g. Adan & Kaye[278]). There are also well established risk factors (genetic, environmental, and developmental)[279,33] and there is increasing interest in the possibility that gene - environment interactions (G×E) are involved not only in the aetiology of eating disorders,[177] but also that they could contribute to their phenotypic heterogeneity and pathoplasticity. There is also interest in the possibility that early environmental factors such as maternal nutritional state and/or level of stress will contribute to the risk of an eating disorder in the offspring.

The increased interest in G×E in relation to eating disorders has coincided with the increase in epigenetic studies in psychiatry in part because of advances in technology but also because of the possibility that epigenetic changes will provide at least some of the biological underpinnings for G×E.[31]

9.2 Epigenetics: An introduction

Epigenetics is the study of changes in gene expression that occur without any alteration in the sequence of the DNA. Epigenetic modifications provide a dynamic mechanism for altering gene expression levels at different stages of development and also as a consequence of environmental changes.[280] These changes can occur via DNA methylation, post-translational modifications of histones, and noncoding RNAs (ncRNAs).[31,48] These various epigenetic modifications not only have an effect of their own, but also interact producing multiple dynamic and complex changes.

DNA methylation involves the addition of a methyl group [catalysed by DNA methyltransferases (DNMT)] on the cytosine-phosphate-guanine (CpG) nucleotides on the fifth position of the pyrimidine ring of cytosine residues, resulting in the suppression of transcription. CpG nucleotides concentrated within a GC-rich region (CpG islands) are usually present in the promoter region of approximately 40% of genes.[48] Histone modifications (which include methylation, acetylation and phosphorylation of amino acids) modify higher order chromatin structure by influencing the accessibility of target DNA or by attracting enzymes for DNA manipulation. The effect of histone modifications can be repressing or activating. Histone deacetylation via histone deacetylases (HDACs) is the best-studied mechanism and works in conjunction with histone acetyla-

tion to regulate thenumber of acetyl groups in the tails of histone proteins.[281] There are several classes of ncRNAs which are involved in epigenetic modifications and these act by altering gene transcription. MicroRNAs (miRNAs) are the best known class ofncRNAsandtarget the 3′-untranslated regions of genes. miRNAs have a size of approximately 23 nucleotides and can change gene transcription by post-translational gene silencing.[282]
Epigenetics has progressed at a rapid rate in recent years, in part because of technological advances. This has increased our understanding of epigenetic processes and how they are likely to be involved in problems such as cancer[283] and possibly in complex disorders.[52] Analysis of epigenetic modifications can be achieved in several ways.[284] DNA methylation at a specific locus is the most accessible and widely used method. In the near future, the use of global DNA methylation analysis will increase rapidly because of the availability of next-generation sequencing methods.[285,286,287,288] Until recently, this type of analysis was limited by the fact that all the CpG sites in the genome (≈28 millions) needed to be included in the analysis of all the samples necessary to detect differentially methylated regions (DMRs) and this was not cost-effective.
Progress in the generation of epigenetic databases has been made, and this includes one for the human brain.[289] These datasets will provide a valuable resource for future research, especially since human brain tissue (especially that associated with eating disorders and other psychiatric disorders) is not readily available.

9.3 Epigenetics and eating disorders

Epigenetic investigations in relation to eating disorders are of great interest as they have the potential to address a number of important questions. For example, questions related to perinatal risk factors, such as maternal nutrition (over or undereating, e.g. the effects of Dutch famine[290]), maternal stress, or early (foetal and/or infant) development, may be associated with long-term changes in the epigenome that alter the risk of eating disorders in the offspring. Secondly, it is possible that epigenetic changes are associated with sex and with pubertal events that confer risk. Thirdly, although dietary patterns are associated with global DNA methylation in peripheral blood leukocytes,[291] it is unknown how epigenetic modifications respond to acute changes in the environment, for example, being exposed to highly palatable calorific food or to malnutrition. These might be associated with trajectories into illness, with maintenance of illness or with different illness outcomes.
Lastly, there is emerging interest in the possible role of epigenetic processing in learning and memory:[292] this is of interest given the possibility that learned behaviours contribute to the development and maintenance of eating disorders.
Most epigenetic studies in eating disorders have examined the levels of DNA methylation in peripheral tissue, mainly blood. It can be argued that these will be of value particularly in eating disorders research because peripheral changes

may occur in relation to weight change and overall energy state. In addition, a case can be made for examining tissue directly related to body weight regulation, such as adipocytes.[31,254] However, the lack of ready access to brain tissue has (and will) hamper epigenetic studies in relation to psychiatric disorders. Postmortem brain tissue remains an option, but this has its own problems, for example, the manner, the time and the cause of death, and the possibility of having to make a retrospective diagnosis. Furthermore, it is likely that multiple different epigenetic modifications will occur in different brain regions,[293] and thus a simple whole brain versus peripheral tissue comparison will not resolve the issue of regional specificity.

