NEURAL CIRCUITS UNDERLYING HYPERACTIVITY IN AN ANIMAL MODEL FOR ANOREXIA NERVOSA

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NEURAL CIRCUITS UNDERLYING HYPERACTIVITY IN AN ANIMAL MODEL FOR ANOREXIA NERVOSA

Neuronale systemen betrokken bij hyperactiviteit
in een diermodel voor anorexia nervosa

(met een samenvatting in het Nederlands)

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Linda Adriana Wilhelmina Verhagen
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CONTENTS

LIST OF ABBREVIATIONS

ABA	activity-based anorexia
AgRP	agouti-related peptide
AN	anorexia nervosa
ARC	arcuate nucleus
BW	body weight
CART	cocaine- and amphetamine-related peptide
DMH	dorsomedial hypothalamus
FAA	food-anticipatory activity
FLU	*cis*-flupenthixol
5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine, serotonin
HVA	homovannilic acid
ICV	intracerebroventricular
LH	lateral hypothalamus
LMA	locomotor activity
α-MSH	α-melanocyte-stmulating hormone
NAc	nucleus accumbens
NPY	neuropeptide Y
POMC	pro-opiomelanocortin
PVH	paraventricular nucleus of the hypothalamus
RIA	radioimmunoassay
RWA	running wheel activity
VMH	ventromedial hypothalamus
VTA	ventral tegmental area
WAT	white adipose tissue

Chapter 1

CHAPTER 1

General introduction

Content

A. Anorexia nervosa

Clinical features of anorexia nervosa

Anorexia nervosa (AN) means literally "a nervous loss of appetite" and is derived from the Greek orexis, appetite. In medical literature, the disorder was first described in the 1870's (Gull, 1997). Gull described a strange disease which affected mainly young women and called it "apepsia hysteria". Six years later he brought a revised version of his report in which he changed the name into "anorexia nervosa". William Gull also described the first symptoms of AN including reduced food intake, hyperactivity, hypothermia, amenorrhea and emaciation. Without knowing the history of AN, it is easy

to be influenced by the media's interpretation of AN as nothing more than an illness fueled by a desire to be thin. Upon realizing that AN existed across time and different cultures, it becomes clear that this disorder cannot be explained by such a simplistic mechanism. Nowadays, the fourth edition of the American Psychiatric Association's Diagnostic and Statistic Manual (DSM-IV) describes the accompanied features of AN (see also Table 1.) with only minor modifications as compared to the original description. The main feature of individuals diagnosed with AN is the refusal to maintain body weight at a minimally accepted body weight (body mass index (BMI) ≤ 17.5 kg/m^2). Another essential feature of AN covers the intense fear of becoming fat and display disturbed perceptions of body size and shape. Furthermore, in many cases, AN individuals are amenorrheic. In addition, DSM-IV classification recognizes two clinical subtypes of AN. The restricting type accomplishes severe weight loss by simply fasting and/or excessive exercise, whereas the binge-eating/purging type attempts weight loss through vomiting or the use of laxatives. Moreover, typical personality features of AN individuals include perfectionism, obsessionality, anxiety, harm avoidance, and low self-esteem (Wonderlich et al., 2005). The average prevalence of AN has been reported to be 0.3% (Hoek and van Hoeken, 2003), and AN has the highest mortality rate (>10%) of all psychiatric disorders (Sullivan, 1995; Birmingham et al., 2005).

Excessive physical activity is demonstrated by many, if not most, patients with AN at some point in the course of the disorder, and has been described as a hallmark feature of the syndrome. The prevalence of hyperactivity varies widely between 31-80%, due to a lack of clear definition of hyperactive behavior in AN patients (Hebebrand et al., 2003). Although not part of the DSM IV criteria for AN, hyperactivity seems to be a trait of AN. The high levels of physical activity may take various forms, including a planned excess of sports, walking or other physical activity with the apparent goal of further catabolism. Restless movements that appear to be non-purposive are also observed, however, suggesting that starvation itself might elevate activity levels. Also other species display increased locomotor activity upon starvation suggesting that it might be an expression of foraging behavior.

With the thinness ideal in mind, it seems that AN is a phenomenon of recent years. But this is not true. Doctors described cases of self-thinning already hundreds of years ago. Probably, self-thinning is as old as humanity. Nowadays, it is realized that environmental factors trigger the disease, but that pathophysiological mechanisms underlie the disease.

Table 1. DSM IV criteria for anorexia nervosa

Criteria
• 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.
• Amenorrhea (at least three consecutive cycles) in postmenarchal girls and women. Amenorrhea is defined as periods occurring only following hormone (e.g., estrogen) administration.
Type
• Restricting type: During the current episode of anorexia nervosa, the person has not regularly engaged in binge-eating or purging behavior (self-induced vomiting or misuse of laxatives, diuretics, or enemas).
• 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).

Pharmacotherapy in anorexia nervosa

Pharmacotherapy thus far played only a limited role in the treatment of AN. Several classes of drugs have been studied in the treatment of AN. Some examples are antidepressants (i.e. clomipramine, fluoxetine), mood stabilizers (i.e. lithium), appetite enhancers (i.e. tetrahydrocannabinol), opiate antagonists (i.e. naltrexone), and antipsychotics (i.e. chlorpromazine, pimozide, haloperidol, risperidone) (Mitchell et al., 2003; Attia and Schroeder, 2005; Zhu and Walsh, 2002; Kruger and Kennedy, 2000). Many of these mentioned drugs act on serotonergic, noradrenergic, and/or dopaminergic neurotransmission by binding to the corresponding receptor blocking its activity onto the transporter, and thereby inhibiting neurotransmitter reuptake resulting in increased active levels in the synapse. Selective serotonin reuptake inhibitors (SSRIs) have been studied extensively as well. Studies with SSRIs in malnourished AN patients showed no improvement of treatment (Kaye et al., 1998), and SSRIs seemed to be non-effective in preventing relapse after recovery from AN (Holtkamp et al., 2005). Although several classes of drugs have been tried as therapy for AN, no medication has been demonstrated to be effective in the treatment of AN. When drugs are prescribed to AN patients, this is usually done to treat symptoms that are associated with the

disease, such as to treat psychosis, anxiety, and depression.

Nowadays, development of atypical antipsychotic agents such as olanzapine and risperidone has encouraged the investigation of these drugs in the treatment of AN. Still, the exact mechanism of action is not clear. Olanzapine holds a mixed receptor pharmacology including high affinity for 5-HT_2 serotonin receptors and for dopamine D_2 receptor, and a lower affinity is apparent for most cholinergic and α-adrenergic receptors (Roth et al., 2004). It is known that olanzapine holds antipsychotic effects probably through serotonin antagonism (Barbarich et al., 2004; Dennis et al., 2006; Malina et al., 2003; Powers et al., 2002). Besides, olanzapine is also known to cause body weight gain and to reduce hyperactivity (Hillebrand et al., 2005c; Allison and Casey, 2001). It is not clear which transmitters play a role in the efficacy of atypical antipsychotics for AN treatment. Taken together, the fact that there are no effective drugs to treat AN argues against a straight forward involvement of common neurotransmitters in the etiology of AN. Therefore, I will now introduce briefly the neuronal circuits implicated in normal feeding behavior.

B. Neurobiology

Neural circuits of energy balance in the hypothalamus

Energy homeostasis in the body is tightly regulated. To maintain an adequate body weight, energy intake and energy expenditure are kept in balance even when the total daily amount of ingested food might fluctuate over days. Disturbances in energy balance can result in eating disorders, such as obesity and AN. Taking into account the complexity of energy balance, it is not surprising that many brain areas are involved in regulating food intake and energy expenditure.

During the past decade, research on central mechanisms controlling body weight received much attention due to the obesity epidemic. Already in the 1950's, brain lesioning and stimulation studies identified the hypothalamus as a major brain area controlling energy homeostasis (Stellar, 1954). In the hypothalamus two distinct subsets of neurons within the arcuate nucleus (ARC) are particularly important in feeding regulation. One neuronal subset synthesizes the peptide proopiomelanocortin (POMC) (Gee et al., 1983), which is cleaved to α-melanocyte stimulating hormone (α-MSH) as a neurotransmitter, and the cocaine- and amphetamine-regulated transcript (CART) is located in the same neuron (Douglass and Daoud, 1996). α-MSH acts on other hypothalamic neurons via melanocortin receptors (MC3 and MC4 re-

ceptors) to lower body weight by reducing food intake and increasing energy expenditure. On the other hand, a second neuronal subset synthesizes agouti-related peptide (AgRP) and neuropeptide Y (NPY) (Hahn et al., 1998) to increase food intake and thereby increases body weight and inhibits energy expenditure. The former pathway describes the catabolic, anorexigenic pathway whereas the latter pathway illustrates the anabolic, orexigenic pathway (see also Figure 1.). Hypothalamic areas including the paraventricular nucleus (PVN), perifornical area (PFA) and the lateral hypothalamus (LH) are highly innervated by projectory neurons from the ARC (Schwartz et al., 2000). These two ARC-derived neuronal tracts often occur in parallel and counterbalance one another to maintain an adequate body weight.

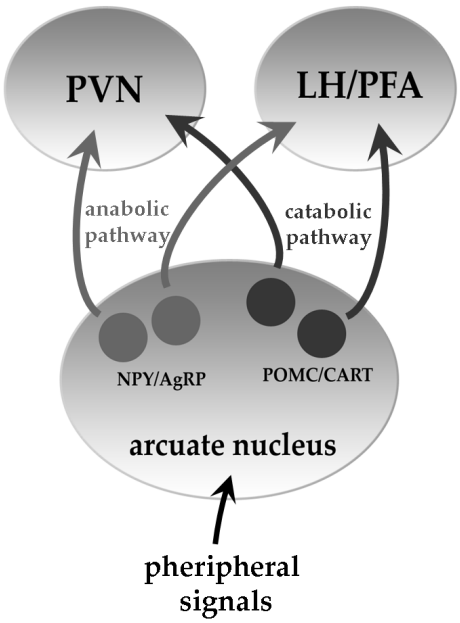

Figure 1.
Hypothalamic projections from the arcuate AgRP/NPY and POMC/CART neurons towards other hypothalamic nuclei to regulate food intake. The ARC senses peripheral signals, such as leptin and ghrelin.

Peripheral signaling

Although adipose tissue was seen as the site to store energy, nowadays it is realized that it also functions as an endocrine organ. It has been shown that adipose tissue is capable of secreting adipokines such as leptin and adiponectin which control energy balance.

Long-term regulation of food intake

Leptin, a 16 kDa adipokine secreted from adipose tissue, circulate in proportion to the amount of adipose tissue in the body. Since its discovery in 1994, leptin has been shown to have an important role in sensing energy balance. Although leptin receptors are found in several brain areas including the ARC, PVN, DMH, VMH, and LH, leptin receptors localized in the ARC have an established role in energy balance (Elias et al., 2000). After binding to its receptors in the ARC, leptin acts on the anabolic neurons (NPY/AgRP) and the catabolic neurons (POMC/CART) to down-regulate the orexigenic neuropeptides and to up-regulate the anorexigenic peptides respectively (Schwartz et al., 2000; Jequier, 2002). During periods of weight gain, leptin levels are high and initiate decreased food intake and increased energy expenditure. Mice lacking leptin (ob/ob mice), or its receptor (db/db mice) are hyperphagic and become severely obese (Trayhurn, 1984). Infusion of leptin to normal (wild-type) and to ob/ob animals decreases body weight by reducing fat mass (Halaas et al., 1995).

Short-term regulation of food intake

Insulin is a peripheral hormone produced by the pancreas and levels of insulin correlate positively with adiposity (Bagdade et al., 1967). Administration of insulin centrally to the brain results in a reduction of food intake and body weight (Air et al., 2002). Mice with genetic deletion of the insulin receptor in the brain obtain a similar obese phenotype (Bruning et al., 2000).

Ghrelin, secreted by endocrine cells of the stomach and the only peripheral orexigenic signal, promotes food intake by acting on many of the same hypothalamic ARC neurons as leptin (described above) (Zigman and Elmquist, 2003). During positive energy balance ghrelin levels are low whereas during negative energy balance (i.e. starvation) ghrelin levels rise. The increase in ghrelin levels just before food ingestion is thought to mediate meal initiation and therefore has a role in the short-term regulation of food intake. Peripheral and central injections of ghrelin promote food intake and body weight gain (Tschop et al., 2000; Wren et al., 2001). Recently, it was reported that ghrelin transgenic mice (with high ghrelin levels) displayed hyperphagia and increased energy expenditure (Bewick et al., 2009).

Non-homeostatic regulation

Food intake is driven by homeostatic feeding based upon energy supplies. However, food intake can also be driven by the palatability or pleasure associated with eating, also referred as "non-homeostatic" feeding (Berthoud, 2004). In recent years, several brain areas were identified to be implicated in non-homeostatic feeding, including the cerebral cortex, hippocampus, amygdala, and the nucleus accumbens (Saper et al.,

2002). The palatability of food seems to be an important aspect of non-homeostatic feeding. For example, obese individuals prefer diets with high levels of fat and carbohydrates as compared to non-obese individuals (Drewnowski et al., 1992).

Eventually the decision to initiate or to stop food intake is affected by anticipation, motivation to eat, food seeking behavior, food intake, satiety, and short- and long-term regulation by peripheral signals. Whereas these processes might be regulated by various brain areas, interaction between these brain systems is essential for normal feeding behavior and body weight regulation. It is clear that different factors that affect food intake do so, via a distributed network of neural circuits in which a coordinated response is organized to control feeding behavior (see Figure 2.). The precise role of each neuron in these circuits in the regulation of food intake is difficult to assess, since behavior is a response of a network, rather than that of single neurons. Thus, little information is known about how input from specific neuronal subsets integrates with each other and with the homeostatic signals derived from the periphery.

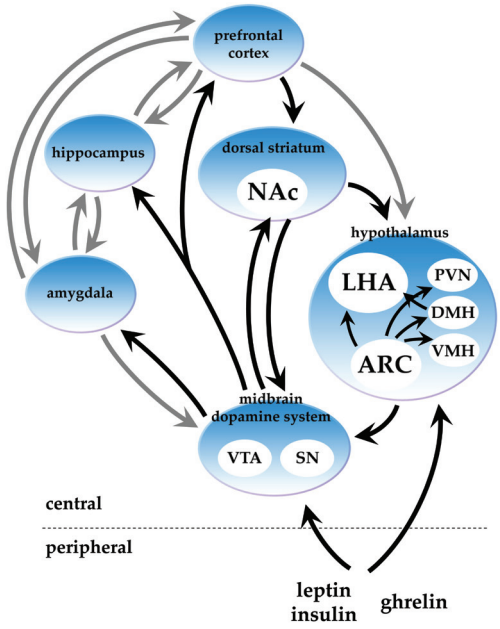

Figure 2.

Overview of the distributed network of neural circuits organizing feeding behavior. Interaction with the homeostatic signals (leptin, insulin and ghrelin) derived from the periphery. ARC, arcuate nucleus; DMH, dorsomedial hypothalamus; LHA, lateral hypothalamus; NAc, nucleus accumbens; PVN, paraventricular nucleus; SN, substantia nigra; VMH, ventromedial hypothalamus; VTA, ventral tegmental area.

Neuroendocrine dysregulation in anorexia nervosa

The endocrine phenotype of AN has been associated with its clinical symptoms. For example, decreased levels of leptin have been associated with amenorrhea (Chan and Mantzoros, 2005). Though almost all of the endocrine changes that occur in AN represent physiological adaptation to starvation, some changes still persist after recovery and therefore might contribute to the susceptibility to the reappearance of AN. Below a selection is made of endocrine changes in AN patients which may be maladaptive.

Adipokines

In underweight AN patients, it has been persistently reported that basal plasma and cerebrospinal fluid levels of leptin are markedly lower as compared with normal-weight healthy controls (Hebebrand et al., 1997; Grinspoon et al., 1996). These low levels of circulating leptin are significantly correlated with the patients' decreased body mass index and reduced body fat content (Holtkamp et al., 2003a; Monteleone et al., 2000; Mantzoros et al., 1997; Ferron et al., 1997). This indicates that low levels of leptin reflect energy supplies in the human body. After long-term recovery to normal body weight, leptin levels have been found to be similar to control patients (Gendall et al., 1999; Haas et al., 2005; Hebebrand et al., 1997; Holtkamp et al., 2003a; Lob et al., 2003). Furthermore, in cases with a too rapid weight restoration during refeeding, leptin levels reach values higher than expected upon their body weight (Lob et al., 2003; Holtkamp et al., 2003a; Hebebrand et al., 1997), which may be an important factor why patients experience difficulties in reaching or maintaining a certain target weight (Hebebrand et al., 2007). However, the possibility that hyperleptinemia during refeeding may prevent body weight stabilization has not been properly tested so far. Therefore, the role of leptin in the process of body weight recovery in AN needs to be further explored.

The excessive physical activity seen in AN patients at referral has been associated with low plasma leptin levels (Exner et al., 2000; Holtkamp et al., 2003b). AN patients rated their motor restlessness the highest when their leptin levels and body weight were the lowest (Exner et al., 2000). During severe AN, it has been shown that hyperactivity is negatively correlated with food intake and contribute to body weight loss (Holtkamp et al., 2004a; Holtkamp et al., 2004b; Kaye et al., 1988b).

Adiponectin, another hormone produced exclusively by adipocytes, has been shown to modulate insulin sensitivity (Havel, 2002). Elevated levels of circulating adiponectin have been reported in underweight AN patients (Pannacciulli et al., 2003; Delporte et al., 2003). On the contrary, other researchers found no difference in concentrations of adiponectin as compared to healthy controls (Iwahashi et al., 2003), and even decreased levels of adiponectin were found (Tagami et al., 2004). Differences in the patients' samples, assay methods and time of the day of blood collection may

be responsible for such a discrepancy among the studies. But in the last four years, increased levels of adiponectin in AN patients has been confirmed by other research groups (Bosy-Westphal et al., 2005; Housova et al., 2005). Furthermore, adiponectin has been found to be strictly correlated to the patients' nutrional status, since an inverse correlation between hormone concentrations and both BMI and percent body fat mass was demonstrated (Dostalova et al., 2007; Housova et al., 2005). Still, the physiologic relevance of high adiponectin levels in AN is unclear. Elevated circulating adiponectin concentrations might represent a compensatory mechanism for the increased insulin sensitivity in patients with AN, since a negative correlation between plasma adiponectin and insulin has been found in these patients (Harris et al., 2005).

Moreover, other adipose tissue-derived hormones like resistin and visfatin were not altered in plasma of AN patients as compared to controls (Haluzikova et al., 2008; Dostalova et al., 2008). Possibly, further investigation is needed to clarify the possible role of resistin and visfatin in eating disorders and/or its metabolic complications.

Gut-related hormones

Plasma ghrelin levels are inversely correlated with body weight and increase following body weight loss (Cummings et al., 2002). Enhanced ghrelin levels tend to normalize during body weight recovery. In addition, the dynamics of ghrelin secretion after food intake have been studied with contradictory results. In underweight AN patients, it has been found that the food-induced suppression of circulating ghrelin was almost completely absent (Nedvidkova et al., 2003), suggesting that a single meal is not able to suppress the drive to eat in order to counteract the negative energy balance seen in AN. Two other studies reported that in AN levels of ghrelin, although suppressed by food ingestion in percentages similar to normal subjects, remained significantly higher than in controls (Misra et al., 2004; Stock et al., 2005). Otto and researchers found that ghrelin release in AN patients was not different from healthy subjects (Otto et al., 2005). Moreover, although morning ghrelin levels progressively declined with the recovery of body weight, the ghrelin response to food intake was not influenced by weight recovery (Otto et al., 2005). One of the major technical issues in ghrelin studies is represented by the rapid degradation of circulating ghrelin into inactive fragments. Therefore, increases in total ghrelin plasma levels in AN may not be representative of an increased active ghrelin production. In fact, it has been shown that, in AN subjects, plasma active ghrelin was increased and normally suppressed after oral glucose administration (Nakai et al., 2003).

Peptide YY (PYY), consisting of two forms PYY_{1-36} and PYY_{3-36}, is secreted from the endocrine gut cells and released into the circulation (Druce et al., 2004). It appears that PYY_{3-36} acts as a satiety signal in order to terminate individual meals, partially by decreasing ghrelin levels. Studies of PYY in AN patients are limited. Baseline PYY_{3-36}

levels have been reported normal or even increased in patients suffering from AN (Misra et al., 2006; Nakahara et al., 2007; Stock et al., 2005). In addition, after body weight recovery, PYY_{3-36} levels are not completely restored (Nakahara et al., 2007).

Preclinical research demonstrated that the gut-related peptide cholecystokinin (CCK) is involved in the control of meal size (Gibbs et al., 1973). CCK acts as neurotransmitter in the central nervous system and is thought to modulate food intake through interactions with brain areas in the hypothalamus, as well as other brain regions (Blevins et al., 2000). Plasma levels of CCK have been reported to be normal as well as increased in AN patients (Tamai et al., 1993; Phillipp et al., 1991; Geracioti, Jr. et al., 1992; Abell et al., 1987). Taken together, given their negative energy balance status, there may be unexpected endocrine responses in AN patients in leptin, ghrelin, PYY, CCK and adiponectin.

Genetics in anorexia nervosa

Over the past 15 years, it has become more clear which molecular and neuronal pathways are relevant for eating behavior and body weight regulation. In addition, advances have been made in determining genetic variation underlying inter-individual differences in body weight. Until now, it has not been demonstrated that AN is caused by genetic variation within a single gene, but complex interactions among multiple genes might contribute to the occurrence and/or development of AN (Bulik et al., 2007).