This is a major issue and it is arguable that the field requires much more data from animal studies, for example, related to blood brain comparisons, related to brain region comparisons, and lastly in relation to environmental and developmental effects associated, for example, with maternal diet and stress.

Animal models can only mimic certain aspects of eating disorders (e.g. physical hyperactivity or behavioural inflexibility); however, they may allow a better understanding of eating disorders or any other psychiatric disorder.[64,294] For example, in contrast to epigenetic studies in heterogeneous patient populations, animal studies can allow the systematic study of epigenetic modifications in relation to time, environment, genetics, and, importantly, in different tissues. In this way, it is possible to study epigenetic modifications in specific brain areas and also to observe the effects of dietary, behavioural, and pharmacological interventions on these modifications in this target tissue. Further, animal studies can accelerate our understanding of critical phases of illness development as the reproduction and lifetime of, for example, mice, is much shorter than of humans.

9.4 Clinical studies

Despite recent advances in epigenetic research, the number of epigenetic studies in eating disorders is very limited. These have focussed on DNA methylation of promoter regions of candidate genes associated with eating disorders. Here, we review these studies in detail.

In an early study using lymphocytes,[59] significant global DNA hypomethylation was reported to be present in a group of anorexia nervosa patients but not in a bulimia nervosa group. In addition, hypermethylation of the promoter region of the alpha synuclein gene was reported in the anorexia nervosa group: the bulimia nervosa group showed a trend towards similar hypermethylation but this did not reach significance after correcting for multiple testing, possibly because the number of participants ($n = 24$) was low. The same research group has reported data from their epigenetic studies of several candidate genes.[59,58,60,57] These included vasopressin and atrial natriuretic peptide (ANP).[58]

No differences were observed in the promoter region methylation of the vasopressin gene; however, they observed hypermethylation of the ANP promoter

region in bulimia nervosa but not anorexia nervosa patients. Furthermore, patients who showed purging behaviour (from both the anorexia nervosa and bulimia nervosa groups) also had hypermethylation in the ANP promoter region. No difference in methylation was observed for patients showing binging behaviour, suggesting that specific epigenetic modifications are associated with specific phenotypes.
The pro-opiomelanocortin (POMC) gene has been analysed in a cross-sectional study with a group of acutely underweight anorexia nervosa patients (acAN), and longitudinally, where a subset of the patients had achieved weight recovery (recAN) after treatment. Levels of POMC promoter region methylation were not different between the acAN, recAN, and the control groups), but differences were found in POMC gene expression levels,[57] indicating that methylation and gene expression levels are not always directly related. Interestingly, the recAN patients were not different from the control groups, suggesting that the expression levels were associated with the state of the illness. In a subsequent study,[60] these authors assessed the methylation of the promoter region of dopaminergic genes: the dopamine transporter (DAT) and dopamine receptors D2 (DRD2) and D4 (DRD4). Although no differences (in methylation and gene expression levels) were observed for DRD4, both anorexia nervosa and bulimia nervosa groups showed increased methylation and gene expression of DAT gene relative to healthy controls. Further, only the anorexia nervosa group showed hypermethylation levels for DRD2 gene, but both anorexia nervosa and bulimia nervosa showed downregulation of the DRD2 gene. This again suggests that methylation is not always related to gene expression levels. These results show differences between the two eating disorders in the dopaminergic system. Although the DAT gene was similar in methylation and gene expression, DRD2 was hypermethlyated only in the anorexia nervosa group which had a lower BMI than the bulimia nervosa group.
These results suggest that epigenetic modifications are present in different stages of illness (acute versus recovered) and also with specific phenotypes (purging versus binging).