The heritability of AN has been well established. Relatives of AN patients have a higher risk to become anorectic, with approximately ten-fold greater risk than relatives of unaffected individuals (Strober et al., 2000; Lilenfeld et al., 1998). Unfortunately, a disadvantage of family studies is that it is difficult to distinguish between genetic and/or environmental factors underlying this familial aggregation. On the contrary, twin studies are a powerful tool in the quantification of genetic and environmental factors for a given phenotype. Differences between monozygotic twins result from environmental factors only, whereas differences between dizygotic twins could be affected by both genetic and environmental factors. Studies in twin pairs with AN determined a greater concordance in monozygotic twins compared to dizygotic twins (concordance rate of 0.7 and 0.1, respectively) (Holland et al., 1984). From these family and twin studies, we can conclude that genetic factors strongly contribute to the susceptibility of AN (Bulik et al., 2007). Probably due to the rather low prevalence of AN, no adoption studies have been reported so far.

Molecular genetic studies aiming to identify chromosomal regions and possible candidate genes use two different methods; linkage and association (i.e. case-control or within-family design). After a linkage study, one nominates “candidate genes” in

the region under the linkage signal, and performs an association study on alleles in the genes. In this way, a specific gene, or even a specific allele, can be identified as playing a possible causal role in AN. With the current technology to genotype hundreds of thousands of alleles in parallel, it is now possible to perform association studies on the level of the whole (human) genome. Linkage studies in AN thus far only identified suggestive linkage. For a complete impression of all performed linkage and association studies, I refer to recent reviews by Monteleone & Maj (Monteleone and Maj, 2008) and Bulik and researchers (Bulik et al., 2007). Taken together, although the heritability of AN is high, genetic research thus far did not identify major genes. Therefore, genetic studies do not clarify which molecular pathways underlie AN.

Imaging in anorexia nervosa

Research utilizing brain imaging techniques has generated important information about the neurobiology underlying AN. Besides the structural differences observed using computerized tomography and magnetic resonance imaging (Dolan et al., 1988; Katzman et al., 1997; Kornreich et al., 1991; Krieg et al., 1989; Lambe et al., 1997; Miwa et al., 2004; Wagner et al., 2006), several other differences were examined using functional imaging among AN patients. For example, increases in cerebral blood flow within numerous brain areas including the dorsolateral prefrontal cortex, posterior cingulated cortex and precuneus have been demonstrated using single positron emission computerized tomography (SPECT) imaging (Matsumoto et al., 2006). In addition, several studies have demonstrated temporal lobe hypoperfusion, which is even persistent after body weight recovery (Gordon et al., 1997).

Research with positron emission tomography (PET) showed that regional blood flow normalizes after long-term body weight recovery from AN (Frank et al., 2007). However, following body weight recovery, the serotonin system (related to anxiety and impulse control) and the dopaminergic system (related to motor activity and reward) remained disturbed in recovered AN patients (Bailer et al., 2004; Bailer et al., 2005; Frank et al., 2005). For example, in a PET study determining binding potential of D_2/D_3 receptors in recovered AN patients compared to healthy controls, increased binding of the D_2/D_3 receptors have been found suggesting increased availability of these receptors (Frank et al., 2005). Another PET study revealed a reduced 5-HT_{2A} receptor binding after recovery in AN patients (Frank et al., 2002). Furthermore, Delvenne and researchers showed hypometabolism of glucose in cortical regions (frontal and parietal cortex) in AN patients as compared to controls (Delvenne et al., 1999). Finally, functional MRI studies demonstrated changes of brain activation in several brain regions (Uher et al., 2004). Thus, imaging studies indicate permanent changes in serotonin and dopamine neural circuits in patients recovered from AN.

C. Animal models for anorexia nervosa

Endocrine, genetic and imaging studies in AN have not provided clear insights in what is underlying AN. Without knowledge on what causes AN, scientist have turned to animal models mimicking important features of the eating disorder. Major characteristics of AN investigated with animal models are female predominance, reduction of food intake, body weight loss, hyperactivity, and abnormal endocrine function. Each animal model mimics one or more aspects to our understanding of AN. In this paragraph I will focus on commonly used animal models applied in research on eating behavior and body weight regulation, with special focus on reduced feeding behavior and hyperactivity.

Genetic mouse models

Anorexia mouse, anx/anx mouse

Lethal mutation in mice located on chromosome 2 called anorexia, gene symbol anx, causes starvation in mice (Maltais et al., 1984b). After birth the *anx/anx* mice appear to be normal, but after one week the amount of food ingested by *anx/anx* mice is not sufficient to sustain normal body weight regulation, while the ability to eat remains normal (Maltais et al., 1984a). In addition, the *anx/anx* mice display abnormal behavior including tremors, hyperactivity and uncoordinated gait. Eventually, the *anx/anx* mice will die within 3-5 weeks after birth depending on the genetic background. The *anx/anx* mouse model has been subjected to comprehensive investigations. Several studies have revealed changes in the dopaminergic (Johansen et al., 2001), serotonergic (Jahng et al., 1998b; Son et al., 1994), and noradrenergic systems (Jahng et al., 1998a) that may contribute to the eating- and/or motor-disturbances seen in these animals. Histochemical analysis on the *anx/anx* revealed alterations in hypothalamic signaling pathways. The *anx/anx* mice show abnormalities in the orexigenic (NPY/AgRP neurons) and the anorexigenic (POMC/CART neurons) pathways.

Dopamine deficient mouse

Mice unable to synthesize dopamine (DA) specifically in dopaminergic neurons were created by inactivating the tyrosine hydroxylase (TH) gene followed by restoration of

TH function in noradrenergic cells. After birth, these dopamine deficient (DD) mice become hypoactive and stop feeding after a couple of weeks (Zhou and Palmiter, 1995). Finally, these DD mice will die by 3-4 weeks of age. Daily administration with 3,4-dihydroxy-L-phenylalanine (L-DOPA) to DD mice stimulates locomotor activity necessary to seek and ingest food, but they do not eat enough to survive (Szczypka et al., 1999b). Restoring DA signaling in the central caudate putamen of DD mice by bilateral injection of a canine adenovirus engineered to express TH and GTPCH1 gene expression, feeding and locomotor behavior are normalized as compared to controls (Szczypka et al., 1999a).

Additional genetic models

Detailed and informative discussion on environmentally induced models, spontaneous mutations, and genetic knock-out mouse models of AN have been reviewed by Siegfried and researchers (Siegfried et al., 2003). More recently, in a review by Casper and researchers, the relevance of animal models to eating disorders like AN has been discussed. Disruption of a single gene involved in feeding behavior does not often results in a expected phenotype, probably as a result of redundancy and/or compensation in central pathways during development. For example, AgRP and NPY are strongly implicated in feeding behavior (Schwartz et al., 2000). Both neuropeptides stimulate food intake as revealed by pharmacological and physiological studies. Despite the support of this strong evidence, AgRP deficient mice nor AgRP/NPY double-knockout mice suffer no obvious feeding or body weight deficits and maintain a normal response to starvation (Qian et al., 2002). Therefore, absence of a lean phenotype in a knockout animal does not mean that the gene does not play a role in feeding behavior in AN.

Only for a few genes implicated in feeding behavior, disruption resulted in a anorectic phenotype (Casper et al., 2008; Siegfried et al., 2003). For example, the endocannabinoid (CB) system has been suggested to be involved in appetite regulation in rodents (Berry and Mechoulam, 2002) and humans (Siegfried et al., 2004). Endocannabinoid receptor deficient mice (CB_1-receptor deficient) are hypoactive and show increased mortality whereas food intake and body weight regulation is normal during ad libitum feeding (Zimmer et al., 1999). Nevertheless, when these CB_1-receptor deficient mice are exposed to food restriction, these mice eat less than wild type littermates (Di, V et al., 2001) supporting the endocannabinoid signaling pathway as candidate for implication in AN.

The melanin-concentrating hormone (MCH) is an orexigenic neuropeptide produced by neurons of the lateral hypothalamic area. Genetic MCH deficiency induces hypophagia, loss of body fat, and increases metabolic rate (Mystkowski et al., 2000; Shimada et al., 1998). But MCH deficient mice still show similar activity levels

as compared to wild type mice in the open field test (Shimada et al., 1998). Taken together, it remains to be determined whether genetic defects in these mouse models, that result in an anorectic phenotype, model AN.

Environmental manipulation

Dietary model

Food restriction alone leading to a catabolic state has been modeled in animals and can serve to study the starvation-induced physiological and endocrine changes and their reversibility (Mahoney et al., 2006; Fetoui et al., 2006; Avraham et al., 1996). The literature describing experimental undernutrition in animals is large and has been extensively studied (i.e. (Luz et al., 1995; Barbarich-Marsteller et al., 2005). Recent studies describing the involvement of several genes in regulating differences in the physiological adaptations to dietary restriction offer possibilities for investigating variations in the human response to fasting. Still, other features of AN such as hyperactivity are not considered in these dietary models.

Stress model

Acute stress by environmental manipulations include tail pinching, cold swimming, and immobilization results in hypophagia and/or reduced body weight (Shimizu et al., 1989; Pare, 1965; Pare, 1964; Antelman and Szechtman, 1975). Chronic stress models seem to be more beneficial regarding mimicking AN in animals. A chronic stress model is the separation model (van Leeuwen et al., 1997). In this model, physical separation acts as a stressor to induce a depression-like condition with decreased feeding, weight loss, and cognitive changes. Mice or rats are housed in one cage but separated by Plexiglass partitions, where they can see and smell each other, and are transferred to a common cage for a feeding period of 1 hour. Separation reduces 1 hour food intake and causes severe weight loss. Although this model resembles voluntary food restriction resulting in body weight loss, it still does not model the increased activity described in AN patients.

Activity models

Several animal models that focus on essential features of AN, exercise or activity, have been proposed to investigate AN. In this section, activity models and their various types of validity are discussed briefly.

Contingent (voluntary) wheel running is mainly applied to situations where humans associate physical health, derived from exercise and body weight reduction, with psychological health. Already in 1986, Hayes and Ross argued that there is a strong link

between exercise and well-being (Hayes and Ross, 1986). In this model of contingent wheel running, exercise is seen as an adaptive behavior, unlike AN, for which exercise exacerbates and maintains the disorder. Therefore, this model lacks face validity as it merely establishes a correlation between activity and food intake.

Another model that mimics activity, forced exercise model, uses an acute forced running paradigm. In this paradigm, rats are trained to run on a flat-belt treadmill. The intensity of activity can be modified by the speed or the angle of the treadmill. In order to avoid that an animal stops running, small electric shocks are applied. Forced exercise has similarities to a training regime in humans, and therefore has face validity. However, the exercise in this model is not voluntary and seems to be one type of activity (treadmill), unlike what is observed in AN patients. Furthermore, the metabolic and physiological changes are consistent with other AN models. Thus, the forced exercise model lacks construct validity.

Rodents placed in cages with running wheels will increase their daily locomotor activity. To compensate for increased energy expenditure due to increased physical activity these rodents will eat more (Scheurink et al., 1999). When exposed to scheduled feeding, rats in running wheel cages decrease food intake, become hyperactive and show immense body weight loss. These well-established observations were first described by Hall and Hanford in 1954 (Hall and Hanford, 1954) and often referred as activity-based anorexia, activity stress or activity anorexia (Dixon et al., 2003; Routtenberg and Kuznesof, 1967). The activity-based anorexia (ABA) model is a unique model since it resembles reduced food intake as well as (voluntary) increased activity levels in rodents similar to starvation and high physical activity levels seen in patients suffering from AN. Thus, this model involves chronic and voluntary wheel running and meets many of the validity criteria for a model of AN.

ABA in rats is dependent upon the interaction between food restriction and running wheel activity. Chronic food restriction, in the absence of running wheels, induces only a transient decrease in food intake and body weight. Within 2-3 days, food-restricted rats maintain their body weight and then begin to gain body weight despite limited access to food. Additionally, female rats maintained on a chronic food restriction schedule show minimal or no disruption of the ovarian cycle. This illustrates that the hyperactivity expressed by food restricted rats is a fundamental component of the ABA model.

Rats with starvation-induced hyperactivity show similarities to symptoms of AN seen in humans. Such symptoms include reduced food intake, weight loss, and hyperactivity. However, caution must be used when generalizing starvation-induced hyperactivity from rats to humans. AN patients may experience drive for thinness, anxiety to gain weight, and other psychopathological features which can not be assessed in animals. Therefore, research on this topic can be helpful to gain a better

understanding for the biological aspects of starvation-induced hyperactivity but caution must be used when generalizing findings to humans because of the psychological features that occur in AN patients (Hebebrand et al., 2003).

As shown in previous studies, the robust changes in behavioral and metabolic responses to the ABA model can not be explained by differences in hypothalamic signaling (de Rijke et al., 2005a; Hillebrand et al., 2005a; Hillebrand et al., 2006a; Hillebrand et al., 2006c). Most of the observations at this level are associated with the homeostatic response to a negative energy balance. Only few alterations in hypothalamic gene expression levels were observed, such as the up-regulated POMC mRNA levels in the ARC (de Rijke et al., 2005a). ABA rats treated with leptin (Hillebrand et al., 2005b) or olanzapine (Hillebrand et al., 2005c) have reduced hyperactivity and thereby diminishing the development of ABA. In contrast, treatment with amphetamine stimulates the development of anorectic behavior in particularly by increasing activity (Glavin et al., 1981). In a study combining voxel-based microPET imaging with behavioral measurements, metabolic changes in brain areas regulating food intake and locomotion were found to be related to the energy status in ABA rats as compared to healthy control rats (van Kuyck et al., 2007). For example, metabolic changes in the ventral striatum, especially the nucleus accumbens, were observed to be negatively correlated with relative body weight loss in rats exposed to the ABA model. This confirms that there is a complex interaction between neuronal circuits involved in the regulation of food intake and locomotion. Thus, not only hypothalamic signaling pathways may be involved in the development of ABA (reduced food intake) but other systems like the dopaminergic reward system (increased activity) might underlie the ABA model as well. Therefore, it is of great interest to examine the role of neuronal pathways regulating food intake and hyperactivity in response to changes in the release of peripheral signals during starvation.

D. Scope of the thesis

The neural mechanisms underlying AN remain obscure. Disturbances in dopamine and serotonin neurotransmission and to a limited extent in some peripheral hormones (i.e. leptin and PYY) have been described in AN. Studies on the frequently used animal model for AN, the activity-based anorexia model, suggest a normal starvation response at the level of the hypothalamus in the brain.

The regulation of energy balance involves complex interactions between peripheral signals and neural circuits that influence metabolism and behavior. Dysfunction of this energy balance regulatory system may underlie eating disorders, such as AN. In the present thesis we focused on regulatory neuronal pathways underlying anorectic behavior and the possible involvement of endocrine signaling with special emphasis on the involvement of the mesolimbic dopaminergic system. We thereby concentrate on starvation-induced hyperactivity, since this trait appears prominent in patients suffering from AN.

The overall aim of this thesis was to identify the neural mechanism(s) underlying starvation-induced hyperactivity in order to better understand the pathophysiology seen in AN patients. Specific aims were to investigate;

- which specific brain areas are involved in starvation-induced hyperactivity.
  Chapter 2
- the involvement of the mesolimbic dopaminergic system in starvation-induced hyperactivity.
  Chapter 3 & 4
- the contribution of peripheral signals (leptin and ghrelin) in starvation-induced hyperactivity.
  Chapter 5 & 6
- genetic loci and possible candidate genes underlying starvation-induced hyperactivity.
  Chapter 7

Finally, in ***chapter 8***, the main findings of these studies are summarized and discussed in relation to the general understanding of the pathophysiology seen in AN patients and implications in future perspectives.

Chapter 2

CHAPTER 2

Anticipation to meals during restricted feeding increases activity in hypothalamic neurons

Linda A.W. Verhagen, Mieneke C.M. Luijendijk, Jan-Willem de Groot,
Linda G.P. van Dommelen, Anne G.N. Klimstra, Tom A.P. Roeling, Roger A.H. Adan

Abstract

Rats exposed to fixed restricted meals develop anticipation to food. They increase their activity levels in the hours preceding food access, which has been described as food-anticipatory activity (FAA).

In the present study, we determined the possible involvement of brain areas of the hypothalamus and reward system in the early development of food-anticipatory hyperactivity in rats exposed to the activity-based anorexia (ABA) model. We thereby used two different paradigms, rats exposed to the ABA model (ABA normal) and rats exposed to the same restraint in food access but on a random feeding schedule (ABA random). The latter group of rats were not able to anticipate to food.

Surprisingly, we found a strong correlation between the expression of food anticipation measured by running wheel activity and c-*fos* expression levels in the dorsomedial hypothalamus (DMH) of ABA normal rats, whereas no correlation was found in ABA random rats. On the contrary, only in the randomly fed ABA rats, a strong negative correlation was found between the neuronal activity in the hypothalamic area and the percentage body weight loss. Interestingly, these results imply that food anticipation to meals during food restriction more strongly affects neuronal activation in the hypothalamus than negative energy balance alone. We conclude that during the early stages of development of FAA, the DMH plays a role in anticipation for food during periods of negative energy balance.

Introduction

To maintain an adequate body weight, energy intake and energy expenditure are kept in balance (homeostasis) even when the total amount of ingested food might fluctuate over days. Already in the 1950's, the hypothalamus was identified as a major brain area controlling energy homeostasis (Stellar, 1954). In the hypothalamus, the arcuate nucleus (ARC) is the central brain area sensing signals form the periphery and projecting to other hypothalamic areas, such as the paraventricular nucleus of the hypothalamus (PVH), dorsomedial hypothalamus (DMH), ventromedial nucleus (VMH), and the lateral hypothalamus (LH) (reviewed in Harrold, 2004; Morton et al., 2006). These hypothalamic areas have been implicated in energy balance and food intake (reviewed in Schwartz et al., 2000). In order to maintain energy homeostasis, during negative energy balance food intake is increased and following overconsumption food intake is decreased (Strubbe and van Dijk, 2002).

Food availability is unpredictable and animals often deal with periods of food scarcity. Therefore, the ability to seek and find food is crucial for survival. Periods of food shortage are accompanied by periods of increased activity when animals have to forage for food. In the laboratory, food scarcity can be replicated by exposing rodents to restricted feeding schedules (Routtenberg and Kuznesof, 1967; Dixon et al., 2003; Hall et al., 1953; Hall and Hanford, 1954) which trigger behavioral changes such as reduced food intake accompanied by increased movement and foraging behavior also recognized as food-anticipatory activity (FAA). FAA is regarded as the increase in locomotor activity levels during 2-3 hours prior to the scheduled meal time (Mistlberger, 1994). It has been shown that food anticipation is anatomically and functionally uncoupled from the light-entrainable circadian system as revealed by lesioning studies of the suprachiasmatic nucleus (Stephan et al., 1979; Krieger et al., 1977). Thereafter, many attempts have been made to identify the anatomical and functional substrate controlling food anticipation by non-specific lesioning of other hypothalamic brain nuclei in the central nervous system (Davidson et al., 2001; Landry et al., 2006; Landry et al., 2007a; Landry et al., 2007b; Mistlberger et al., 2003; Davidson et al., 2000; Davidson and Stephan, 1999; Mistlberger and Rechtschaffen, 1984; Krieger, 1980) or by blocking peripheral pathways signaling to the brain (Moreira and Krieger, 1982; Comperatore and Stephan, 1990; Davidson and Stephan, 1998). There is still debate on which brain nucleus or nuclei are involved in FAA, with the DMH, LH, and nucleus accumbens as strong candidates. During food anticipation, a neuronal network rather than a specific brain area is more likely to be disrupted (reviewed in Mistlberger, 1994; Strubbe and Woods, 2004).

In the present study, we determined the neuronal involvement of selected brain areas proposed to be involved in FAA in previous studies (Angeles-Castellanos et al.,

2004; Mendoza et al., 2005) in the early development of food-anticipatory hyperactivity in the activity-based anorexia (ABA) model using expression of c-*fos*, a so-called "immediately early gene" as a marker of neuronal activation. In the ABA model, rats are given free access to a running wheel and fed once per day for a limited period of time (1-2 hr food access). Exposure to the ABA model leads to a chronic catabolic state caused by a reduced food intake and increased activity (Routtenberg and Kuznesof, 1967). The fixed meal time elicits FAA characterized by increased running wheel activity and locomotor activity before scheduled meals. We focused on the possible involvement of hypothalamic areas including the ARC, PVH, DMH, VMH and LH, and on the reward system consisting of the NAc and VTA. Moreover, this study takes into account the individual differences in behavioral and physiological parameters and addressed whether these affected neuronal activation during FAA in the ABA model. Rats exposed to the ABA model (=ABA normal) were compared with rats subjected to the same restraint in food access with time points of food access at random (=ABA random). Thus, both groups of rats were food restricted to a similar extent, with the only difference that the ABA normal rats could anticipate, whereas the ABA random rats could not anticipate mealtime. This study design made it possible to determine neuronal changes during the early phase of food anticipation without the influence of factors such as prolonged times of arousal and changes in metabolic diurnal rhythmicity, drinking behavior and body temperature.

Material and methods

Animals

Adult female outbred Wistar WU rats (n=16) (Harlan, Horst, The Netherlands) weighing 155-165 gram upon arrival were individually housed in a ambient temperature- and humidity-controlled room (21°C ± 2°C) under a 12-hour dark-light cycle, lights on at 13.00. All described procedures were approved by the ethical committee on the use and care of animals of the University of Utrecht, The Netherlands. For ethical reasons, it was decided that rats were to be removed from the experiment when their body temperature was lower than 33°C before feeding, or when rats lost more than 25% of their initial body weight.