9.5 Animal studies

To date, most of the animal studies on epigenetic changes related to eating disorders have been performed in the context of high-fat diet exposure leading to obesity.[295,296,297] Diet has been found to have an effect on the hypothalamus, where histone deacetylase expression levels were modified in response to fasting or high-fat diet.[295] The epigenetic effects of a high-fat diet on the μ-opioid receptor have been investigated with a combination analysis of promoter region DNA methylation, antimethyl CpG-binding protein 2, histone modification analysis, and expression analysis in specific brain areas.[297] A high-fat diet led to decreased gene expression in the reward-related brain areas. The combined analysis of these processes gives a better understanding of the epigenetic

changes as a whole rather than as separate events. This also shows the dynamic effect of the environment (dietary change) in adulthood via epigenetic modifications. These epigenetic modifications have been shown to be transmitted to following generations which, when faced with environmental stimuli, are either predisposed or protected towards that environmental stimuli (e.g. resistance to diet induced obesity[298]).
Another example of complex epigenetic changes, even though not in eating disorders can be seen in the epigenetic status of glial-cell-derived neurotrophic factor (Gdnf) gene in mice.[299] In this study, the epigenetic changes of the Gdnf promoter acted as repressive or activating markers of transcription in the nucleus accumbens, which resulted in a different response to the same stressor between the two strains that were tested. This shows the complexity of epigenetic regulation, which can be dependent on the genetic background, and more research is necessary under controlled conditions to unravel these complex mechanisms.

9.6 Conclusion

Epigenetics research is rapidly expanding in part because of increased understanding of the processes involved,[48,281,282] in part because of the likely role of epigenetic changes in problems such as cancer[283] and lastly, because of technological developments such as the ability to conduct genome-wide screens of the epigenome.[285,286] This latter issue is important in part because most genetic studies in eating disorders indicate that the disorders are likely to be polygenic and thus it is likely that variably methylated regions (VMRs) will be more relevant to complex phenotypes, for example, in a study from Feinberg, BMI was associated with VMRs that were stable over time.[55]
Epigenetics progress in relation to eating disorders has been relatively slow. This is because of a number of reasons, the main one being the lack of access to brain tissue. Access to postmortem brain samples from patient groups will improve the knowledge of epigenetic modifications in brain regions and will help to establish if there are differences between patients and healthy controls (e.g. Dempster et al.[300]). However, as described above, postmortem studies have their own problems. Nonetheless, having access to brain material will be an important next step for epigenetic studies related to psychiatric disorders.
It is important to recognize that epigenetic studies, in relation to psychiatry, are still in their infancy. For example, studies to date have focussed on DNA methylation and there are very few studies of histone modification, of the enzymes that control such processes (e.g. DNMTs and HDAC), or of the effects of epigenetic changes on gene expression (e.g. a study for the FTO gene in obesity,[54] combined results at the level of genotype, phenotype, DNA methylation, and gene expression).
In the immediate future it is likely that, data from epigenetic studies in animals will guide psychiatric studies. Animal studies using established models

of eating disorders[64] will allow the systematic analysis of epigenetic modifications associated with the disorder in controlled genetic and environmental backgrounds,[297,299] that is, will increase our understanding of the biological underpinnings of gene-environment interactions. Animal studies will also allow the comparison of epigenetic changes in peripheral and central systems. This information is critically important for the future use of peripheral tissue in psychiatric investigations.

9.7 Acknowledgments

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Samenvatting in het Nederlands

Anorexia nervosa en het Activity Based Anorexia muismodel

Overmatig veel lichaamsbeweging, één van de kenmerken van anorexia nervosa, speelt een belangrijke rol in de ontwikkeling, de instandhouding en het verloop van deze ernstige psychiatrische aandoening. Juist dit kenmerk van de ziekte kan worden nagebootst in het Activity Based Anorexia (ABA) muismodel. In dit model vertonen de dieren tijdens voedselrestrictie een paradoxaal gedrag. Hoewel er weinig voedsel voorradig is, worden de dieren hyperactief en verliezen zij in snel tempo veel lichaamsgewicht. Met dit model hebben wij de achterliggende genetica, de verantwoordelijke genen en de interactie tussen de genen en omgeving onderzocht tijdens de verschillende stadia van dit gedragsfenotype.

Chromosoom Substitutie Stammen en het in kaart brengen van de verantwoordelijke genen

Bij Chromosoom Substitutie Stammen (CSS) is één enkel chromosoom van de C57BL/6J muis vervangen door hetzelfde chromosoom van de A/J muis. Na screening van deze stammen en hun ouderstammen in het ABA model, bleek dat vooral de chromosomen 4, 12 en 13 een rol spelen in de ontwikkeling van hyperactiviteit tijdens voedselrestrictie. De waargenomen hyperactiviteit is geassocieerd met onregelmatige activiteit in de lichtfase (Zeitgeber tijd 0 tot 7), waarin normaal de C57BL/6J dieren rusten. Dit fenotype werd al vanaf dag 1 van voedselrestrictie waargenomen in CSS12 en CSS13 en vanaf dag twee in CSS4 dieren. Dit gedrag leidde tot zeer versneld lichaamsgewichtverlies.
Hoewel het lichaamsgewicht van deze CSS dieren bij aanvang van de proef lager was dan dat van C57BL/6J dieren, was dit niet geassocieerd met hyperactiviteit. Andere stammen, zoals CSS2 en CSS5, hebben ook een lager lichaamsgewicht maar vertonen niet de hyperactiviteit. We kunnen dus concluderen dat de genetiche loci op de chromosomen 4, 12 en 13 de ontwikkeling van de verstoorde activiteit in respons op de voedselrestrictie reguleren en dat dit onafhankelijk is van het lichaamsgewicht. Een ander interessant aspect van deze hyperactiviteit in de drie chromosoom stammen is dat dit niet gevonden is in de ouderstammen (A/J en C57BL/6J) en dat de hyperactiviteit alleen tijdens bepaalde

omgevingsfactoren ontstaat. Dit suggereert mogelijk "transgressive segregation" onder het nageslacht van de twee ouderstammen. Het kenmerk hiervan is het ontstaan van nieuwe of extreme fenotypen in de hybride populaties.
Om de plaats van het locus op chromosoom 12 te bepalen, hebben we een vrouwelijke F_2 populatie gegenereerd (n = 186). Deze populatie is gegenotypeerd en in het ABA gescreend. Er is een locus gevonden in het proximale gedeelte van het chromosoom en de eerste resultaten duiden op positieve "heterosis". Dit is wanneer het fenotype van het heterozygote genotype die van beide homozygote genotypen (A/J en C57BL/6J) overstijgt. Analyse van overerving geeft aan dat het hier een overdominant effect van het fenotype betreft. De huidige plaats van het gevonden locus is ook in andere muizenstammen belangrijk gebleken voor de regulatie van activiteit. Er is echter nog aanvullend onderzoek nodig om de verantwoordelijke genen te identificeren. Doordat er ook "heterosis" in het spel is, zal dit geen makkelijke taak zijn.

Kandidaatgenen

Wanneer een gen wordt uitgeschakeld, kunnen de geobserveerde fenotypen direct gekoppeld worden aan dat gen. Een gedeelte van dit proefschrift is gericht op het onderzoek bij Neuropeptide Y (NPY) Y1 en Y2 receptor knockout muizen. Om de rol van deze genen in de regulatie van gedragsactiviteit te onderzoeken hebben wij de dieren en hun controle wild type nestgenoten in het ABA model getest. Deze genen zijn op basis van eerdere experimenten, waarbij NPY infusies een effect hadden op activiteit tijdens voedselrestrictie, geselecteerd.
De data laat zien dat de twee receptoren elk een specifieke functie hebben. Receptor Y2 reguleert het dagelijks energie verbruik (loopwielactiviteit) en receptor Y1 reguleert de anticipatie op het voedsel. Dit suggereert dat niet de Y1 receptor verantwoordelijk is voor hyperactiviteit. Mogelijk zijn het de Y2 receptoren, waarvan aangenomen wordt dat dit autoreceptoren zijn gelegen op NPY neuronen, die de afgifte van NPY reguleren. Verlies van deze receptoren kan hebben geleid tot hyperactiviteit via verminderde remming van afgifte van NPY. Verdere studies moeten de verdere rol van deze Y2 receptor uitwijzen.

Het samenspel tussen genen en omgeving

Het samenspel tussen genen en omgeving in neuropsychiatrische stoornissen treedt de laatste tijd steeds vaker op de voorgrond. In dit proefschrift zijn experimenten beschreven waarin getracht is de effecten van de maternale omgeving (via zogenaamde cross-fostering) op lichaamsgewicht en ABA gevoeligheid te bestuderen.
Hiervan zijn de resultaten voor de CSS4 stam opvallend te noemen. De verandering in maternale omgeving gaf, ongeacht de genetische achtergrond van de pleegmoeder, een betere prestatie in het ABA model op latere leeftijd in de anders zo ABA gevoelige CSS4 dieren. Er werd geen effect van maternale omgeving gevonden voor de laag gevoelige C57BL/6J stam, wat suggereert dat het effect