Surgical procedures

One week after arrival, all rats received transmitters (TA10TA-F40 Data Sciences International, St. Paul, Minnesota) in the abdominal cavity under fentanyl/fluanisone (0.1 ml/100 g body weight, IM; Hypnorm, Janssen Pharmaceutica, Beerse, Belgium) and midazolam (0.05 ml/100 gram body weight, IP; Dormicum, Hoffman-LaRoche,

Mijdrecht, The Netherlands) anesthesia. After surgery, rats were treated with carprofen (0.01 ml/100 gram body weight, s.c.; Rimadyl, Pfizer Animal Health, Capelle a/d Ijssel, The Netherlands) and saline (3 ml, s.c.) and allowed to recover for two weeks.

Experimental set-up

After recovery, rats were randomly assigned to experimental groups; ABA normal group (n=9) and ABA random group (n=7). All rats were housed in novel cages containing a running wheel for a training period of ten days (from day -10 to day 0). Running wheel activity (RWA) was continuously registered using a Cage Registration Program (Department Biomedical Engineering, UMC Utrecht, The Netherlands). At the end of day -4, transmitters were switched on by magnetic field induction to allow continuous assessment of (baseline) body temperature and locomotor activity (LMA). Until the beginning of day 0, animals were fed ad libitum and water was continuously available. After 1 hour at the beginning of the dark phase at day 0, food was removed for the experimental groups and animals were placed on the scheduled feeding of 1 hour per day (see also Figure 1.A.). The ABA normal group had daily food access for one hour at the beginning of the dark phase (from 13.00-14.00). In contrast to the ABA normal group, the ABA random group obtained daily food access at different time points during the dark phase (day 0, food was removed for the first time at 14.00; day 1 from 19.00-20.00; day 2 from 16.00-17.00; day 3 from 23.00-24.00). The following parameters were assessed daily: body weight, running wheel activity, locomotor activity, body temperature, food intake, and water intake. Body weight was measured prior to the dark phase, and just before food access during the food-restriction period in both experimental groups. Based on a review by Mistlberger (Mistlberger, 1994), food-anticipatory activity (FAA) was defined as the RWA four hours prior to food access on days of scheduled food access. Thus, FAA comprised of total wheel revolutions during four hours preceding the dark phase from day 0 till day 3. End the end of day 3, when FAA has clearly developed (de Rijke et al., 2005b), rats were sacrificed by intracardiac perfusion. Rats were injected with sodium pentobarbital and perfused transcardially with 250 ml of 0.9% saline solution followed immediately by 250 ml of 4% paraformaldehyde (PFA) containing 0.05% glutaraldehyde in 0.01M phosphate buffered saline (PBS). Brains were removed and post-fixated in 4% PFA overnight at 4 °C. Finally, brains were cryoprotected in 30% sucrose solution and stored at 4 °C.

Immunohistochemistry

Coronal brain sections of 16 µm thickness were cut with a freezing microtome (Micron HM 500, Germany) and stained for c-*fos*. Sections were fixated with 75% acetone and 25% ethanol and rinsed in 0.01 M phosphate-buffered saline (PBS) (pH 7.4, 3× 10'). Endogenous peroxidase activity was blocked with 3% H_2O_2 in 0.01 M PBS (30'). The

section were rinsed in PBS, blocked with 5% normal goat serum (NGS) in PBS, and incubated overnight at room temperature with primary antibody (polyclonal rabbit c-*fos* antiserum, 1:2000; Santa Cruz Biotechnology, USA) diluted in 2% NGS in PBS. Next day, after rinsing, section were incubated with 2% NGS in PBS and incubated for 1 hour with secondary antibody (biotinylated goat anti-rabbit, 1:800; Dako, Denmark) diluted in 1% NGS in PBS. Following rinsing in PBS, sections were treated with a complex of avidine-biotin solution for 1 hour. To visualize the peroxidase reaction, the signal was developed by diaminobenzidine solution containing 0.25% nickelammoniumsulphate. Finally, sections were dehydrated with alcohol, cleared with xylene and covered with Entellan (Merck, Germany).

Quantification of labeled cells

Sections were analyzed by counting the number of c-*fos* positive cells in the different areas throughout the brain using the Kontron KS 400 image analysis system. Sections were matched using the stereotaxic brain atlas from Paxinos and Watson (1998) and a blind study was performed when counting the cells. Using the fornix as a reference point, the hypothalamus was divided into different quadrants containing the ARC, DMH, VMH, LH, and PVH (see also Figure 1.B.). Besides brain areas of the hypothalamus, the reward system consisting of the NAc and VTA were analyzed as well. From each animal, 3 sections were analyzed.

Data analysis

Data were analyzed using SPSS 11.5 for Windows and were controlled for normality and homogeneity. Body weight, food intake, body temperature, and activity levels are expressed as mean ± standard error of the mean. For all behavioral measurements, baseline levels were not significantly different between the experimental groups. Within group differences were analyzed using the Student's t-test. Furthermore, association between neuronal activation and physiological parameters during ABA was investigated using Pearson's bivariate correlation analysis. The Bonferroni correction was executed on the outcome of the correlation analysis to control for multiple testing. Statistical significance was set at $p<0.05$.

Results

Physiological parameters during the development of ABA

Baseline body weight was not significantly different between rats exposed to ABA normal and rats exposed to ABA random (ABA normal 224.3 ± 4.3 gram, ABA random 229.7 ± 3.6 gram, n.s.). During food restriction, body weight decreased in both ABA

normal and ABA random rats. As depicted in Figure 2.A., ABA random rats had a significantly higher body weight loss as compared to ABA normal rats ($p<0.001$). In addition, for the period of scheduled feeding (ABA normal and ABA random), total food intake was not significantly different in rats exposed to ABA random as compared to ABA normal rats (see Figure 2.B.).

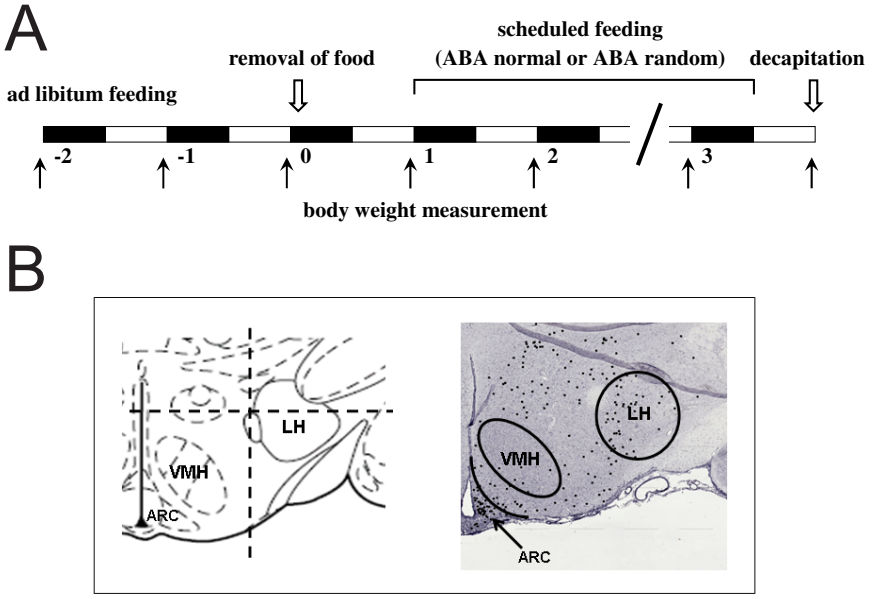

Figure 1.

(A) Scheme of experimental set-up. Filled bars indicate the dark phase, open bars the light phase. At day 0, food was removed and rats were placed on scheduled feeding (ABA normal received 1 hour food access at the beginning of the drak phase; ABA random received 1 hour food access randomly during the dark phase). Food intake and body weight were measured daily. (B) Schematic representation of the analysis for c-*fos* expression in several selected brain areas.

Behavioral parameters during the development of ABA

Running wheel activity (RWA), as percentage of baseline in each rat, was significantly increased in rats exposed to ABA random (Figure 2.C., $p=0.002$). As depicted in Figure 2.D., a similar significant increase was observed for locomotor activity (LMA) in ABA random rats. On the contrary, in ABA normal rats, RWA tended to increase after exposure to restricted feeding but this increase in RWA did not reach significance (Figure 2.C., $p=0.057$). In addition, no difference in LMA as compared to baseline

was found in ABA normal rats (see also Figure 2.D.). Thus, food intake was similar in both groups, but rats exposed to the random feeding schedule displayed increased activity levels.

Total FAA of individual ABA normal and ABA random rats expressed as total wheel revolutions four hours before food access are shown in Figure 3. Although the average FAA in ABA normal rats and ABA random rats did not significantly differ, a tendency towards increased FAA over three days in ABA normal rats was observed.

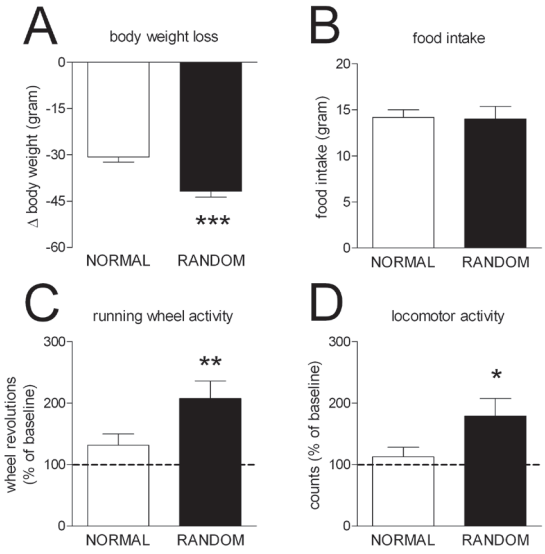

Figure 2.

Behavioral parameters over the course of the experiment; (A) body weight loss, (B) food intake, (C) running wheel activity, and (D) locomotor activity of ABA normal rats (open bar, n=9) and ABA random rats (black bar, n=7). Asterisks indicate significant differences; significant differences between groups for body weight loss, significant differences from baseline for running wheel activity and locomotor activity, *$p<0.05$, **$p<0.01$, ***$p<0.001$, Student's t-test.

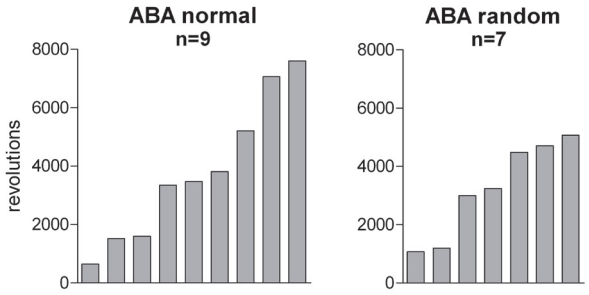

Figure 3.
Total food anticipation (measured by wheel revolutions) in individual rats exposed to the ABA normal (n=9, left panel) and ABA random (n=7, right panel) paradigm.

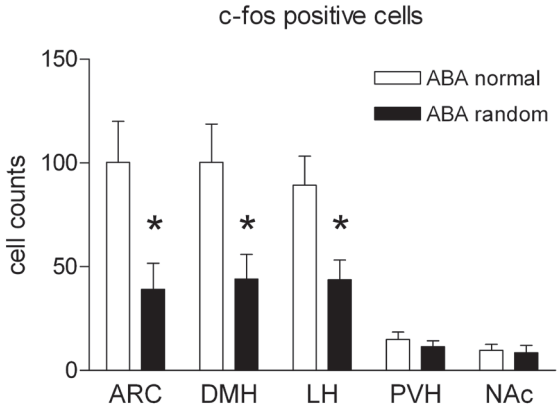

Figure 4.
C-*fos* expression levels (positive cell counts) in various brain areas of ABA normal rats (open bar, n=9) and ABA random rats (black bar, n=7). Note that the c-*fos* expression levels in the ventromedial hypothalamus (VMH) and ventral tegmental area (VTA) were too low for further analysis. ARC, arcuate nucleus; DMH, dorsomedial hypothalamus; LH, lateral hypothalamus; NAc, nucleus accumbens. Asterisks indicate significant differences, *$p<0.05$, Student's t-test.

C-fos expression levels in brain areas of ABA normal rats and ABA random rats
To investigate the possible involvement of brain areas during the initiation of FAA, c-*fos* expression levels were determined in various brain areas of rats exposed to ABA normal or ABA random (see Figure 4.). Rats exposed to ABA random were not able

to anticipate to food, while food access was presented at variable timepoints during the restricted feeding schedule.

In the ARC, DMH and LH, levels of c-*fos* expression were significantly increased in ABA normal rats as compared to ABA random rats. In addition, no significant changes were found for c-*fos* expression levels in the PVH and NAc in ABA normal rats compared to rats exposed to ABA random. In both experimental groups only few c-*fos* positive cells were found in the VMH and VTA. In total, less than 10 positive cells per rat were counted in the VMH or VTA and therefore these brain nuclei were excluded for further investigation.

Correlation analysis in ABA normal rats and ABA random rats

In order to determine whether the variability in c-*fos* expression could be explained by FAA (as measure of expressed anticipatory activity) or by body weight loss (as a measure of extent of negative energy balance), correlation analysis between these physiological and behavioral parameters and the c-*fos* expression levels in the brain areas of rats exposed to ABA normal or ABA random was performed (see Table 1.). In Figure 5.A. and 5.B., total amount of FAA measured by wheel revolutions is plotted against the c-*fos* expression in the DMH for both experimental groups presenting a strong positive association found in ABA normal rats anticipating for food, whereas association is lacking in ABA random rats. Furthermore, no association was found in ABA normal rats between the c-*fos* expression levels in various brain areas and percentage body weight loss (%BW), total food intake (total FI), and total running wheel activity (total RWA) (see Table 1.).

As indicated in Table 1., c-*fos* expression levels in all analyzed brain areas of ABA random rats were not correlated with total FAA. Although c-*fos* expression of the hypothalamic brain areas were not correlated with FAA, the expression levels in the ARC were strongly negatively correlated with percentage body weight loss in ABA random rats (see Figure 5.C and 5.D.). Note that correlation between the c-*fos* expression levels and percentage body weight in the DMH and LH of ABA random rats almost reached significance.

Table 1. Correlation analysis of c-*fos* expression levels in various brain nuclei and behavioral, physiological parameters in rats exposed to ABA normal or ABA random.

	ABA normal (n=9)				ABA random (n=7)			
	%BW	total FI	total RWA	total FAA	%BW	total FI	total RWA	total FAA
ARC	r = 0.432	r = -0.199	r = -0.133	r = 0.318	**r = -0.985**	r = -0.635	r = 0.607	r = -0.107
	p = 0.245	p = 0.607	p = 0.732	p = 0.404	**p < 0.001**	p = 0.091	p = 0.110	p = 0.820
DMH	r = 0.388	r = -0.093	r = -0.065	**r = 0.809**	r = -0.848	r = -0.476	r = 0.285	r = 0.090
	p = 0.302	p = 0.812	p = 0.869	**p = 0.008**	p = 0.016	p = 0.233	p = 0.494	p = 0.849
LH	r = 0.403	r = -0.040	r = -0.077	r = 0.382	r = -0.852	r = -0.428	r = 0.409	r = 0.090
	p = 0.282	p = 0.918	p = 0.844	p = 0.311	p = 0.015	p = 0.290	p = 0.315	p = 0.848
PVH	r = -0.132	r = 0.200	r = 0.310	r = 0.454	r = -0.746	r = -0.277	r = 0.488	r = 0.279
	p = 0.735	p = 0.605	p = 0.418	p = 0.220	p = 0.054	p = 0.506	p = 0.266	p = 0.544
NAc	r = 0.425	r = 0.003	r = 0.166	r = 0.587	r = -0.776	r = 0.103	r = 0.764	r = 0.935
	p = 0.401	p = 0.995	p = 0.754	p = 0.221	p = 0.123	p = 0.846	p = 0.077	p = 0.020

Values for correlation between c-*fos* expression levels in brain nuclei and parameters in rats exposed to ABA normal (n=9) or ABA random (n=7). Percentage body weight loss (%BW), total food intake (total FI), total running wheel activity (total RWA), and total food-anticipatory activity (total FAA). ARC, arcuate nucleus; DMH, dorsomedial hypothalamus; LH, lateral hypothalamus; PVH, paraventricular nucleus of the hypothalamus; NAc, nucleus accumbens. Significant correlations are written in bold. Following Bonferroni correction, statistical significance was set at $p \leq 0.0125$.

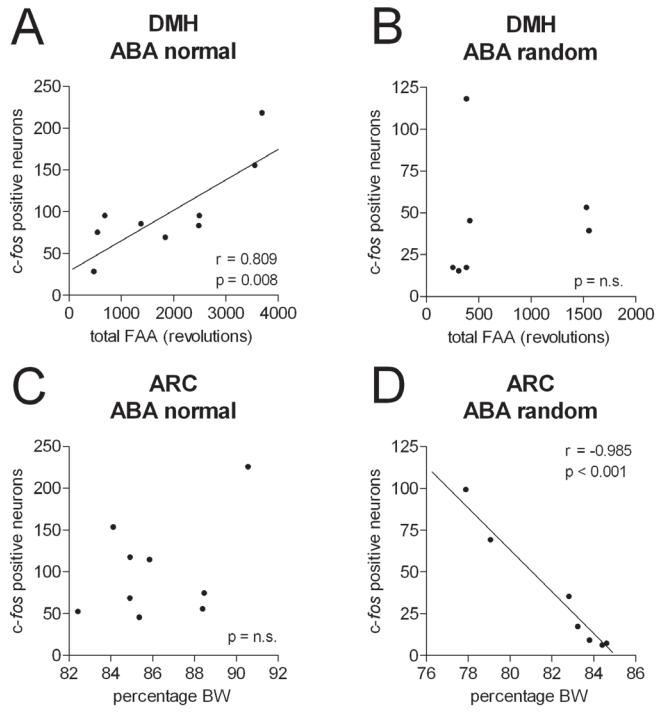

Figure 5.

Correlation grpahs between the total amount of food anticipation measured by wheel revolutions (total FAA) and c-*fos* expression levels in the dorsomedial hypothalamus (DMH) in rats exposed to ABA normal (A) or in rats exposed to ABA random (B). A positive association for total FAA was observed in rats exposed to the ABA normal paradigm, whereas no correlation was found in ABA random rats. In addition, correlation graphs between body weight (plotted in percentage of body weight at the start of the ABA model) and c-*fos* expression levels in the arcuate nucleus (ARC) in rats exposed to ABA normal (C) or in rats exposed to ABA random (D). A strong negative association for percentage body weight was observed in rats exposed tot the ABA random paradigm, whereas no correlation was found in ABA normal rats. Significant correlations were analyzed by Pearson's bivariate correlation analysis with the Bonferroni correction. Statistical significance was set at $p \leq 0.0125$.

Discussion

In this study, we investigated the neuronal involvement of selected brain areas in the early development of food-anticipatory activity in the activity-based anorexia (ABA) model using two different paradigms, ABA normal and ABA random. It was

supposed that ABA normal rats could anticipate to food, whereas ABA random rats received food access on variable timepoints during the dark phase and, therefore, were not able to anticipate to food. Even though ABA random rats were in a more negative energy balance most presumably caused by increased activity levels resulting in a more rapid body weight loss, in ABA normal rats the hypothalamic nuclei ARC, DMH, and LH known to be involved in the regulation of food intake had increased c-*fos* expression levels during food anticipation as compared to ABA random rats suggesting augmented levels of neuronal activity in these specific brain areas. Interestingly, correlation analysis in brain areas of the hypothalamus and the reward system (in particular the NAc) that were previously associated with anticipation to food only showed a strong positive association between neuronal activity in the DMH and total FAA in ABA normal rats, whereas this correlation was absent in ABA random rats. These data support a role for the DMH in increasing locomotor activity as expression of food anticipation during restricted scheduled feeding. Interestingly, in the random fed group, despite the lower total amount of c-*fos* expression in the ARC, the variability of c-*fos* expression within this group was correlated with the extent of body weight loss, whereas this was not the case in the ABA normal group.

In recent research studies, discrepancies on the involvement of the DMH in FAA were reported. For example, Gooley and researchers showed an increase in c-*fos* expression levels throughout the DMH at scheduled mealtime in rats (Gooley et al., 2006). In addition, DMH-lesioned rats reduced the increase in locomotor activity and wakefulness just before mealtime. On the contrary, in a study by Landry and researchers, FAA was still persistent following DMH lesion in rats (Landry et al., 2006). Despite the reported opposite results, accumulating research studies (Angeles-Castellanos et al., 2004; Caba et al., 2008; Gooley et al., 2006; Mieda et al., 2006) and this study suggest that the DMH plays a role in the anticipation for food when animals are exposed to food restriction. A recent study in rats showed that a peripheral injection of ghrelin, which would mimic a peripheral "hunger" signal, induces c-*fos* expression in the DMH (Kobelt et al., 2008). In this study, it was shown that the positive c-fos cells were surrounded by Agouti-related peptide (AgRP) fibers suggesting for an involvement of the co-localized AgRP/NPY projection from the ARC to the DMH. Moreover, the highest NPY mRNA levels in the ARC of normal rats can be detected just prior dark phase (Akabayashi et al., 1994) and this peak of NPY mRNA levels in the ARC can shift towards meal time (Yoshihara et al., 1996). Still, the specific neuronal population and coupled pathways of the DMH involved in food anticipation need to be identified, and NPY/AgRP neurons in the ARC are strong candidates.

As already shown by Mendoza and researchers, c-*fos* expression levels in the DMH and LH were lower in ad libitum fed rats than in rats exposed to restricted feeding (Mendoza et al., 2005). In our study, ABA random rats had increased activity levels

most likely resulting in a more rapid body weight loss as compared to ABA normal rats. Thus, ABA random rats were in a more negative energy balance. In ABA rats, however, c-*fos* expression levels in the ARC, DMH, and LH were increased as compared to ABA random rats. This supports that food anticipation results in increased c-*fos* expression observed in the ARC, DMH and LH of ABA rats on top of the increased neuronal activity caused by a negative energy balance. Nonetheless, only expression levels in the DMH were strongly positively correlated with food anticipation when RWA was taken as a measure for FAA. In ABA normal and ABA random rats, the c-*fos* expression levels in the VMH and VTA were too low for further analysis. Indeed, we confirmed that c-*fos* expression levels in the VMH were minimal as shown previously by Mendoza and researchers (Mendoza et al., 2005).

Furthermore, we could not find evidence for the involvement of the NAc. This is in agreement with previous studies performed in our laboratory demonstrating that the extracellular levels of dopamine were not changed during FAA (Verhagen et al. 2009a). However, Mendoza and researchers showed an increase in neuronal activity in the NAc (shell and core) just prior to the time of access to a palatable meal in food-entrained rats (Mendoza et al., 2005). These conflicting data on the involvement of the NAc during FAA might be explained by the differences in physiological state and/or food palatability (Barbano and Cador, 2005) and the extended period of time to induce FAA in other studies (Mendoza et al., 2005). Besides, we use relatively small groups of rats as compared to other studies.

While FAA involves many behavioral patterns, we cannot rule out the possibility that hormonal or other regulatory systems are involved as well. For example, leptin treatment in the ABA model reduced %FAA as compared to saline-treated rats (Verhagen et al., 2009a). Besides, food anticipation in the ABA model occurs at the end of the light phase, a timepoint at which animals wake up and prepare for the upcoming period of activity and to stimulate feeding behavior. In the ABA model, we were not able to find differences in neuronal activation in the PVH suggesting that this nucleus is not involved in the initiation of FAA.

In ABA random rats, a strong negatively correlation was found between the neuronal activity in the ARC and the percentage body weight loss and a tendency towards significance was observed in the other hypothalamic areas, the DMH and LH (see Table 1. and Figure 5. C. and D.). In contrast, no association with percentage body weight could be determined in ABA normal rats. Interestingly, these results might imply that food anticipation to meals more strongly affects neuronal activation in the ARC, DMH and LH than negative energy balance alone.

Taken together, we provide evidence for involvement of the ARC, DMH, and LH in the anticipation to meals and we showed a strong positive correlation between the neuronal activity in the DMH and FAA in rats exposed to the ABA model suggesting a

role for the DMH in anticipation for food during periods of negative energy balance.

Acknowledgements

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Chapter 3

CHAPTER 3

Dopamine antagonism inhibits anorectic behavior in an animal model for anorexia nervosa

Linda A.W. Verhagen, Mieneke C.M. Luijendijk, Jacquelien J.G. Hillebrand, Roger A.H. Adan

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Abstract

Excessive physical activity is commonly described as symptom of Anorexia Nervosa (AN). Activity-based anorexia (ABA) is considered an animal model for AN. The ABA model mimics severe body weight loss and increased physical activity. Suppression of hyperactivity by olanzapine in anorectic patients as well as in ABA rats suggested a role of dopamine and/or serotonin in this trait. Here, we investigated the effect of a non-selective dopamine antagonist in the ABA model. A dose-response curve of chronic treatment with the non-selective dopaminergic antagonist *cis*-flupenthixol was determined in the ABA model. Treatment reduced activity levels in both *ad libitum* fed and food-restricted rats. Treated ABA rats reduced body weight loss and increased food intake. These data support a role for dopamine in anorexia associated hyperactivity. Interestingly, in contrast to leptin treatment, food-anticipatory activity still persists in treated ABA rats.

Introduction

Anorexia nervosa (AN) is a psychiatric disorder with a high mortality rate (Bulik et al., 2007). AN is characterized by a dramatic reduction in caloric intake by excessive dieting, which is accompanied by physiological, biochemical, and behavioral disturbances (Casper et al., 1991; Davis, 1997). Excessive physical activity is commonly described as symptom of AN (Kron et al., 1978). Between 31% and 80% of anorectic patients display abnormally high levels of physical activity and overexercise (Hebebrand et al., 2003). Furthermore, excessive physical activity and caloric restriction reinforce each other in the development of severe weight loss.

Although the exact cause of AN is still unknown, serotonin and dopamine have been implicated in the etiology of AN (Bulik et al., 2007). Human genetic research demonstrated an association between the dopamine D_2 receptor polymorphism with AN (Bergen et al., 2005; Bulik et al., 2007). Regarding the serotonergic receptors, Ricca and researchers found an association with the 5-HT_{2A} receptor gene polymorphism (Ricca et al., 2002). In another study, a functional polymorphism of the serotonergic transporter gene was found in anorectic patients compared to normal weight controls (Fumeron et al., 2001). Besides genetic studies, positron emission tomography imaging studies with selective neurotransmitter radioligands confirmed altered serotonergic and dopaminergic neuronal pathway activities. Altered brain serotonin 5-HT_{1A} and 5-HT_{2A} receptor binding, and increased dopamine D_2/D_3 receptor binding were found in patients after recovery of AN (Bailer et al., 2005; Frank et al., 2005; Frank et al., 2002). Taken together, these studies support an involvement of serotonin and dopamine in AN.

The serotonergic/dopaminergic system has been targeted therapeutically in AN; antipsychotics were among the first agents studied for the treatment of AN. For example, the typical antipsychotic chlorpromazine induces body weight gain in anorectic patients, but long-term treatment provides significant adverse effects (Dally and Sargant, 1966). Another typical antipsychotic, pimozide, has been shown to increase body weight, but did not improve patient's attitude or behavior (Vandereycken and Pierloot, 1982). Both, these drugs antagonize central dopaminergic receptors (Reilly, 1978). Typical antipsychotics as described above have been replaced by newer atypical antipsychotics, such as olanzapine. Olanzapine is prescribed to anorectic patients to reduce agitation and to reduce anxiety about refeeding (Powers et al., 2002; Malina et al., 2003; Barbarich et al., 2004). Additionally, it has been associated with body weight gain (Allison and Casey, 2001). In animal studies, acute injections of olanzapine increase food intake and reduce locomotor activity (Thornton-Jones et al., 2002). In food-restricted running rats, chronic infusion of olanzapine significantly reduced running wheel activity (Hillebrand et al., 2005c). These reduced activity levels were

also observed in anorectic patients when treated with olanzapine (Hillebrand et al., 2005c). Olanzapine has a mixed receptor pharmacology. It has high affinity for 5-HT_2 serotonin receptors and for dopamine receptors, and a lower affinity is apparent for most cholinergic and α-adrenergic receptors (Roth et al., 2004). Although it is unclear via which receptors olanzapine reduces hyperactivity, the serotonergic and dopaminergic receptors are good candidates.

Activity-based anorexia (ABA) is an animal model which mimics a subset of important characteristics of AN, in particular excessive exercise and reduced food consumption (Routtenberg and Kuznesof, 1967). In this model, rats are given free access to a running wheel and fed once per day for a limited period of time (1-2 hrs). These food-restricted animals increase their activity levels and decrease their food intake, whereas *ad lib* fed animals regain their body weight after a short period of body weight loss when given access to a running wheel. The hyperactive behavior observed upon exposure to the ABA model has been explained in terms of foraging behavior, anticipation, reward and stress (Mistlberger, 1994; Watanabe et al., 1992; Casper et al., 2008). Hyperactivity and reduction of food intake have also been associated with dopamine function (Leibowitz and Brown, 1980; Barry and Klawans, 1976). This is in agreement with animal experiments. Dopamine-deficient mice (DD) lacking the dopamine synthesizing enzyme tyrosine hydroxylase (TH) in dopaminergic neurons become hypoactive, hypophagic, and will die of starvation (Zhou and Palmiter, 1995). DD mice become more active and immediately eat following L-dopa administration (Zhou and Palmiter, 1995; Szczypka et al., 1999b). Furthermore, restoring dopamine production in DD mice by viral-mediated gene transfer results in feeding (Szczypka et al., 1999a).

In the present study, we determined whether antagonism of dopamine receptors counteracts anorectic behavior. A dose-response of chronic non-selective dopaminergic antagonist *cis*-flupenthixol treatment on development of ABA in rats was determined. We discuss the effect of dopamine antagonism in comparison to the effect of antipsychotics and leptin in the ABA model, in order to determine whether hyperactivity and other anorectic behaviors were affected in a similar manner.

Experimental procedures

Animals

Fifty-six female outbred Wistar WU rats (Harlan, Horst, The Netherlands) weighing 155-165 gram upon arrival were individually housed in a ambient temperature- and humidity-controlled room (21°C ± 2°C) under a 12-hour dark-light cycle, lights on at 2 am. All described procedures were approved by the ethical committee on the use

and care of animals of the University of Utrecht, The Netherlands. For ethical reasons, it was decided that rats were to be removed from the experiment when their body temperature was lower than 33°C, or when rats lost more than 25% of their initial body weight.

Drugs
The non-selective dopaminergic D_1/D_2 receptor antagonist *cis*-flupenthixol (Sigma-Aldrich, Zwijndrecht, The Netherlands) was dissolved in sterile isotonic saline and was chronically infused (continuous for 7 days, 12 µl/day, s.c.) with osmotic pumps (Alzet model 1007D, DURECT, Cupertino California). A dose-response curve was determined using doses ranging from 0.03 -1.0 mg/day. Experimental food-restricted groups treated with different dose of *cis*-flupenthixol are displayed as follows; saline, FLU 0.03, FLU 0.1, FLU 0.25, and FLU 1.0. Each experimental group consisted of 8 animals.

Surgical procedures
One week after arrival, all rats (n=56) received transmitters (TA10TA-F40 Data Sciences International, St. Paul, Minnesota) in the abdominal cavity under fentanyl/fluanisone (0.1 ml/100 g body weight, IM; Hypnorm, Janssen Pharmaceutica, Beerse, Belgium) and midazolam (0.05 ml/100 gram body weight, IP; Dormicum, Hoffman-LaRoche, Mijdrecht, The Netherlands) anesthesia. After surgery, rats were treated with carprofen (0.01 ml/100 gram body weight, s.c.; Rimadyl, Pfizer Animal Health, Capelle a/d Ijssel, The Netherlands) and saline (3 ml, s.c.) and allowed to recover for two weeks. After recovery, rats were anesthetized by isoflurane, and osmotic minipumps containing vehicle or *cis*-flupenthixol were placed s.c. into the flank region of the rat after overnight incubation in saline at 37°C.

Experimental set-up
Animals were individually housed in cages with running wheels for a training period of ten days (from day -10 to day 0). Running wheel activity (RWA) was continuously registered using a Cage Registration Program (Department Biomedical Engineering, UMC Utrecht, The Netherlands). At the end of day -4, transmitters were switched on by magnetic field induction to allow continuous assessment of (baseline) body temperature and locomotor activity. Until the beginning of day 0, animals were fed ad libitum and water was continuously available. At the end of day -1, animals were divided into experimental groups (food-restricted rats: saline, FLU 0.03, FLU 0.1, FLU 0.25, and FLU 1.0; *ad lib* fed rats: saline and FLU 0.1; each individual group with n=8), matched for body weight (average vehicle: 216.9 ± 3.8 g, *cis*-flupenthixol: 218.0 ± 3.2 g, n.s.) and baseline RWA. Baseline RWA was determined as average RWA dur-

ing four days prior to the start of infusion (day -4 to day -1) (average vehicle: 6511 ± 1052 revolutions, *cis*-flupenthixol: 6392 ± 1093 revolutions, n.s.). In all rats, osmotic pumps were implanted as indicated above, just before the start of experimental day 0. After 1.5 hour at the beginning of the dark phase at day 0, food was removed for the restricted experimental groups and animals were placed on the scheduled feeding of 1.5 hours per day. Body weight of all animals was measured just prior the dark phase, and just before food access in the food-restricted experimental groups. The animals were sacrificed by decapitation at the end of the light phase of day 5 (see also Figure 1). Trunk blood was collected into lithium-heparin (Sarstedt, Nümbrecht, Germany) containing tubes with 83 μmol EDTA and 1 mg aprotonin. Tubes were collected on ice until centrifugation (20 min. at 3000 rpm 4˚C), subsequently plasma was stored at -20˚C until assay. Brains were quickly removed, frozen and stored at -80˚C. Interscapular brown adipose tissue (BAT), white adipose tissue surrounding the oviducts (PGAT), retroperitoneal white adipose tissue (RPAT), subcutaneous white adipose tissue (SCAT), adrenals, and kidneys were collected, weighed, frozen and stored at -80˚C.

Measurements in plasma samples

Plasma levels of leptin and insulin were measured by radioimmuno assay (RIA). From the samples, 2x 50 μl was taken for measurement in duplo. Plasma leptin and insulin were measured using commercially available RIA kits, according to the manufacturer's protocol (Linco Research, St. Charles Missouri, USA).

Statistical analysis

Body weight, food intake, body temperature, activity levels, fat depots, and hormonal plasma levels are expressed as mean ± standard error. Data were analyzed using SPSS 11.5 for Windows and were controlled for normality and homogeneity. For all measurements (*ad lib* fed and food-restricted rats), baseline levels were not significantly different between all experimental groups. The significance of the changes as a consequence of the chronic treatment with different *cis*-flupenthixol concentrations was evaluated by ANOVA with repeated measures (i.e. Treatment (5 levels; saline, FLU 0.03, FLU 0.1, FLU 0.25 and FLU 1.0)) using Huynh Feldt correction for Mauchly's Sphericity effects. When appropriate, the ANOVA was followed by *post-hoc* testing (Tukey HSD) if significant effects were detected. Within group differences were analyzed using the Student's t-test. Statistical significance was set at $p<0.05$.

Results

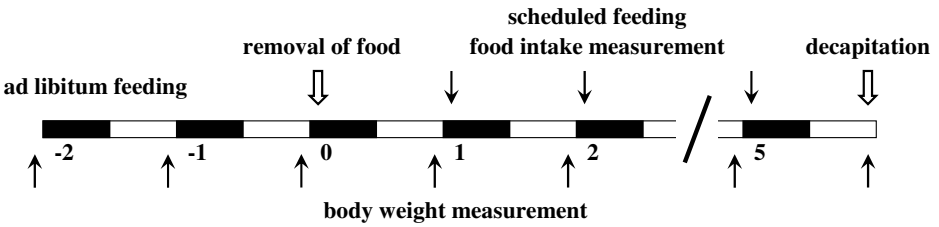


Figure 1.

Scheme of experimental set-up. Filled bars indicate the dark phase, open bars the light phase. At day 0, food was removed for the restricted experimental groups and placed on scheduled feeding of 1.5 hour at the beginning of the dark phase. Food intake and body weight were measured daily.

Dose-response curve in activity-based anorexia

To determine the effective dose for chronic treatment with the dopaminergic antagonist *cis*-flupenthixol, we analyzed the effect of different dosages on body weight, food intake, basal body temperature and total RWA in rats exposed to the ABA model. During the restricted feeding schedule, all animals decreased their body weight. Treatment with FLU 0.03 showed a similar body weight loss as compared to control animals (see figure 2.A.). Statistical analysis revealed a significant decrease in body weight loss over time in food-restricted rats treated with FLU 0.1 ($p=0.011$). The FLU 0.1 treated food-restricted rats had a significantly higher body weight and food intake from the third day onwards as compared to saline-treated food-restricted rats. In comparison with control animals, food-restricted rats treated with highest doses FLU 0.25 and FLU 1.0 immediately decreased their body weight rapidly. The same decreased effect was observed for measurements of food intake (Figure 2.B.). Likely to the rapid decrease in body weight and food intake, these animals also significantly decreased their body temperature. This probably reflects starvation-induced hypothermia, a defense mechanism to spare energy. Due to ethical reason, rats treated with FLU 0.25 and FLU 1.0 were removed from the experiment at the end of day 3.

All measurements of relative body weight, food intake, basal body temperature, activity levels (Figure 2.) were not significantly different between vehicle and the lowest dose FLU 0.03 of *cis*-flupenthixol. In FLU 0.03 treated rats, the amount of fat depots (total WAT (% of body weight); 0.71 ± 0.12 gr) and plasma levels of leptin and of insulin (leptin 0.18 ± 0.03 ng/ml; insulin 0.12 ± 0.03 ng/ml) did not differ from saline-treated rats. In order to determine whether the effect of treatment with *cis*-flupenthixol on activity, body weight and food intake was dependent on the imposed

food restriction, we pursued next experiments in non-food-restricted rats with the effective dose of 0.1 mg/day since this dose had an effect on body weight, food intake and activity levels.

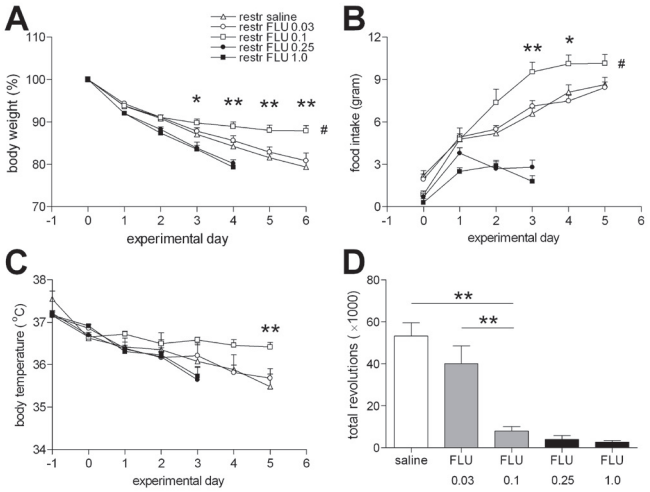


Figure 2.

Dose response curves of relative body weights (A), food intake (B), basal body temperature measured during inactivity in the early light phase (C) and total RWA (D) of food-restricted rats treated with vehicle or *cis*-flupenthixol 0.03, 0.1, 0.25 or 1.0 mg/day. Each experimental group consisted of 8 animals. Due to ethical reasons, rats treated with FLU 0.25 and FLU 1.0 were removed from the experiment at the end of day 3. Significant differences over time are indicated by symbol #, whereas asterisks indicate significant differences per individual day (vehicle-treated food-restricted vs. FLU-treated food restricted rats), *p<0.05, **p<0.01, Student's t-test.

Effects of cis-flupenthixol on body weight, and food intake

As shown in figure 3, relative body weight at the final experimental day (end of day 5) was not different between ad lib fed groups that were treated with vehicle or cis-flupenthixol. Food-restricted rats treated with FLU 0.1 had a significantly higher relative body weight as compared to food-restricted vehicle-treated rats ($p<0.001$) at the final day of the experiment. This difference in food-restricted rats was already significant at day 3 of the experiment ($p=0.02$) (Figure 3).

Cumulative food intake from day 1 till day 5 was similar between ad lib fed saline- and FLU-treated rats, whereas cumulative food intake was significantly increased in restricted FLU-treated rats in comparison with restricted saline-treated rats (see Table 1.). Regarding each individual day, food intake was significantly increased on

day 3 and 4 of the experiment (p=0.004 and p=0.028, respectively) in food-restricted FLU-treated rats.

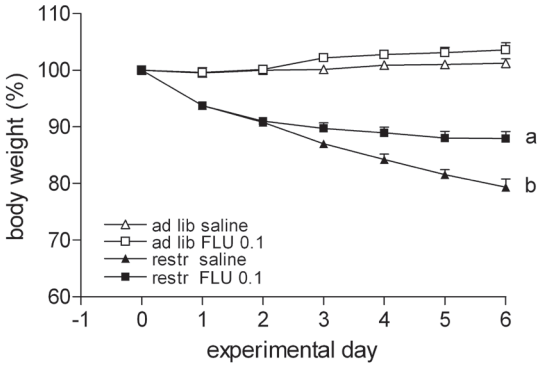


Figure 3.

Relative body weights of *ad lib* fed and food-restricted rats, treated with vehicle or *cis*-flupenthixol 0.1 mg/day. Each experimental group consisted of 8 animals. Significant differences in body weight loss over time are shown as follows; vehicle vs. *cis*-flupenthixol treatment in (a) food-restricted rats; (b) vehicle-treated *ad lib* fed rats vs. food-restricted rats.

Body composition in ad lib fed and food-restricted rats treated with cis-flupenthixol

After 5 days, rats were decapitated and fat pads were removed. Visceral fat pads were still present in food-restricted rats treated with FLU 0.1, while almost all fat pads had disappeared in food-restricted rats treated with vehicle. As shown in Table 1, significantly more total white adipose tissue (WAT) was present in FLU-treated rats as compared to control (ad lib fed 133.3 ± 10.7%; food-restricted 201.5 ± 24.2%). Further analysis revealed that the percentage of abdominal fat pads (AWAT) were significantly higher (p=0.02) in food-restricted rats treated with cis-flupenthixol, and a similar increase was also observed in ad lib fed FLU-treated rats. In addition, plasma leptin and insulin levels were significantly increased when rats were treated with cis-flupenthixol. However, these levels of leptin and insulin were still significantly lower as compared to ad lib fed running rats (see Table 1.).