van vroege gebeurtenissen op ABA gevoeligheid afhankelijk is van de genetische achtergrond van het dier.
De effecten van maternale omgeving op lichaamsgewicht waren daarintegen anders. Er werd een interactie waargenomen tussen ontwikkelingsstadium en genetische achtergrond en maternale omgeving. Wanneer moeder en pup eenzelfde genetische achtergrond hadden, werden CSS4 dieren beïnvloed door de fostering, die een verdere afname in lichaamsgewicht op dag 14 (postnataal) als gevolg had. CSS4 dieren met een CSS4 moeder, al dan niet ge-cross-fostered, hadden een vergelijkbaar lichaamsgewicht wanneer zij volwassen waren, maar hun gewicht was nog steeds lager dan die van volwassen C57BL/6J dieren. Wanneer moeder en pup een verschillende genetische achtergrond hadden, werd het lichaamsgewicht op dag 14 bepaald door de genetische achtergrond van de pleegmoeder. Deze vinding geldt vooral voor de CSS4 dieren. Het lichaamsgewicht van ge-cross-fosterde CSS4 dieren met een C57BL/6J pleegmoeder was significant hoger. Deze resultaten wijzen op een complexe regulatie van lichaamsgewicht tijdens alle stadia van het leven.
De interactie tussen gen en omgeving kan biologische processen beïnvloeden via epigenetische modificaties. We hebben daarom de DNA methylatie in de verschillende CSS4 groepen onderzocht. Verandering van maternale omgeving is sterk geassocieerd met veranderingen in DNA methylatie. Verschillende genen die in verband zijn gebracht met een aantal psychiatrische stoornissen hadden regionen met zogenaamde hypermethylatie van het DNA. Ook het gen Fam155a werd hierbij opgemerkt. Dit gen wordt in humane studies zeer sterk in verband gebracht met anorexia nervosa. Natuurlijk is verder onderzoek noodzakelijk om het causale verband tussen gen en omgeving, methylatie en gedrag te vinden. Maar wij hopen hier toch voor een nieuwe link tussen dier en mens te hebben gezorgd. Dit benadrukt ook de belangrijke rol van studies met dieren om de humane situatie beter te leren begrijpen.

Factoren die de ABA gevoeligheid beïnvloeden

Uit de screening van de chromosoom substitutie stammen kwam naar voren dat stammen met een hogere hyperactiviteit tijdens de restrictiefase ook een versnelde afname van het lichaamsgewicht vertoonden. Daardoor bereikten zij eerder het zogenaamde 'humane eindpunt' en werden uit de proef genomen. Omdat voorkomen beter is dan genezen stelden wij ons de volgende vraag: "Wat zijn de factoren die de ABA gevoeligheid beïnvloeden of voorspellen?" Om deze vraag te beantwoorden hebben wij in plaats van hybride dieren een panel van 11 inteeltstammen gescreend in het ABA model. In beginsel is dit panel samengesteld uit stammen met een sterk verschillend lichaamsgewicht, activiteit en mate van voedselinname.
We hebben stammen gevonden met verschillende niveaus in ABA gevoeligheid. Vergelijkbaar met de screening van de chromosoom substitutie stammen, was een hogere activiteit tijdens de restrictiefase geassocieerd met een versnelde afname van het lichaamsgewicht. De basisfactoren: lichaamsgewicht, voed-

selinname en activiteit beïnvloeden tot op zekere hoogte de ABA gevoeligheid. Uit de beschikbare literatuur over lichaamsvet, leptine, insuline en glucose voor deze stammen kwam geen correlatie met ABA gevoeligheid naar boven.
Van alle basisfactoren was activiteit de factor met de hoogst voorspellende waarde. De interactie tussen lichaamsactiviteit en voedselinname leek een effect te hebben op ABA gevoeligheid, maar dit was te wijten aan één van de stammen die voor beide factoren hoog scoorde. Het effect van deze factoren is ook sterk afhankelijk van de genetische achtergrond. Andere basisfactoren of combinaties hiervan spelen een grotere rol dan activiteit alleen. Deze bevindingen staan aan het begin van verder onderzoek naar de onderliggende neurobiologische mechanismen en de manier waarop alle factoren elkaar onderling beïnvloeden.

Klinische implicaties

Hyperactiviteit is één van de belangrijkste kenmerk van anorexia nervosa en bepaald voor een groot gedeelte de ontwikkeling, instandhouding en verloop van de ziekte. Om de genetische basis hiervan beter te begrijpen zijn we gestart met het screenen van verschillende muizenpanels in het ABA model in de zoektocht naar de rol van de kandidaatgenen en om de rol van hyperactiviteit bij de gevoeligheid voor ABA te onderzoeken. We hebben ook het samenspel van gen en omgeving onderzocht. Hoewel verder onderzoek nodig is om al deze mechanismen te ontrafelen zijn met de huidige resultaten een paar relevante uitgangspunten boven water gekomen.
Het zou bijvoorbeeld interessant zijn om de relatie tussen verhoogde lichaamsbeweging, NPY en NPY Y2 receptor mutaties in acuut zieke anorexia nervosa patienten te onderzoeken. Vooral omdat in een recente studie een verhoogd NPY level in mensen met bulimia nervosa is beschreven. Deze levels stegen des te meer naarmate de patienten meer lichaamsbeweging kregen. NPY levels, in cerebrospinale vloeistof, waren lager in patienten die al enige tijd genezen waren. Dit suggereert een relatie tussen de ernst van de ziekte en circulerend NPY.
Ten tweede suggereren onze bevindingen dat verhoogde activiteit vlak voor het voedselrestrictie geassocieerd is met een verhoogde gevoeligheid voor ABA. Deze bevindingen geven aan dat verhoogde activiteit voorafgaand aan het ziektebeeld een belangrijk gegeven is. Dit is een zogenaamde proximale risicofactor die men in overweging zou moeten nemen. Daarbij verklaart wellicht de genetische component (chromosoom substitutie stam data) waarom niet alle patienten met anorexia nervosa de verhoogde lichaamsactiviteit vertonen. We kunnen speculeren dat voorafgaand aan het ziektebeeld andere factoren, zoals het verlangen om af te vallen en zorgen over hoe men eruit ziet, een trigger zijn om dit excessieve gedrag, afhankelijk van de genetische achtergrond, te veroorzaken.

Quantitatieve promotor DNA methylatie van vier anorexia nervosa kandidaatgenen

In deze pilotstudie hebben we vier kandidaatgenen die het risico op eetstoornissen kunnen veranderen onderzocht. Er was een verschil in de mate van DNA methylatie voor de verschillende genen. Maar er werd geen verschil gevonden tussen anorexia patienten en gezonde mensen voor elk van de genen of voor de specifieke CpG sites na correctie voor "multiple testing". Dit is waarchijnlijk te wijten aan het lage aantal waarnemingen.
De deelnemers werden willekeurig geselecteerd uit de Maudsley eetstoornissen database, wat natuurlijk beperkingen met zich meebrengt. Door deze beperkingen en het lage aantal patiënten kunnen we de invloed van epigenetische modificaties niet uitsluiten. Een goede volgende stap in dit onderzoek zou een studie zijn met meer patiënten en een analyse op genotype en genexpressie. Deze combinatie zou ons begrip vergroten over de onderlinge samenhang tussen genotype, epigenetica en fenotype voor deze ziekte.

Eindopmerkingen

De studies in dit proefschrift geven aan dat het traject van anorexia nervosa waarschijnlijk gedefinieerd wordt door het voortdurende samenspel tussen genen en omgeving. Dit begint al op een heel vroege leeftijd. Vlak voordat de ziekte zich uit, wordt verhoogde lichaamsbeweging als risicofactor meer prominent en dit zou ook als een maat voor de ernst van de ziekte kunnen fungeren. De resultaten vragen om verdere studies naar welke neuronale veranderingen tezamen met epigenetische veranderingen in de ontwikkeling van een mens leiden tot psychiatrische stoornissen.

Curriculum Vitae

Eneda Pjetri was born on 8th of July 1980 in Fier, Albania. After completing her secondary education in 1998 in Tirana, Albania, she started her studies at the Medical Faculty of Marmara University, Istanbul, Turkey, graduating as MD in 2004. Continuing her studies in the Master Neuroscience and Cognition in Utrecht, The Netherlands, she gained research experience in the Department of Psychiatry, Section of Structural Neuroimaging Section, under the supervision of Drs. Jiska Peper and Dr. Hilleke Hulshoff Pol. Her second internship, was in Rudolf Magnus Institute of Neuroscience, Utrecht, The Netherlands, under the supervision of Dr. Cathy Fernandes and Dr. Martien Kas. Following graduation in 2007, she started her PhD research under the supervision of Dr. Martien Kas, Prof. Dr. Berend Olivier and Prof. Dr. Roger Adan. The subject of her research was to understand the neurobiology underlying hyperactivity in anorexia nervosa, which concluded with this thesis.

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