RWA and FAA in ad lib fed and food-restricted rats treated with cis-flupenthixol

Baseline running wheel activity (RWA) was not significantly different before treatment (on day -1) in ad lib fed and food-restricted rats. In ad lib fed and food-restricted animals, treatment with *cis*-flupenthixol significantly reduced the hourly RWA during

Table 1. Body composition and behavioral parameters in *ad lib* and food/restricted rats, treated with vehicle or *cis*-flupenthixol (0.1 mg/day).

	ad libitum fed		restricted	
	saline	FLU 0.1	saline	FLU 0.1
Final body weight (% of initial weight)	101.21 ± 0.81	103.61 ± 1.25	79.34 ± 1.47 [c]	87.92 ± 1.28 [b]
Cum. food intake (day 1-5, gram)	98.21 ± 3.44	95.38 ± 2.83	33.30 ± 1.77 [c]	41.93 ± 2.97 [b]
Total WAT (% of body weight)	0.84 ± 0.06	1.12 ± 0.09 [a]	0.66 ± 0.10	1.33 ± 0.16 [b]
Total abdominal WAT (% of body weight)	0.62 ± 0.05	0.80 ± 0.07 [a]	0.44 ± 0.04 [c]	0.84 ± 0.13 [b]
Leptin (ng/ml)	1.26 ± 0.18	2.16 ± 0.31 [a]	0.14 ± 0.02 [c]	0.35 ± 0.07 [b]
Insulin (ng/ml)	1.35 ± 0.32	2.22 ± 0.45	0.07 ± 0.01 [c]	0.34 ± 0.06 [b]
Total RWA (revolutions)	35494 ± 5939	9272 ± 5066 [a]	53227 ± 6275	7892 ± 2182 [b]
Total LMA (counts)	10370 ± 1908	4590 ± 1676 [a]	14233 ± 4280	3175 ± 522 [b]
FAA (% of total RWA)	1.21 ± 0.82	3.25 ± 2.66	18.01 ± 2.25 [c]	23.18 ± 5.48

Final body weight (% of initial body weight at day 0), cumulative food intake, weight of fat depots (% total WAT, and % total abdominal WAT), plasma leptin and insulin levels, total running wheel activity (RWA), total locomotor activity (LMA), and food-anticipatory behavior (FAA, % of total RWA, day 0 - day 4) in *ad lib* fed and food-restricted rats, treated with vehicle or *cis*-flupenthixol (0.1 mg/day). Significant differences are shown as follows; vehicle vs. *cis*-flupenthixol in (a) *ad lib* fed rats and (b) food-restricted rats; (c) vehicle-treated *ad lib* fed rats vs. food-restricted rats.

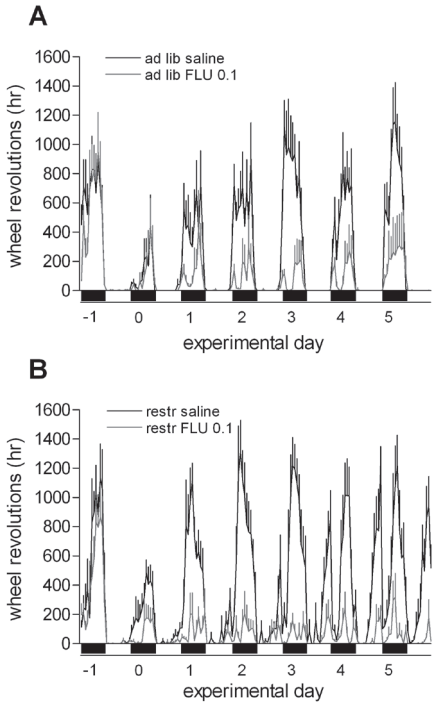


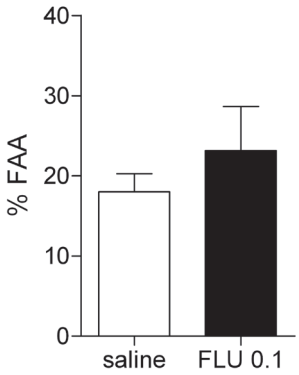
Figure 4.

Hourly running wheel activity (RWA) of *ad lib* fed (A) and food-restricted rats (B), treated with vehicle (Black solid line) or *cis*-flupenthixol 0.1 mg/day (gray solid line). Each experimental group consisted of 8 animals. Black bars indicate dark phase, and white bars indicate light phase. Hourly RWA is presented as mean ± SEM.

dark phase (see Figure 4). General locomotor activity (LMA), as measured by telemetric devices, was also significantly reduced by treatment. Cumulative LMA (day 0-5) was significantly decreased in treated *ad lib* fed and food-restricted rats (see Table 1). When rats are fed for only 1.5 hour at the beginning of the dark phase, animals become more active during hours preceding food access. In contrast to ABA rats, *ad lib* fed running rats show stable levels of RWA. Based on a review by Mistlberger (Mistlberger, 1994), food-anticipatory activity (FAA) was defined as the RWA four hours prior to food access on days of scheduled food access. Since, under restriction conditions the food was given during the first 1.5 hours of the dark phase, the RWA in the last four hours of the light phase was taken into consideration for the evaluation of FAA. In figure 5, total FAA is presented as percentage of total RWA during these days. No significant differences in percentage FAA of total RWA were found between vehicle-

treated and FLU-treated rats during ad lib condition nor during food restriction.

Figure 5.



Percentage food-anticipatory activity (%FAA = total FAA as percentage of total RWA) in ABA rats treated with *cis*-flupenthixol 0.1 mg/day compared with vehicle-treated ABA rats. Each experimental group consisted of 8 animals. No significant differences were determined.

Discussion

The present study shows the effect of the non-selective dopaminergic antagonist cis-flupenthixol in rats exposed to the ABA model. Treatment with *cis*-flupenthixol (0.1 mg/day) increased food intake in ABA rats but not in ad lib fed rats. Furthermore, treatment with *cis*-flupenthixol reduced activity levels and increased the percentage of total (abdominal) fat mass under both ad lib fed and food-restricted conditions. Interestingly, food-anticipatory activity remained in ABA rats treated with *cis*-flupenthixol.

No differences on behavioral parameters were found between food-restricted rats treated with *cis*-flupenthixol 0.03 mg/day and vehicle-treated food-restricted rats, whereas treatment with *cis*-flupenthixol 0.1 mg/day caused a significant reduction of body weight loss and a significant increase in food intake over time. Treatment with the two highest doses of *cis*-flupenthixol, specifically 0.25 and 1.0 mg/day, caused a general disruption of behavior. Rats treated with FLU 0.25 and FLU 1.0 strongly decreased their body weight, food intake, body temperature and activity levels. Clearly, these doses of *cis*-flupenthixol were too high and promoted emaciation.

The effects of chronic treatment with *cis*-flupenthixol (0.1 mg/day) on RWA and LMA in both *ad lib* fed and food-restricted rats are reliable and robust. These findings on RWA and LMA in *ad lib* fed as well as food-restricted running rats are similar to pre-

vious results on studies examining the effects of the antipsychotics chlorpromazine, pimozide, and olanzapine (Hillebrand et al., 2005c; Routtenberg, 1968; Lambert and Porter, 1992). Although pimozide (0.25 mg/kg) suppressed RWA less than *cis*-flupenthixol (0.1 mg/day) in our study, this low dose of pimozide did not reduce RWA during *ad libitum* conditions. Higher doses of pimozide (0.5-1.0 mg/kg) reduced RWA in both *ad libitum* fed and food-restricted running rats, similar to our measurements with *cis*-flupenthixol. Since *cis*-flupenthixol similarly affect activity levels in rats exposed to the ABA model, antagonism of dopamine receptors is a likely explanation for the efficacy of these antipsychotics on activity. Besides, rats receiving pimozide did not increase food intake as compared to controls, while in our study FLU-treated rats consumed significantly more than saline-treated rats only under food-restricted conditions.

Vehicle-treated food-restricted rats increased RWA following the start of scheduled feeding. Total daily wheel revolutions increased and the distribution of activity changed too. In the hours prior to food access, high levels of activity were displayed (= food-anticipatory activity). In the literature, the role of dopamine in anticipatory activity remains uncertain. Several studies carried out with palatable food do show involvement of dopamine in anticipatory activity (Mistlberger and Mumby, 1992; McCullough and Salamone, 1992; Barbano and Cador, 2007). We are not aware of reports on dopamine release when normal chow is presented to food-deprived animals. However, while general activity levels are strongly reduced after treatment with *cis*-flupenthixol, food-restricted rats still exhibit food-anticipatory activity (Figure 5.). If *cis*-flupenthixol effectively antagonized dopamine receptors in the rats in the present study, then this argues against involvement of dopamine in anticipatory activity in this model. This is in agreement with a study by Mistlberger and Mumby. They found that treatment with a selective dopamine D_2 receptor antagonist, haloperidol, reduced activity levels but food anticipation maintained (Mistlberger and Mumby, 1992). We extended this finding by showing that also a mixed D_1/D_2 dopamine antagonist has a similar effect.

Leptin treatment attenuates hyperactive behavior in ABA rats as well (Hillebrand et al., 2005b). Leptin, however, did not suppres RWA in *ad lib* fed running rats. Thus, the effect of leptin in suppressing hyperactivity is specific for animals displaying food-restriction induced hyperactivity. Therefore, we propose that lack of leptin contributes to induction of hyperactivity. Interestingly, lack of leptin signaling in the VTA was recently shown to increase locomotor activity (Hommel et al., 2006). It was also demonstrated that leptin suppressed dopamine neuronal activity (Hommel et al., 2006). Taken together, we hypothesize that lack of leptin signaling in the VTA may result in disinhibition of dopaminergic activity which would underlie increased locomotor activity.

Since the effect of *cis*-flupenthixol on total RWA was similar to the effect of

leptin and olanzapine (Hillebrand et al., 2005b; Hillebrand et al., 2005c), we also analyzed percentage FAA of leptin- and olanzapine-treated rats in these earlier performed studies, in order to determine whether FAA was affected similarly. Interestingly, the percentage FAA was significantly reduced when ABA rats were chronically treated with leptin as compared to vehicle-treated ABA rats (see figure 6.; leptin-treated 54.20 ± 8.84%, p=0.0084) whereas in olanzapine-treated rats FAA remained unaffected (see figure 6.; olanzapine-treated 82.55 ± 17.57%, n.s.) similarly to the effect of *cis*-flupenthixol reported here (Figure 5.). This suggests involvement of leptin rather than dopamine in FAA in the ABA model.

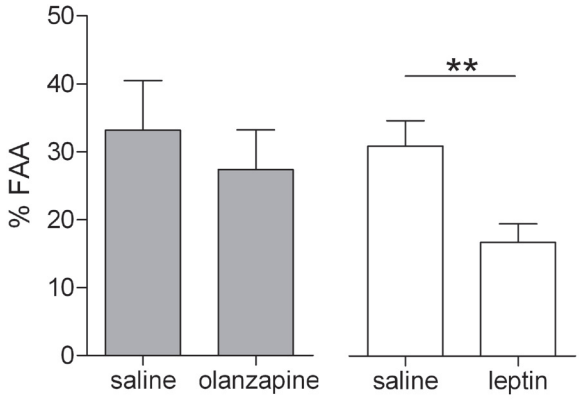


Figure 6.

Percentage food-anticipatory activity (%FAA = total FAA as percentage of total RWA) in ABA rats treated with olanzapine (7.5 mg/kg, n=7) or leptin (4 μg/day, n=7) compared with vehicle-treated ABA rats (respectively n=7, n=8) (Hillebrand et al., 2005c; Hillebrand et al., 2005b). Asterisks indicate significant differences, **p<0.01, Student's t-test.

Cumulative food intake remained unchanged in ad lib fed rats following cis-flupenthixol treatment, to some extent it is decreased compared to ad lib fed vehicle-treated rats (not significant). Previous studies examining the effects of chlorpromazine, pimozide and olanzapine during ABA showed no significant effects on food intake (Routtenberg, 1968; Lambert and Porter, 1992; Hillebrand et al., 2005c). Thus, the significant increase in food intake following treatment was specific for ABA rats treated with *cis*-flupenthixol, although we can not exclude that differences in experimental set-up underlie the different results. Food-restricted rats treated with *cis*-flupenthixol decreased their RWA and LMA. Hence, food-restricted treated rats saved energy by

reducing energy expenditure and this was reflected by the higher precentage total amount of fat tissue and increased plasma leptin levels as compared to food-restricted vehicle-treated rats. It is known that leptin administration reduces appetite and promotes weight loss in obese, genetically leptin-deficient humans and rodents (Myers et al., 2007). The arcuate nucleus of the hypothalamus is an important target of leptin's effect on food intake and energy expenditure where leptin inhibits neuropeptide Y and agouti-related peptide and activates melanocortins. Leptin treatment also decreases food intake when rats are exposed to the ABA model (Hillebrand et al., 2005b), indicating that leptin even inhibits feeding behavior during a negative energy balance. Despite the increased plasma leptin levels and preservation of adipose tissue as compared to vehicle-treated rats, rats treated with *cis*-flupenthixol and exposed to the ABA model showed increased ingestive behavior. Thus although the FLU-treated food-restricted rats have a more positive energy balance than vehicle-treated rats, as reflected by their increased percentage adipose tissue and plasma levels of leptin and insulin, food intake is still increased compared to saline-treated food-restricted rats. Thus, compared to saline-treated food-restricted rats, FLU-treated rats have lesser orexigenic drive, since they have higher levels of leptin, and leptin would stimulate hypothalamic anorexigenic neural circuits (Porte, Jr. et al., 2002). This supports the notion that antagonism of dopaminergic receptors stimulates food intake downstream of the hypothalamic circuits that respond to peripheral satiety signals.

Anorectic patients often display abnormally high physical activity levels (Kron et al., 1978), which might prolong the process of recovery (Holtkamp et al., 2004a; Kaye et al., 1988b). The present data of dopamine antagonism and the comparison with treatment of antipsychotics in ABA rats from earlier studies suggest that hyperactive behavior and reduced food intake observed in anorectic patients may be targeted by dopamine receptor antagonism. In conclusion, these data suggest that antipsychotics may be considered in the treatment of AN patients when reduction of hyperactive behavior is expected to accelerate recovery.

Acknowledgements

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Chapter 4

CHAPTER 4

Dopamine and serotonin release in the nucleus accumbens during starvation-induced hyperactivity

Linda A.W. Verhagen, Mieneke C.M. Luijendijk,
Gerdien A.H. Korte-Bouws, S. Mechiel Korte, Roger A.H. Adan
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Abstract

Activity-based anorexia (ABA) is considered an animal model for anorexia nervosa (AN). By scheduled feeding and voluntary wheel running, it mimic severe body weight loss and increased physical activity in AN. Pharmacological, genetic and imaging studies implicate dopamine and serotonin in the regulation of feeding behavior, food-anticipatory activity, and food reward. Previous studies propose that the nucleus accumbens (NAc) plays an important role in these food-related processes. Here we determined dopamine and serotonin levels in the NAc upon exposure to the ABA model. Surprisingly, the release of dopamine and serotonin in the NAc were not increased during the initiation of food-anticipatory behavior in ABA rats. Dopamine release in the NAc was increased during feeding behavior in ABA rats. During ABA, levels of serotonin were low and circadian activity is blunted. We conclude that during the early stages of development of food-anticipatory activity, increased dopamine does not trigger hyperactivity.

Introduction

Anorexia nervosa (AN) is a psychiatric disorder with a high mortality rate. AN is characterized by a dramatic reduction in caloric intake and by excessive dieting, which results in physiological, biochemical, and behavioral disturbances (Casper et al., 1991; Davis, 1997; Kron et al., 1978). Excessive physical activity is commonly described as a symptom of AN. Between 31% and 80% of anorectic patients display abnormally high levels of physical activity and overexercise (Hebebrand et al., 2003). Moreover, excessive physical activity and caloric restriction might reinforce each other in the development of severe weight loss and it is not known what drives these behaviors.

Atypical antipsychotics are frequently prescribed drugs for treatment of AN to reduce agitation, obsessionality, and anxiety about refeeding (Barbarich et al., 2004; Bissada et al., 2008; Malina et al., 2003). Treatment with these antipsychotics have been associated with reduced physical activity levels and increased body weight gain (Allison and Casey, 2001; Hillebrand et al., 2005c). It is not known via which mechanism these antipsychotics act. Both dopamine and serotonin are good candidates, since several lines of evidence support an involvement of altered 5-HT and DA signaling in AN. Low levels of the serotonergic metabolite 5-hydroxyindoleacetic acid (5-HIAA) were found in the cerebral spinal fluid of AN patients as compared to healthy normal women, and these low levels of 5-HIAA in AN patients normalize after weight gain (Kaye et al., 1988a). Genetic research demonstrated an association with the 5-HT_{2A} receptor gene polymorphism (Ricca et al., 2002), and the serotonergic transporter gene compared to normal weight controls (Fumeron et al., 2001). Bailer and researchers reported an increase in 5-HT_{1A} and normal 5-HT_{2A} receptor activity in ill AN patients (Bailer et al., 2007). Regarding dopamine involvement, an association between the dopamine D_2 receptor polymorphism with AN was found (Bergen et al., 2005; Bulik et al., 2007). Furthermore, imaging studies confirmed altered serotonergic and dopaminergic neuronal pathways activities after recovery from AN (Bailer et al., 2005; Frank et al., 2002; Frank et al., 2005).

Activity-based anorexia (ABA) is an animal model for AN, which mimics important characteristics of AN, in particular excessive exercise and reduced food consumption (Routtenberg and Kuznesof, 1967). In this model, rats are given free access to a running wheel and fed once per day for a limited period of time (1-2 hrs). Exposure to the ABA model leads to a chronic catabolic state caused by a reduced food intake and increased locomotor activity. This hyperactive behavior includes increased activity levels before scheduled meals, called food-anticipatory activity (FAA).

A fundamental role of the nucleus accumbens (NAc) during food-antic-

ipatory activity was shown by Mendoza and researchers, who showed a food-entrained pattern of c-*fos* expression in the NAc with high levels prior to meal-time of palatable food in rats suggesting a fundamental role of the NAc during food-anticipatory activity (Mendoza et al., 2005). Selective dopaminergic lesions in the NAc or local administration of dopamine antagonists strongly decrease this locomotor behavior (Kelley et al., 2005). Interestingly, DA depletion or DA receptor blockade in the NAc was able to decrease food-anticipatory activity (Barbano and Cador, 2006; McCullough and Salamone, 1992). Although DA in the NAc plays an important role in food reward and feeding, it has been reported that 5-HT release might be involved as well. Rats increased activity levels and reduced food intake when treated with a selective 5-HT_{1B} receptor agonist (Lee et al., 2002). Stimulation of 5-HT_{2C} receptors also reduced food intake (Hewitt et al., 2002; Clifton et al., 2000). Moreover, direct pharmacological stimulation of the serotonin receptor in the NAc, particularly the 5-HT_4 receptor, reduced food intake in food-deprived mice (Jean et al., 2007).

Given the fact that antipsychotics reduce hyperactivity and the role of the NAc in food-anticipatory activity and its associated hyperactivity, we here examined the hypothesis that increased dopamine and/or serotonin in the NAc accompanies feeding, food-anticipatory activity, and hyperactivity in rats exposed to the ABA model.

Experimental procedures

Animals

Female outbred Wistar WU rats (n=24) (Harlan, Horst, The Netherlands) weighing 155-165 grams upon arrival were individually housed in a ambient temperature- and humidity-controlled room (21°C ± 2°C) under a 12-hour dark-light cycle, lights on at 2 am. All described procedures were approved by the ethical committee on the use and care of animals of the University of Utrecht, The Netherlands. For ethical reasons, it was decided that rats were to be removed from the experiment when their body temperature was lower than 33°C before feeding, or when rats lost more than 25% of their initial body weight.

Surgical procedures

All rats received transmitters (TA10TA-F40 Data Sciences International, St. Paul, Minnesota) in the abdominal cavity under fentanyl/fluanisone (0.1 ml/100 g body weight, IM; Hypnorm, Janssen Pharmaceutica, Beerse, Belgium) and midazolam (0.05 ml/100 gram body weight, IP; Dormicum, Hoffman-LaRoche,

Mijdrecht, The Netherlands) anesthesia. After surgery, rats were treated with carprofen (0.01 ml/100 gram body weight, s.c.; Rimadyl, Pfizer Animal Health, Capelle a/d Ijssel, The Netherlands) and saline (3 ml, s.c.) and rats were allowed to recover for two weeks.

We used intracerebral microdialysis to obtain repeated measurements on the release of extracellular levels of neurotransmitters and their metabolites. Experimental animals were anaesthetized as described above and were stereotaxically provided unilateral with a microdialysis probe. The dialysis probe consisted of a dialysis fiber (molecular weight cutoff 10 kDa, 4 mm in total length), glued into the ends of parallel lengths of 27-gauge stainless steel needles. The U-shaped tip of the microdialysis membrane was positioned just lateral to the nucleus accumbens shell (NAc; coordinates: 1.7 mm anterior to bregma, 1.0 mm lateral from midline, and 7.5 mm below the surface of the brain; with toothbar set to obtain flat skull position). We used dental cement to secure the probe to two stainless steel screws inserted in the skull. Twenty-four animals were operated according to this protocol.

Experimental set-up

After one week acclimatization to the animal facility, animals received transmitters. After recovery of two weeks, animals were individually housed in cages with running wheels for a training period of ten days (from day -10 to day 0). Running wheel activity (RWA) was continuously registered using a Cage Registration Program (Department Biomedical Engineering, UMC Utrecht, The Netherlands). At the end of day -4, animals were provided with a microdialysis probe as described above and transmitters were switched on by magnetic field induction to allow continuous assessment of (baseline) body temperature and locomotor activity. Until the beginning of day 0, animals were fed *ad libitum* and water was continuously available. After 1.5 hour at the beginning of the dark phase at day 0, food was removed for the restricted experimental group and animals were placed on the scheduled feeding of 1.5 hours per day. Body weight of all animals (n=24) was measured just prior the dark phase. All animals were allowed to recover for at least one week before the connection to swivel and perfusion catheters. Overall, the food-restricted experimental group consisted of 15 animals, and the *ad libitum* fed experimental group of 9 animals.

Subsequently perfusion of the dialysis probes was started with Ringer solution at a constant flow of 2.0 µl/min with a high precision pump (Harvard Scientific, MA, USA), and animals were left undisturbed for at least 6 hours before collection of the samples started. Hourly samples were collected from the beginning of the light period at day 3 until the end of the dark phase on

day 4 (see figure 1). 40 µl of each individual dialysate sample was passed onto a vial containing 14 µl 0.1 N acetic acid. Samples were immediately stored at -80 °C until analysis by high performance liquid chromatography electrochemical detection (HPLC-ECD). All animals were sacrificed by decapitation at the end of the light phase of day 4. Trunk blood was collected into lithium-heparin (Sarstedt, Nümbrecht, Germany) containing tubes with 83 µmol EDTA and 1 mg aprotonin. Tubes were collected on ice until centrifugation (20 min. at 3000 rpm 4°C), and subsequently plasma was stored at -20°C until assay. Brains were quickly removed, frozen and stored at -80°C. Interscapular brown adipose tissue (BAT), white adipose tissue (WAT) surrounding the oviducts (PGAT), retroperitoneal WAT (RPAT), subcutaneous WAT (SCAT), and adrenals were collected, weighed, frozen and stored at -80°C.

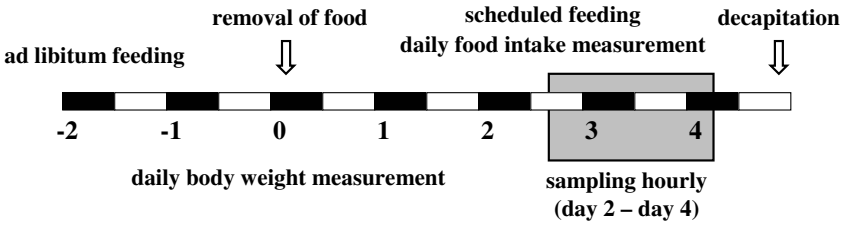

Figure 1.

Scheme of experimental set-up. Filled bars indicate the dark phase, open bars the light phase. At day 0, food was removed for the food-restricted experimental group and placed on scheduled feeding (1.5 hour food access at the beginning of the dark phase). Food intake and body weight were measured daily. As indicated by the gray area, hourly microdialysis sampling was performed from the middle of light phase at day 2 until the middle of dark phase at day 4.

Measurements in plasma samples

Plasma levels of leptin and insulin were measured by radioimmuno assay (RIA). From the samples, 2x 50 µl was taken for measurement in duplo. Plasma leptin and insulin were measured using commercially available RIA kits, according to the manufacturer's protocol (Linco Research, St. Charles Missouri, USA).

HPLC-ECD determination of monoamines and their metabolites in microdialysate

Microdialysis samples were stored at -80 °C until analysis. Neurotransmitters, dopamine (DA) and serotonin (5-HT) and their metabolites, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic

acid (5-HIAA) respectively were detected simultaneously by HPLC with electrochemical detection using an Alexys 100 LC-EC system (Antec Leyden, The Netherlands). The system consisted of two pumps, one autosampler with a 10 port injection valve, two columns and two detector cells. Column 1 (ALF 105 C18 1 x 50 x mm, 3 µm particle size) in combination with detector cell 1, separated and detected DA and 5-HT. Column 2 (ALF 115 C18 1 x 150 mm, 3 µm particle size) in combination with detector cell 2, separated and detected the metabolites. The mobile phase for column 1 consisted of 50 mM phosphoric acid, 8 mM KCL, 0.1 mM EDTA (pH 6.0), 12 % Methanol and 500mg/L OSA. The mobile phase for column 2 consisted of 50 mM phosphoric acid, 50 mM citric acid, 8 mM KCl, 0.1 mM EDTA (pH 3.2), 10 % methanol and 500 mg/L OSA. Both mobile phases were pumped at 50 µl/min. Samples were kept at 8 °C during analysis. From each microdialysis sample 5 µl was injected simultaneously onto each column. The neurotransmitters were detected electrochemically using µVT-03 flow cells (Antec Leyden, The Netherlands) with glassy carbon working electrodes. Potential settings were for DA and 5-HT +0.30 V versus Ag/AgCl and for metabolites +0.59 V versus Ag/AgCl. The columns and detector cells were kept at 35 °C in a column oven. The chromatogram was recorded and analyzed using the Alexys data system (Antec Leyden, The Netherlands). The limit of detection was 0.03 nM (S/N ratio 3:1).

Histology

Frozen brains were sectioned (coronal, 20µm) through the NAc and stained with cresyl violet in order to check the accuracy of the probe placements. Probe placement was defined appropriate when positioned in the NAc, based on Paxinos brain atlas (Paxinos and Watson, 1998). Rats with incorrect probe placements were removed from further analysis.

Statistical analysis

All data are presented as mean ± standard error. Data were analyzed using SPSS 11.5 for Windows and were controlled for normality and homogeneity. For all behavioral measurements (*ad lib* fed and food-restricted rats), baseline levels were not significantly different between all experimental groups. The significance of the changes as a consequence of scheduled feeding was evaluated by ANOVA with repeated measures (i.e. condition (2 levels; ad lib fed and food-restricted)) using Mauchly's Test of Sphericity. When appropriate, within group differences were analyzed using the Student's t-test. Statistical significance was set at $p<0.05$.

A total of twenty-four animals were used in this study. Data of animals

(n=9) had to be discarded because of incorrect probe placements, a blockade of the microdialysis probe or an incomplete data set.

Results

Physiological parameters in ad lib fed and food-restricted running rats
Following introduction to scheduled feeding of 1.5 hours at the beginning of the dark phase at day 0, relative body weight was significantly decreased over time in comparison with *ad lib* fed rats (day $F(3,36)=84.7$, $p<0.0001$; day × condition $F(3,36)=138.3$, $p<0.0001$). Daily food intake of food-restricted rats was significantly decreased from day 0 until the end of the experiment (day $F(4,28)=3.3$, $p=0.026$) (see figure 2). As shown in table 1, cumulative food intake of food-restricted rats was significantly less compared with *ad lib* fed rats (food-restricted 27.3 ± 2.4 %; $p<0.01$). Similar to the rapid decrease in body weight and food intake, food-restricted animals also significantly decreased their body temperature. This probably reflects starvation-induced hypothermia, a biological mechanism to reduce energy expenditure. In addition, no difference in cumulative water intake between *ad lib* fed and food-restricted rats was observed. These results confirm earlier studies using this ABA model.

Activity measurements in ad lib fed and food-restricted running rats
Baseline running wheel activity (RWA) was not significantly different on day -1 in *ad lib* fed and food-restricted rats. Total daily RWA was significantly increased over time (day $F(4,52)=4.5$, $p=0.003$; day × treatment $F(4,52)=4.6$, $p=0.003$) in food-restricted rats. This significant increase in total daily RWA was primarily due to an increase in cumulative RWA during the light phase (day 0-4; $p<0.0001$), whereas cumulative RWA during the dark phase did not reach any significance (day 0-4; n.s.). When rats are fed for only 1.5 hour at the beginning of the dark phase, animals become more active during hours preceding food access. Based on a review by Mistlberger (Mistlberger, 1994), food-anticipatory activity (FAA) was defined as the RWA four hours prior to food access on days of scheduled food access. Since, under restriction conditions the food was given during the first 1.5 hours of the dark phase, the RWA in the last four hours of the light phase was taken into consideration for the evaluation of FAA. In figure 2, total FAA of each individual day is presented. Significant increases in FAA were found in food-restricted rats from day 0 till day 3. At the end of day 4 the rats were sacrificed during the period of anticipation, therefore the increased FAA on the last day did not reach significance.

General locomotor activity (LMA), as measured by telemetric devices, was also significantly increased by restricted conditions. Cumulative LMA (day 0-5) was significantly increased in food-restricted rats as compared to ad lib fed rats (see Table 1). Like RWA, cumulative LMA during the light phase is significantly increased in food-restricted rats as compared to *ad lib* fed rats (day 0-4; $p<0.001$).

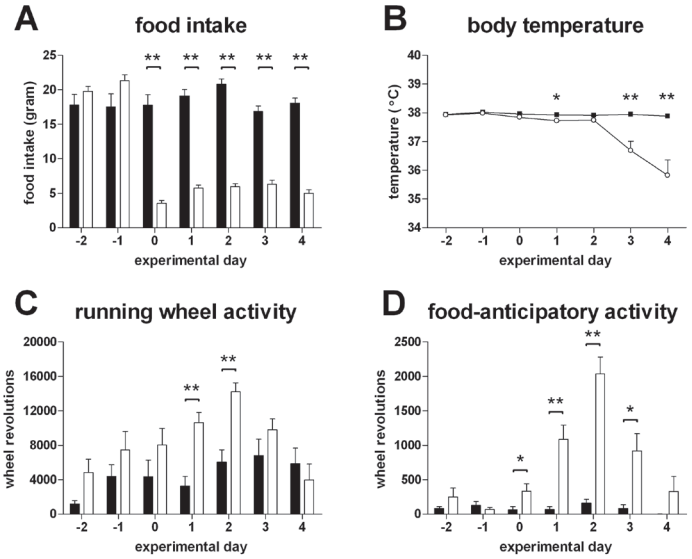

Figure 2.

Behavioral parameters; (A) food intake, (B) body temperature, (C) total running wheel activity per day, and (D) food-anticipatory activity of *ad lib* fed (black bar, black square, n=7) and food-restricted (open bar, open circle, n=8) rats. From day 0, food-restricted rats were placed on scheduled feeding. Microdialysis was performed from the middle of light phase at day 2 until the middle of dark phase at day 4. Asterisks indicate significant differences, *$p<0.05$, **$p<0.01$, Student's t-test.

Effects of feeding schedule on extracellular DA, DOPAC and HVA levels in the NAc

The effects of scheduled feeding on extracellular concentration of DA and its metabolites DOPAC and HVA in the NAc are shown in figure 3. In food-restricted rats the DA levels reached a peak 60 minutes after the initiation of food access at the beginning of the dark phase on day 3. On experimental day 4, a similar peak was seen after the initiation of food access. No differences were

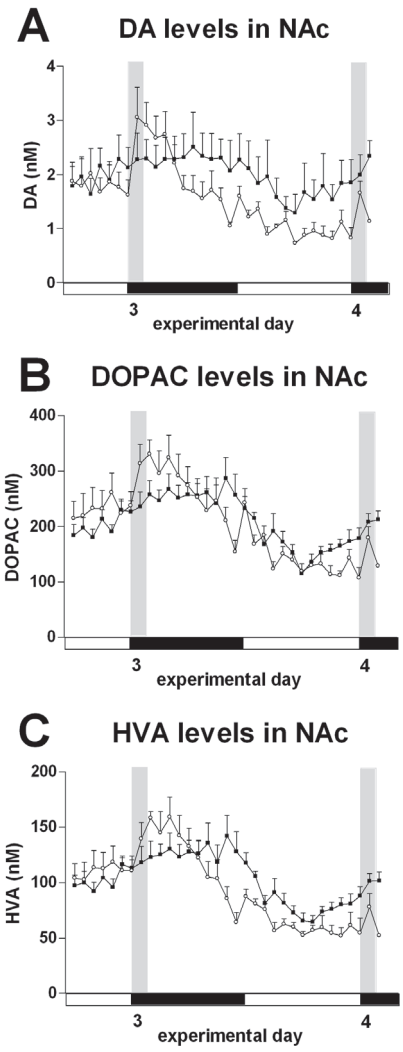

Figure 3.

Pofile of microdialysis samples of the neurotransmitter dopamine (DA) and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovannilic acid (HVA) measured in the nucleus accumbens (NAc), and detected by HPLC with electrochemical detection. Filled black bars indicate the dark phase, open bars the light phase. Hourly microdialysis sampling was performed from the middle of light phase at day 2 until the middle of dark phase at day 4. As indicated by the gray area, food-restricted rats received food access for 1.5 hour at the beginning of the dark phase. Black squares represent *ad lib* fed rats (n=7), open circles represent food-restricted rats (n=8).

found in the average DA, DOPAC and HVA levels in food-restricted rats as percentage of *ad lib* fed rats during the dark phase. However, extracellular levels of DA, DOPAC and HVA levels were decreased during the light phase on day 3 (DA 58.35 ± 3.23%, $p<0.0001$; DOPAC 82.10 ± 4.09%, $p=0.007$; HVA 75.57 ± 3.23%, $p=0.0001$).

During food-anticipatory activity (FAA) the average DA release in food-restricted rats as percentage of average DA release in *ad lib* fed rats is significantly reduced (food-restricted; 81.58 ± 2.50%, $p=0.007$) whereas no differences were found for the release of its metabolites DOPAC and HVA. When comparing the release of DA, DOPAC and HVA during FAA at the end of the light phase on day 2 with the 4 hours at the beginning of the dark phase on day 3, thus including 1.5 hour of food access, significant increases over time were determined for all three measurements ($F_{DA}(7,63)=6.7$, $p<0.001$; $F_{DOPAC}(7,49)=9.0$, $p<0.001$; $F_{HVA}(7,49)=8.1$, $p<0.001$, respectively). There were also significant effects of treatment (ad lib fed vs. food restriction) ($F_{DA}(7,63)=4.8$, $p<0.001$; $F_{DOPAC}(7,49)=2.9$, $p=0.013$; $F_{HVA}(7,49)=3.2$, $p=0.007$, respectively).

Effects of feeding schedule on extracellular 5-HT and 5-HIAA levels in the NAc

As depicted in figure 4, the effects of scheduled feeding on extracellular concentration of 5-HT and its metabolite 5-HIAA in the NAc are shown. In *ad lib* fed rats, 24-hour rhythms of extracellular 5-HT and 5-HIAA were observed with high levels during the dark phase whereas in food-restricted rats this 24-hour rhythm was less pronounced.

A significant decline in average 5-HT and 5-HIAA levels was observed during the dark phase of day 3 in ABA rats (food-restricted; 5-HT 46.44 ± 3.36%, $p<0.0001$; 5-HIAA 66.56 ± 3.07%, $p<0.0001$). A similar decrease in 5-HT and 5-HIAA release was observed during the light phase of day 3. During FAA, levels of extracellular 5-HT and 5-HIAA in food-restricted rats were significantly decreased as compared to *ad lib* fed rats (food-restricted; 5-HT 61.98 ± 2.89%, $p=0.01$; 5-HIAA 75.50 ± 1.43%, $p=0.003$).

Body composition in ad lib fed and food-restricted running rats

At the end of the light phase of day 4, all rats were sacrificed by decapitation and fat pads were removed. Visceral fat pads were still present in *ad lib* fed rats, while almost all fat pads had disappeared in food-restricted rats. As shown in Table 1, a significantly smaller amount of total white adipose tissue (WAT) was present in food-restricted rats as compared to *ad lib* fed rats (food-restricted; 18.12 ± 2.17%). In addition, plasma leptin levels were significantly decreased when rats were exposed to the restricted feeding schedule ($p<0.001$) compared

to *ad lib* fed rats.

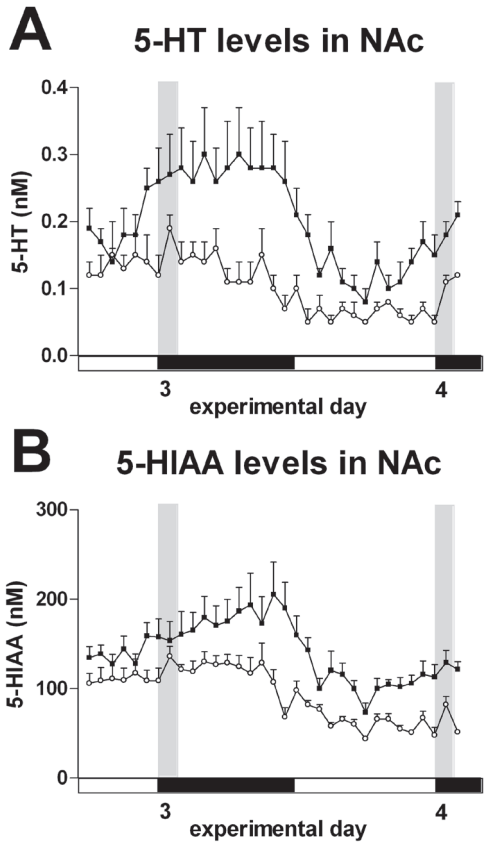

Figure 4.

Profile of microdialysis samples of the neurotransmitter serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) measured in the nucleus accumbens (NAc), and detected by HPLC with electrochemical detection. Filled black bars indicate the dark phase, open bars the light phase. Hourly microdialysis sampling was performed from the middle of light phase at day 2 until the middle of dark phase at day 4. As indicated by the gray area, rats received food access for 1.5 hour at the beginning of the dark phase. Black squares represent *ad lib* fed rats (n=7), open circles represent food-restricted rats (n=8).

Table 1. Body composition and behavioral parameters in ad lib fed and food-restricted rats.

	ad lib fed	restricted
Final body weight (% of initial weight)	102.63 ± 0.88	78.93 ± 0.76 **
Cum. food intake (day 0-4, g)	87.86 ± 1.49	24.00 ± 2.14 **
Cum. water intake (day 0-4, ml)	132.9 ± 17.8	151.2 ± 17.4
Total WAT (% of body weight, mg)	1.38 ± 0.16	0.25 ± 0.03 **
Total abdominal WAT (% of body weight, mg)	0.99 ± 0.12	0.25 ± 0.03 **
Leptin (ng/ml)	0.85 ± 0.07	0.26 ± 0.03 **
Insulin (ng/ml)	1.89 ± 0.32	0.77 ± 0.44 #
Total RWA (revolutions)	26308 ± 6666	46591 ± 3772 *
Total LMA (counts)	6217.5 ± 731.1	9353.2 ± 1067.9 *
FAA (% of total RWA, revolutions)	1.43 ± 0.28	14.17 ± 2.50 **

Final body weight (% of initial body weight at day 0), cumulative food intake, cumulative water intake, % total WAT, and % total abdominal WAT, plasma leptin and insulin levels, total running wheel activity (RWA), total locomotor activity (LMA), and food-anticipatory activity (FAA; % of total RWA, day 0 – day 4) in ad lib fed and food-restricted rats. Significant differences between ad lib fed and food-restricted rats are indicated with an asterisk, *$p<0.05$, **$p<0.01$. A trend towards significance is indicated with #.

Discussion

In this study, the release of extracellular DA, 5-HT and their metabolites in the NAc was determined upon exposure to ABA. Food intake was significantly decreased in ABA rats which resulted in a rapid decline of body weight as well as in the percentage of total (abdominal) fat mass. Furthermore, significant increases in activity levels were found in ABA rats as compared to *ad lib* fed running rats. In ABA rats, DA release was increased during food intake, but not during food-anticipatory activity (the period immediately preceding food delivery). Moreover, levels of DA and its metabolites DOPAC and HVA were significantly decreased during the light phase on day 3. 5-HT release was decreased and serotonergic circadian activity was blunted in ABA rats compared to *ad lib* fed running rats. Levels of 5-HT and 5-HIAA were both significantly decreased in ABA rats during the dark and light phase of day 3.

Food intake has been associated with dopamine function (Barry and Klawans, 1976; Leibowitz and Brown, 1980). This is in agreement with animal experiments. Dopamine-deficient mice (DD) lacking the dopamine synthesizing enzyme tyrosine hydroxylase (TH) in dopaminergic neurons become hypophagic, and will die of starvation (Zhou and Palmiter, 1995). Already in 1988, Hernandez and Hoebel showed an increase in DA turnover in microdialysis samples collected from the NAc of rats bar pressing when food was available (Hernandez and Hoebel, 1988). Here we confirmed that the release of DA, DOPAC, and HVA in the NAc were increased when ABA rats consumed food. Thus, in these studies dopamine release was only observed in conditions where food was delivered, not when food was expected.

Animal studies have shown that 5-HT has a suppressive effect on food intake (Simansky, 1996). Rats reduce food intake when treated with selective serotonergic agonists (Clifton et al., 2000; Lee et al., 2002). Likewise, clinical studies reveal association between food restriction and a decrease in 5-HT metabolism, especially in individuals with eating disorders (Anderson et al., 1990). In our study, we confirmed a decrease in release of 5-HT in the NAc. ABA rats maintained low levels of 5-HT and 5-HIAA and circadian activity was blunted. This is in agreement with the fact that underweight anorectic patients have been reported to have low basal levels of 5-HIAA in cerebrospinal fluid (Kaye et al., 1988a). These low levels may be caused by malnutrition resulting in low levels of protein intake containing the essential amino acid tryptophan, the precursor of 5-HT. Kaye and researchers have hypothesized that low levels of 5-HT, due to insufficient tryptophan intake may be causal to depressive symptoms in AN patients (Weltzin et al., 1995; Kaye et al., 2003). We cannot exclude that low

levels of 5-HT in other brain regions besides the NAc play a role in anorectic behaviors. However, treatment with chronic d-fenfluramine, a 5-HT releaser and 5-HT reuptake inhibitor, failed to influence the ABA model (Hillebrand et al., 2006a). ABA rats treated with fenfluramine displayed an accelerated weight loss since these rats reduced their food intake (Atchley and Eckel, 2005). These different results might be explained by variation in experimental set-up, such as time and duration of treatment, concentration of (d-)fenfluramine, duration of food access, adaptation to the running wheel, and initial body weight).

ABA rats increased activity levels following the start of scheduled feeding. Total daily RWA and LMA were increased as compared to *ad lib* fed running rats. While increasing activity levels in ABA rats, DA release in the NAc was still somewhat lower than in *ad lib* fed running rats. Thus, accumbal DA release is unlikely to contribute to food-anticipatory activity in this model. In ABA rats, total daily activity increased but also the distribution of activity changed. High levels of food-anticipatory activity (FAA) were displayed in the hours prior to food access. We have shown that DA levels rise when ABA rats had access to their food, but DA levels did not increase during the hours preceding food intake. In contrast, several studies carried out with palatable food do show involvement of dopamine in anticipatory activity (Barbano and Cador, 2006; McCullough and Salamone, 1992; Mistlberger and Mumby, 1992). Note however that in most of these studies, animals were trained on these food schedules for extended periods of time. Furthermore, as mentioned in the introduction, Mendoza and researchers found high levels of c-*fos* expression in the NAc prior to mealtime of a palatable food in food-entrained rats (Mendoza et al., 2005). These conflicting data on the involvement of the NAc during food-anticipatory activity might be explained by the differences in physiological state and/or food palatability (Barbano and Cador, 2005) and again the extended period of time to induce food-anticipatory activity in other studies (Mendoza et al., 2005).

The fact that dopamine release is not associated with food-anticipatory activity when animals are placed on the scheduled chow diet is congruent with the result that ABA rats treated with a non-selective dopaminergic antagonist (Verhagen et al., 2008, chapter 3). Although 24-h activity levels were reduced, ABA rats treated with the dopaminergic antagonist still exhibit food-anticipatory activity. Similarly to DA, 5-HT does not seem to play an important role in the initiation of food-anticipatory activity.

Taken together, the present study showed that DA is released in the NAc during feeding behavior in freely moving rats exposed to the ABA model. During starvation-induced hyperactivity, levels of 5-HT were low and circadian activity was blunted. The release of DA and 5-HT in the NAc do not seem to be

involved in the initiation of food-anticipatory behavior when rats are placed on a restricted feeding schedule with chow diet.

Acknowledgements

The authors thank Henk te Biesebeek of the Instrumental Department of the UMC Utrecht for his assistance in the preparation of the experimental set-up for intracerebral microdialysis. L.A.W. Verhagen was supported by NWO Grant No. 90339175, The Netherlands.

Chapter 5

CHAPTER 5

Lack of leptin signaling in the ventral tegmental area accelerates starvation-induced hyperactivity

Linda A.W. Verhagen, Mieneke C.M. Luijendijk, Marijke W.A. de Backer,
Max Koppers, Keith M. Garner, Ralph J. DiLeone, Roger A.H. Adan

Abstract

In anorexia nervosa, low leptin levels have been associated with hyperactivity. However, it is not known how leptin is associated with this hyperactive behavior. Using an animal model that mimics features of anorexia nervosa, including food-restriction induced hyperlocomotion, we demonstrate that local injections of leptin into the VTA suppress running wheel activity (RWA). Furthermore, attenuated leptin signaling achieved by leptin receptor knockdown in the VTA exaggerates this increased RWA. Central injections of leptin are ineffective in inhibiting RWA when leptin receptors are knocked down in the VTA.

The results support that declining levels of leptin, that accompany caloric restriction, result in increased locomotor activity because of decreased leptin signaling in the VTA. Since leptin has been shown to suppress neuronal firing of dopaminergic VTA neurons, we propose that increased activity of the mesolimbic system underlies starvation-induced hyperactivity.

Introduction

Between 31% and 80% of patients suffering from anorexia nervosa (AN) display abnormally high levels of physical activity and excessive exercise (Hebebrand et al., 2003). This excessive physical activity has been associated with low plasma leptin levels (Exner et al., 2000; Holtkamp et al., 2003b). AN patients rated their motor restlessness the highest when their leptin levels and body weight were the lowest (Exner et al., 2000). During severe AN, it has been shown that hyperactivity is negatively correlated with food intake and to contribute to body weight loss (Holtkamp et al., 2004b; Holtkamp et al., 2004a; Kaye et al., 1988b; Schwartz et al., 2000).

The hormone leptin, derived from adipose tissue and sensed by the brain, regulates energy balance and neuroendocrine function (Leinninger and Myers, Jr., 2008; Morton et al., 2006; Myers et al., 2008; Schwartz et al., 2000). In ad libitum fed rodents, leptin suppresses food intake and, by stimulating thermogenesis and locomotor activity, increases energy expenditure (Teske et al., 2008). Obese leptin-deficient ob/ob mice were characterized by having an increased food intake, reduced thermogenesis, and reduced locomotor activity, which were improved by replacement of leptin in these animals (Weigle et al., 1995; Levin et al., 1996; Halaas et al., 1995; Hwa et al., 1996; Pelleymounter et al., 1995). Recently, it was shown that daily leptin treatment intracerebroventricular (ICV) in *ad libitum* fed rats resulted in reduced food intake, increased body weight loss, and a significant increase in locomotor activity (Choi et al., 2008).

Most research has focused on leptin receptors within the hypothalamus, particularly in the arcuate nucleus (Schwartz et al., 2000). Recent data identified leptin receptors on dopaminergic neurons of the ventral tegmental area (VTA) in the mesolimbic midbrain, a brain area known to be involved in reward and locomotion (Figlewicz et al., 2003; Fulton et al., 2006; Hommel et al., 2006). DiLeone and researchers showed that leptin in dopaminergic VTA neurons inhibited neuronal firing and feeding behavior (Hommel et al., 2006). Moreover, lack of leptin signaling in the VTA stimulated locomotor activity when food was abundant. This stimulation of locomotor activity by knockdown of leptin receptors in the VTA contrasts with the stimulatory effect on locomotor activity by peripheral and central administered leptin (Choi et al., 2008). We therefore explored further the role of leptin in an animal model for AN.

Activity-based anorexia (ABA) is an animal model for AN, mimicking important characteristics of AN, in particular excessive exercise and reduced food consumption (Routtenberg and Kuznesof, 1967). In this model, rats are given free access to a running wheel and fed once per day for a limited period of time (1-2 hrs). Exposure to the ABA model leads to a chronic catabolic state caused by a reduced food intake and increased running wheel activity. The increase in running wheel activity coin-

cides with low plasma leptin levels (de Rijke et al., 2005b) as increased hyperactivity does in AN patients. As shown by Exner and researchers, continuously administered leptin through an osmotic minipump reduced hyperactivity in rats exposed to the starvation-induced hyperactivity model (Exner et al., 2000). An additional study using the ABA model showed that chronic ICV leptin treatment prevented ABA rats to develop starvation-induced hyperactivity while food intake was suppressed by leptin (Hillebrand et al., 2005b). Leptin, however, did not suppress hyperactivity in *ad libitum* fed running rats. Thus, the effect of leptin in suppressing hyperactivity is specific for animals displaying food-restriction induced hyperactivity.

The low leptin levels and associated hyperactivity seen in AN patients as well as the ABA rats (Hebebrand et al., 2003), together with the increased activity seen with reduced leptin signaling in the VTA under *ad libitum* feeding conditions (Hommel et al., 2006), let us to hypothesize that lack of leptin signaling in the VTA results in increased locomotor activity during negative energy balance. In this study, we determined the effect of leptin in the VTA during exposure to the ABA model. Furthermore, to elucidate whether the leptin receptors in the VTA were responsible for the suppression of hyperactivity by leptin, rats with local knockdown of leptin receptors in the VTA were generated using viral-mediated RNA interference and exposed to the ABA model.

Materials and methods

Animals

Female outbred Wistar WU rats (n=64) (Harlan, Horst, The Netherlands) weighing 155-165 gram (experiment 1 and 2) and 190-200 gram (experiment 3) upon arrival were individually housed in an ambient temperature- and humidity-controlled room (21°C ± 2°C) under a 12-hour dark-light cycle, lights on at 2 am. All described procedures were approved by the ethical committee on the use and care of animals of the University of Utrecht, The Netherlands. For ethical reasons, it was decided that rats were to be removed from the experiment when their body temperature was lower than 33°C before feeding, or when rats lost more than 25% of their initial body weight.

Experimental set-up (experiment 1 and 2)

One week after arrival, all rats received transmitters (TA10TA-F40 Data Sciences International, St. Paul, Minnesota) in the abdominal cavity under fentanyl/fluanisone (0.1 ml/100 g body weight, IM; Hypnorm, Janssen Pharmaceutica, Beerse, Belgium) and midazolam (0.05 ml/100 gram body weight, IP; Dormicum, Hoffman-LaRoche, Mijdrecht, The Netherlands) anesthesia. After surgery, rats were treated with carpro-

fen (0.01 ml/100 gram body weight, s.c.; Rimadyl, Pfizer Animal Health, Capelle a/d Ijssel, The Netherlands) and saline (3 ml, s.c.) and allowed to recover for two weeks. After recovery, animals were individually housed in cages with running wheels for a training period of ten days (from day -10 to day 0). Running wheel activity (RWA) was continuously registered using a Cage Registration Program (Department Biomedical Engineering, UMC Utrecht, The Netherlands). At the end of day -4, transmitters were switched on by magnetic field induction to allow continuous assessment of (baseline) body temperature and locomotor activity. Until the beginning of day 0, animals were fed *ad libitum* and water was continuously available. Animals were divided into experimental groups, matched for body weight and baseline RWA. Baseline RWA was determined as average RWA during four days prior to the start of infusion (day -4 to day -1). Body weight of all animals was measured just prior to food access at the beginning of the dark phase (see figure 1). During the restricted feeding period, animals received 1.5 hour of food access.

Experiment 1

As previously described, it was shown that chronic leptin treatment reduced hyperactivity and food intake in ABA rats (Hillebrand et al., 2005b). In order to investigate whether a single leptin injection could suppress activity behavior and food intake, we administered leptin acutely in the brain via an intracerebroventricular (ICV) cannula (Plastics One Inc., Roanoke). At day -10 just before the start of training period in running wheel cage, animals (n=16) were anesthetized (as indicated above) and provided with an ICV cannula placed into the lateral ventricle; coordinates were 1.0 mm posterior from bregma, 1.0 mm lateral from midline, 4.5 mm below the surface of the brain, and finally fixed in place with two anchor screws and dental cement. At the end of day 4 of the experiment, just before animals normally display their daily hyperactivity, leptin (concentration 1 µg/3 µl injection volume, n=8) or control (saline, 3µl injection volume, n=8) was injected. Murine recombinant leptin was a kind gift from Dr. A.F. Parlow as part of the National Hormone and Peptide Program of the National Institute of Diabetes and Digestive and Kidney Diseases. At the end of day 5, animals were sacrificed.

Experiment 2

In the first experiment we showed that an acute leptin injection suppressed hyperactivity, but had no effect on food intake. In order to investigate whether this effect of leptin was mediated via the VTA, we administered leptin bilaterally in the VTA. At day -10 just before the start of training period in running wheel cage, rats (n=32) were anesthetized (as indicated above) and provided with two guide cannulae which were implanted just above the VTA; coordinates were 4.3 mm posterior from bregma, 2.6

mm lateral from midline, and 8.2 mm ventral below the surface of the brain; cannulae were placed under a 10° angle and toothbar set to obtain flat skull position. Finally, cannulae were fixed in place with three anchor screws and dental cement. At the end of day 4 of the experiment, just before animals displayed their daily hyperactivity, leptin (concentration 0.1, 0.25, 1.0 μg/μl, 1 μl injection volume, n=8 per experimental group) or control (saline, n=8) was injected with the use of an internal cannula connected to a Hamilton syringe. At the end of day 5, animals were sacrificed.

Experiment 3

After one week acclimatization to the animal facility, anesthetized rats (n=16) received transmitters in their abdominal cavity (as indicated above) and were subsequently mounted into a stereotaxic apparatus. Hamilton syringe needles were bilaterally targeted to the VTA and a total of purified rAAV expression virus (1×10^9 genomic copies/μl), containing either a short hairpin (sh)RNA targeting the leptin receptor mRNA (=$LEPR^{VTA}$, n=8) or control (=$CTRL^{VTA}$, n=8) , was delivered over a period of five minutes (0.2 μl/min, total volume 1μl), after which the needles were kept in place for ten minutes before removal. Subsequently, rats were provided with an ICV cannula placed into the lateral ventricle (as described for experiment 1). Directly after the injection and ICV cannula placement, transmitters were switched on to allow continuous assessment of body temperature and locomotor activity. Until three weeks after viral injections (postinjection day 0 – day 21), body weight, food intake, body temperature and locomotor activity were recorded. Body weight gain and food intake were measured daily. After 3 weeks, rats were individually housed in cages with running wheels for a training period of ten days (from ABA day -10 till ABA day 0) after which the ABA procedure was initiated. On day 0, animals were placed on the restricted feeding schedule (= ABA model) as described earlier for experiment 1 and 2 (see also Figure 1.).

Collection of tissues

At the end of each experiment, animals were sacrificed by decapitation. Interscapular brown adipose tissue (BAT), white adipose tissue surrounding the oviducts (PGAT), retroperitoneal white adipose tissue (RPAT), subcutaneous white adipose tissue (SCAT), and adrenals were collected, weighed, frozen and stored at -80˚C. Brains were quickly removed, frozen and stored at -80˚C.

Design short-hairpin RNA, viral production and purification

HEK293T cells were cultured in fifteen 150 × 150 mm cell culture dishes. The cells were then transfected with pAAV-shLEPR or pAAV-shCTRL (described in Hommel J.D. et al. neuron 2006)) and the helper plasmid pDp1 (Plasmid factory, Germany) at molar

ratio of 1:1 using polyethylenimine (PEI). Sixty hours post-transfected cells were collected, pelleted and resuspended in an ice-cold lysis solution (150mM NaCl, 50 mM Tris, pH 8.4) and stored at -20°C. To lyse the cells, two additional freeze-thaw cycles were performed between dry ice-ethanol (100%) and a 37°C water bath. Subsequently, Benzonase (Sigma, cat# E1014-25KU, 50 units/ml, final), a DNAse, was added and the mixture was incubated at 37°C for 30 minutes. The AAV vectors were then centrifuged at 4000x g for 20 minutes at room temperature and the pellet was discarded. The supernatant was then loaded into an Optiseal tube (Beckman, CA, USA) containing a 15%, 25%, 40%, and 60% iodixanol (optiprep, Lucon bioproducts, cat# 7011804) step gradient. The gradient was centrifuged at 70,000 rpm for 1 hour at 20°C and the 40% fraction was collected. This 40% fraction was added up to 20 ml with Buffer A (20mM Tris, 15mM NaCl, pH 8.5) and was used for anion exchange chromatography with 5 ml HiTrapQ columns (GE Healthcare, UK, Cat# 17-1154-01). A gradient with buffer A and buffer B (20mM Tris, 500mM NaCl, pH 8.5) was created to elute the virus from the column and 2 ml fractions were collected and stored at 4°C. These fractions were screened by PCR and the positive fractions were pooled and transferred to a Centricon Plus-20 Biomax-100 concentrator column (Millipore, MA, USA, Cat# UFC2BHK08 100K) to concentrate the virus and exchange the buffer to phosphate buffered saline. The purified virus was then stored in aliquots at -80°C. Before use, AAV vector titers (genome copies/ml) were determined by real-time quantitative PCR in a LightCycler (Roche) using primers for GFP (forward primer; CTGACCCTGAAGTTCATCTGCAC CAC; reverse primer: TCCAGCAGGACCATGT GATC; PCR product 542 bp).

Localization of injections

Series of 16 μm coronal sections of the brain were sliced using a cryostat (Leica, Rijswijk, The Netherlands), thaw-mounted onto RNAse free Superfrost slides (Menzel, Germany) and stored at -80°C until processing.

Localization of leptin injection, ICV or VTA

For the localization of ICV leptin injections, brain sections (16 μm) were stained with cresyl violet. To visualize the injections in the VTA, brain slices were stained for the rate-limiting enzyme of dopamine synthesis, Tyrosine Hydroxylase (TH), using immunohistochemistry. Sections were fixed in 4% paraformaldehyde (PFA) in 0.01 M phosphate-buffered saline (PBS) for 20 min. Slices were washed with tris-buffered saline (TBS), incubated with 0.3% H2O2 in TBS to reduce endogenous peroxidase activity, washed in TBS blocked with 4% fetal calf serum in TBS, washed again, and incubated overnight at room temperature with polyclonal rabbit anti-TH (1:1000; Pelfreeze) in 0.5% Triton in TBS. The next day, sections were washed and incubated with biotinylated goat anti-rabbit immunoglobulin (1:1000; 1 hour), and washed in

TBS. The slides were then stained with 3,3-diamino-benzidine (DAB) and washed with demineralized water. Finally, slides were dehydrated in ethanol, cleared in xylene and mounted using Entellan. Results from rats with incorrect injections were excluded from further data analysis.

Localization of AAV injection by in situ hybridization and immunohistochemistry
In brain sections, viral infection was localized by in situ hybridization (ISH) with a dioxigenin (DIG)-labeled probe against the EGFP sequence (fragment of 833 bp). Sections were fixed in 4% PFA in 0.01 M PBS for 20 min., washed in PBS, pretreated with 0.25% acetic anhydride in 0.1 M triethanolamine, and washed again in PBS. DIG-labeled RNA probes were prepared and sections were hybridized overnight at 72°C in buffer containing 50% deionized formamide, 2× standard saline citrate (SSC), 10% dextran sulphate, 1× Denhardt's solution, 5mM EDTA and 10mM phosphate buffer, after 5 minutes heating at 80°C. After hybridization, section were rinsed in 2× SSC (short, 72°C) and 0.2× SSC (2 hrs, 72°C). DIG was detected with an alkaline phosphatase-labeled antibody (Roche, Germany) using NBT/BCIP as a substrate. Finally, slides were dehydrated in ethanol, cleared in xylene and mounted using Entellan. To visualize the VTA neurons, adjacent brain slices were stained for TH using immunohistochemistry (as described above). Results from rats with incorrect injections were excluded from further data analysis.

Statistical analysis
All data are presented as mean ± standard error. Data were analyzed using SPSS 11.5 for Windows and were controlled for normality and homogeneity. For all behavioral measurements, baseline levels were not significantly different between all experimental groups. In experiment 1 and 2, measurements of running wheel activity and food intake are presented as percentage of the day before. Differences were analyzed using the Student's t-test. Statistical significance was set at $p<0.05$.

Results

Verification of leptin and AAV injections
Correct placement of cannulas for injections with leptin, ICV or VTA, were determined by cresyl violet staining. Injections with pAAV-shLEPR or pAAV-shCTRL were verified by in situ hybridization and immunohistochemistry (see also Figure 2.). Incorrect injections were excluded from further analysis. In total, two rats from ICV injections, eight rats from VTA injections, and one rat from AAV injections were not included in data analysis.

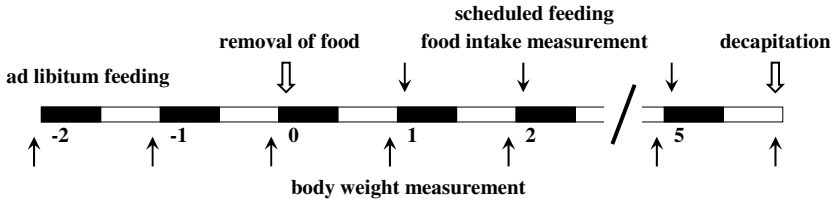

Figure 1.

Scheme of experimental set-up. Filled bars indicate the dark phase, open bars the light phase. At day 0, food was removed and rats were paced on scheduled feeding (1.5 hour food access at the beginning of the dark phase). Food intake and body weight were measured daily. Intracerebroventricular (ICV) injections or injections directly in the ventral tegmental area (VTA) were performed just prior to the dark phase of day 5.

Figure 2

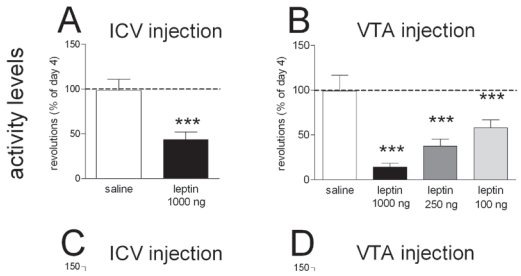

Figure 2.

Localization of bilateral AAV injections in the VTA by *in situ* hybridization with a dioxigenin-labeled probe against the EGFP sequence. Photomicrograph represents the ventral part of the midbrain in a coronal brain section. Regions were defined at -5.00 mm anterior-posterior from bregma using Paxinos and Watson brain atlas. Incorrect injections were excluded from further analysis. VTA, ventral tegemental area; SN, substantia nigra.

Effect of acute ICV leptin injections on activity and food intake in ABA rats

In ABA rats, direct leptin injection (1 μg) in the lateral ventricle prior to day 5 resulted in significantly decreased running wheel activity (RWA; 43.27 ± 9.34%) as compared to activity levels on day 4 (see Figure 3.A.). A similar significant reduction was observed for locomotor activity (LMA; 70.85 ± 10.34%). No differences in activity levels, RWA and LMA, were found in vehicle-injected ABA rats. While affecting activity levels,

acute ICV leptin injection had no effect on daily (restricted) food intake as shown in Figure 3.B. Thus, an acute ICV leptin injection suppressed LMA and RWA but not food intake in rats exposed to the ABA model.

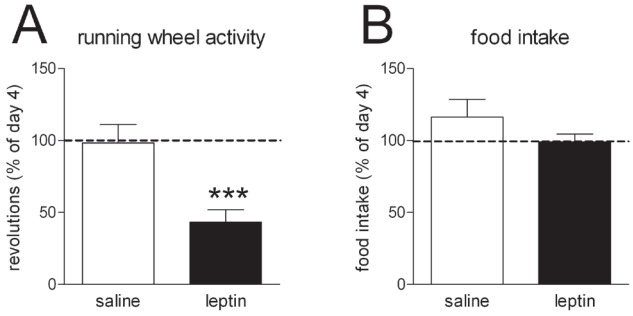

Figure 3.

Effect of acute ICV saline (n=6) or leptin (1000ng, n=8) injection just prior to day 5 on (A) running wheel activity and (B) food intake in ABA rats. Both running wheel activity and food intake are presented as mean percentage compared to day 4 as shown by dashed line. Significant differences are indicated by asterisks, ***p<0.001, Student's t-test.

Acute VTA leptin injections inhibit activity but not food intake in ABA rats

In order to investigate whether leptin inhibited hyperactive behavior via the VTA, we administered leptin bilaterally in the VTA in a dose-dependent manner. As depicted in Figure 4.A., acute leptin injection (100, 250, and 1000 ng) in the VTA significantly suppressed running wheel activity, respectively 14.02 ± 5.29%, 37.73 ± 7.76% and 58.29 ± 8.64%, whereas vehicle VTA injections did not affect activity levels. Consistent with experiment 1, food intake was not affected by acute leptin injections in the VTA (Figure 4.B.) compared with saline-injected ABA rats or to food intake of the day before as depicted in Figure 4.B. by the dashed line. Thus, increasing leptin signaling in the VTA suppressed hyperactive behavior in rats exposed to the ABA model.

Physiological parameters in rats with conditional knockdown during ad libitum feeding

To investigate whether lack of leptin signaling in the VTA contributes to the development of hyperactivity, we injected rAAV shLEPR to generate local knockdown of the leptin receptor in the VTA (=$LEPR^{VTA}$). Before rats were exposed to the ABA model, we analyzed body weight gain, daily food intake, and activity levels (LMA and RWA) in $LEPR^{VTA}$ rats and control rats during *ad libitum* feeding. During analysis, we made a distinction in periods when the rats were still in their home cage (*ad libitum* fed

sedentary; postinjection day 16 till day 20) and during periods in a cage with running wheel (*ad libitum* fed running; ABA day -8 till ABA day -4). The physiological parameters analyzed during *ad libitum* fed sedentary and *ad libitum* fed running phases in LEPR^{VTA} and CRTL^{VTA} rats are summarized in Table 1.

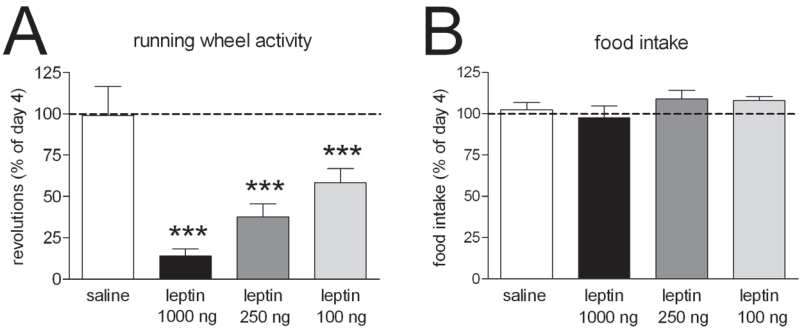

Figure 4.

Effects of acute saline (n=6) or leptin (100 ng (n=6), 250 ng (n=6), or 1000 ng (n=6)) administration in the VTA on (A) running wheel activity and (B) food intake. Both running wheel activity and food intake are presented as mean percentage compared to ABA day 4 as shown by dashed line. Significant differences are indicated by asterisks, ***p<0.001, Student's t-test.

Local knockdown LEPR^{VTA} during ad libitum fed sedentary phase

During postinjection day 16 till day 20, delta body weight was not significant different in *ad libitum* fed sedentary LEPR^{VTA} rats as compared to control rats. Furthermore, no effect on daily food intake was observed in *ad libitum* fed sedentary LEPR^{VTA} compared to control rats.

During the sedentary phase, locomotor activity levels were significantly higher in *ad libitum* fed LEPR^{VTA} rats (LEPR^{VTA}, 182.4 ± 15.0%, p<0.001). Relative to control rats, total LMA levels were already increased in *ad libitum* fed sedentary LEPR^{VTA} rats from postinjection day 14. These results confirms that knockdown of leptin receptors in the VTA increases locomotor activity in *ad libitum* fed rats, and is consistent with the onset of expression from AAV vectors injected in the brain (Reimsnider et al., 2007).

Local knockdown LEPR^{VTA} during ad libitum fed running phase

When rats were placed in running wheel cages, LEPR^{VTA} rats significantly reduced body weight gain (*ad libitum* fed running, p=0.01) in comparison to *ad libitum* fed running

CTRL^{VTA} rats (see Table 1). No effect on daily food intake was found in *ad libitum* fed running LEPR^{VTA} compared to control rats.

Total LMA significantly increased in both *ad libitum* fed running LEPR^{VTA} and CTRL^{VTA} rats as compared to *ad libitum* fed sedentary LEPR^{VTA} and CTRL^{VTA} rats (respectively) (Table 1.). Similarly to the sedentary phase, LMA increased more in *ad libitum* fed running LEPR^{VTA} rats than in control rats (LEPR^{VTA}, 147.2 ± 18.7%, p=0.02). Besides the increase of LMA in the running phase, *ad libitum* fed running LEPR^{VTA} rats also showed a four fold significant increase of RWA (LEPR^{VTA}, 393.6 ± 63.4%, p=0.003) relative to control rats (see Table 1.).

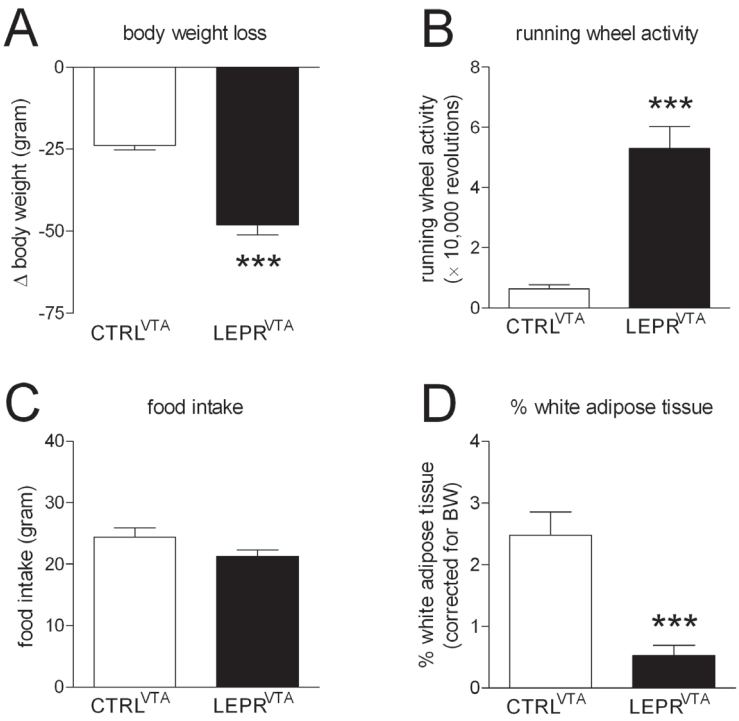

Figure 5.

Behavioral measurements in ABA rats with conditional knockdown of the leptin receptor in the VTA (LEPR^{VTA}, n=7) or control rats (CTRL^{VTA}, n=8). Body weight loss (A), total running wheel activity (B), total food intake (C), and percentage white adipose tissue corrected for body weight (D) in CTRL^{VTA} and LEPR^{VTA} rats. Significant differences are indicated by asterisks, ***p<0.001, Student's t-test.

Table 1. Effect of loss of leptin receptor in VTA neurons on behavioral parameters during different phases; ad libitum fed sedentary and ad libitum fed running.

	ad libitum fed sedentary (postinjection day 16 – 20)		*ad libitum* fed running (day -8 till -4 in running wheel cages)	
	$CTRL^{VTA}$	$LEPR^{VTA}$	$CTRL^{VTA}$	$LEPR^{VTA}$
Δ body weight (gram)	6.30 ± 2.26	3.50 ± 1.14	10.43 ± 1.74	4.25 ± 1.12 [b]
Daily food intake (gram)	18.10 ± 1.20	18.30 ± 0.63	18.90 ± 0.80	18.20 ± 0.86
Total locomotor activity (LMA)	1815.4 ± 180.8	3312.0 ± 272.1 [a]	3994.1 ± 516.3 [c]	6279.9 ± 727.3 [b,d]
Total running wheel activity (RWA)	no running wheel	no running wheel	3473 ± 1170	13670 ± 2203 [b]

Delta body weight (gram), daily food intake (gram), and total activity (*ad libitum* fed sedentary, total locomotor activity (LMA); *ad libitum* fed running, total locomotor and running wheel activity) in rats with conditional knockdown of the leptin receptor in the VTA ($LEPR^{VTA}$, n=7) or control rats ($CTRL^{VTA}$, n=8). Significant differences are shown as follows; $CTRL^{VTA}$ vs. $LEPR^{VTA}$ in (a) *ad libitum* fed sedentary rats and (b) *ad libitum* fed running rats; *ad libitum* fed sedentary vs. *ad libitum* fed running in (c) VTA^{CTRL} rats and (d) VTA^{LEPR} rats.

VTA leptin receptor knockdown in ABA rats

During the restricted feeding schedule, all animals decreased their body weight, similar to observations we published before (de Rijke et al., 2005b; Hillebrand et al., 2005b). At the end of day 3 just prior to decapitation, $LEPR^{VTA}$ lost approximately 20% of their body weight while control animals lost less than 10% of their initial body weight. In Figure 5.A., the relative values for body weight loss are depicted.

Following introduction of scheduled feeding, all rats developed hyperactivity. As shown in Figure 5.B., activity levels in $LEPR^{VTA}$ ABA rats increased drastically over time, resulting in more than six times higher RWA levels during exposure to the ABA model as compared to control-injected rats. Besides, total LMA levels were significantly increased two fold (total LMA; $LEPR^{VTA}$ 11980.8 ± 1306.9 counts, $CTRL^{VTA}$ 6028.7 ± 1100.3 counts, p=0.004). Thus, knockdown of leptin receptors in the VTA strongly increased hyperactivity in rats exposed to the ABA model.

During exposure to the ABA model, no significant changes in total food intake were found between $LEPR^{VTA}$ rats and $CTRL^{VTA}$ rats (see Figure 5.C.). After 4 days of food restriction (ABA day 0–3), rats were decapitated and fat pads were removed. Visceral fat pads were still present in $CTRL^{VTA}$ ABA rats, while almost all fat pads had disappeared in the $LEPR^{VTA}$ rats exposed to the ABA model. As shown in Figure 5.D., significantly less total white adipose tissue (WAT) corrected for body weight was present in $LEPR^{VTA}$ ABA rats as compared to controls ($LEPR^{VTA}$ 0.53 ± 0.16%; $CTRL^{VTA}$ 2.48 ± 0.38%).

Effect of acute ICV leptin injections in $LEPR^{VTA}$ and $CTRL^{VTA}$ rats during ad libitum feeding and the ABA model

In order to determine to what extent suppression of hyperactivity by leptin in rats exposed to the ABA model was dependent on leptin receptors in the VTA, leptin was injected during the *ad libitum* fed running period and the ABA model. Based on results from the first experiment where it was shown that leptin suppressed RWA during ABA, a total of 1 μg of leptin was injected ICV just prior to ABA day -3 and ABA day 4. As depicted in Figure 6.A., leptin did not have an effect on RWA in *ad libitum* fed running rats. Consistent with previous findings in the ABA model, leptin significantly decreased RWA in the control rats during ABA ($CTRL^{VTA}$, 47.04 ± 10.26%) whereas RWA of $LEPR^{VTA}$ rats was not affected by leptin ($LEPR^{VTA}$, 251.88 ± 96.25%) (see also Figure 6.B.). Hence, expression of leptin receptors in the VTA is essential to suppress hyperactivity in rats exposed to the ABA model.

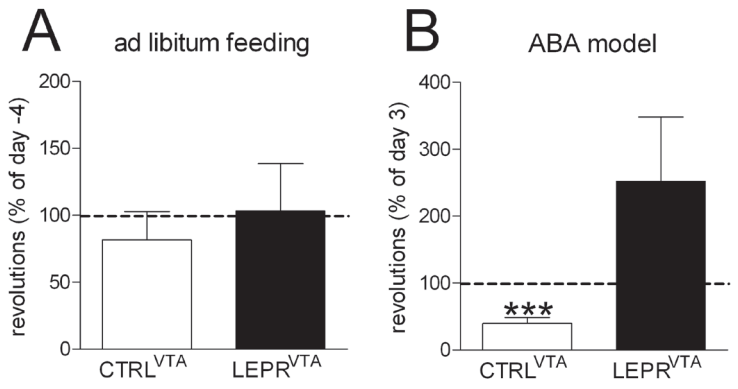

Figure 6.

Effect of acute ICV leptin injection (1000 ng) on running wheel activity during (A) *ad libitum* feeding and (B) ABA model in rats with conditional knockdown of the leptin receptor in the VTA ($LEPR^{VTA}$, n=7) or control rats ($CTRL^{VTA}$, n=8). Running wheel activity is presented as mean percentage compared to respectively ABA day -4 and ABA day 3, as shown by the dashed line. Significant differences are indicated by asterisks, ***p<0.001, Student's t-tes.

Discussion

In an animal model mimicking features of AN (such as food-restricted induced locomotor activity) it was shown before that leptin suppresses hyperactivity (Exner et al., 2000; Hillebrand et al., 2005b). We here demonstrate that acute ICV leptin injections and direct leptin injections in the VTA reduced activity levels in rats exposed to the ABA model, whereas no effect on food intake was observed. Furthermore, local knockdown of leptin receptors in the VTA resulted in increased activity levels in *ad libitum* fed sedentary and running rats, and contributed to remarkably higher levels of starvation-induced hyperactivity compared to controls when exposed to the ABA model. We here provide strong evidence that lack of leptin signaling in the VTA underlies increased locomotor activity during food restriction.

The suppression of RWA by acute leptin injections in the lateral ventricle of rats exposed to the ABA model appears reliable and confirms the reduction of hyperactivity by chronic leptin treatment as observed in the ABA model as well as the semi-starvation induced hyperactivity (SIH) model (Exner et al., 2000; Hillebrand et al., 2005b). In 2003, it has been reported by Figlewicz and researchers that leptin receptors are expressed on dopaminergic neurons in the VTA, a brain area known to be involved in feeding, reward and locomotion (Fulton et al., 2000; Fulton et al., 2006; Hommel

et al., 2006; Figlewicz et al., 2003). Hommel and researchers have demonstrated that leptin decreases neuronal activity in dopaminergic VTA neurons (Hommel et al., 2006). The dopaminergic VTA neurons are part of the mesolimbic dopamine system, which has been implicated in locomotor activity (Jerlhag et al., 2007). The present data support that during starvation lack of leptin signaling increases locomotor activity which has been associated with increased dopaminergic signaling (Carr, 2007).

Acute leptin injections in the VTA in ABA rats resulted in suppression of RWA while food intake was not affected by leptin in the VTA. Interestingly, it has been shown that leptin injections in the VTA of ad libitum fed rats did not affect general activity levels whereas food intake was significantly reduced (Hommel et al., 2006). We also observed that before food restriction, leptin was unable to suppress locomotor activity in ad libitum fed rats. These contrasting results of leptin on activity behavior and food intake might thus be explained by differences in energy status (normal body weight regulation versus starvation). Similarly, the sensitivity to rewards and drugs of abuse is dependent on energy status, with food restriction increasing its sensitivity (Carr, 2007; Fulton et al., 2000).

In *ad libitum* fed rats, direct leptin administration inhibits food intake via its receptors in the brain, in particular the hypothalamus (Elmquist et al., 1999; Schwartz et al., 2000) and VTA (Hommel et al., 2006). In our study, when rats were exposed to food restriction, central leptin injections as well as local VTA injections did not have an effect on food intake. Therefore, we suggest that lack of leptin signaling, due to severe food restriction, leads to a different mode of control in which exogenous leptin no longer suppresses food intake.

Similar to the study of Hommel and researchers, rats with conditional knockdown of leptin receptors in the VTA showed increased activity levels during *ad libitum* feeding while body weight gain remained unaffected (Hommel et al., 2006). On the contrary, Hommel and researchers showed an increase in food intake while in our study total food intake of *ad libitum* fed sedentary $LEPR^{VTA}$ rats was not significant different from control rats. The divergence of leptin receptor knockdown in the VTA on food intake might be explained by gender differences. Female rats are relatively more sensitive to leptin than male rats (Clegg et al., 2003). Whereas Hommel and researchers used male rats, female rats were used in our study. Another possible explanation for the lack of an effect on body weight may be due to the fact that we only measured body weight in *ad libitum* fed sedentary rats until postinjection day 21. Perhaps if we prolonged the sedentary phase, differences in body weight would have been observed in $LEPR^{VTA}$ rats as compared to control rats.

During exposure to the ABA model, all animals decreased their body weight. However, a more rapid body weight loss was observed for $LEPR^{VTA}$ ABA rats than

control ABA rats. The massive increase in activity levels in $LEPR^{VTA}$ ABA rats most likely underlies this difference in body weight loss.

Acute ICV leptin injection in ABA rats with conditional knockdown of leptin receptors in the VTA did not affect RWA, whereas RWA was suppressed in $CTRL^{VTA}$ ABA rats similar to the acute leptin injections in the VTA of ABA rats (Figure 3.). This confirms that leptin receptors in the VTA are essential for leptin to suppress hyperactivity in rats exposed to the ABA model. Moreover, activity levels of $LEPR^{VTA}$ ABA rats tended to increase after an acute ICV leptin injection. Most likely, some $LEPR^{VTA}$ ABA rats were still expanding their activity levels whereas other $LEPR^{VTA}$ ABA rats were already at their maximum levels of wheel revolutions per hour.

Previous studies have shown that leptin alters rewarding aspects of drug seeking and desensitizes brain reward circuitry following food restriction (Shalev et al., 2001; Fulton et al., 2000). This might suggest that running wheel (hyper)activity in the ABA model incorporates rewarding aspects, which can be modulated by leptin. Leptin may thus reduce the rewarding effect of running wheel activity.

Taken together, these results support that declining levels of leptin that accompany caloric restriction result in increased locomotor activity because of decreased leptin signaling in the VTA. Hyperactivity in AN patients is difficult to control and negatively impacts recovery. The results linking lack of leptin signaling in the VTA with increased locomotor activity in rodents provide a mechanism to explain how in anorexia nervosa low leptin levels are related to hyperactivity. Furthermore, it has been shown that leptin suppresses neuronal firing of dopaminergic VTA neurons. We therefore propose that increased activity of the mesolimbic system underlies starvation-induced hyperactivity. Interestingly, antipsychotics prescribed to AN patients have been shown to reduce hyperactivity (Hillebrand et al., 2005c; Pederson et al., 2003). Hence, we hypothesize that reduction of dopamine signaling underlies this effect of antipsychotics.

Acknowledgements

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Chapter 6

CHAPTER 6

Ghrelin antagonism reduces locomotor activity during ad lib feeding and food restriction

Linda A.W. Verhagen, Mieneke C.M. Luijendijk,
Jacquelien J.G. Hillebrand, Roger A.H. Adan

Abstract

Using an animal model that mimics features of anorexia nervosa, including food-restriction induced hyperlocomotion, we demonstrated that plasma ghrelin levels are highly associated with the expression of food anticipation measured by running wheel activity (RWA). Furthermore, we showed that central ghrelin receptor antagonism suppressed RWA, and that this was independent of the energy balance status. Since rats with high levels of activity prior to food access also have high plasma ghrelin levels and that antagonism of ghrelin suppresses RWA, we propose that ghrelin contributes to food-restriction induced hyperlocomotion. Central ghrelin receptor antagonist injections only reduced food intake in ad libitum fed running rats, whereas no effect was observed in ABA rats. We hypothesize that ghrelin antagonism may also reduce hyperactivity in patients suffering from anorexia nervosa.

Introduction

Since its discovery in 1999, the peptide ghrelin secreted from the stomach has emerged as an important signal for the brain to control energy balance (Kojima et al., 1999; Hosoda et al., 2002). Ghrelin receptors are highly expressed in the hypothalamus, in particular the arcuate nucleus (ARC) (Mondal et al., 2005). Caloric restriction increases ghrelin secretion, and subsequent activation of the ghrelin receptor in the ARC has been implicated in stimulation of food intake (Hosoda et al., 2002). Opposite to many endocrine signals, increased plasma ghrelin levels have been associated with the initiation of meals and levels decrease after meals (Cummings et al., 2001). In addition, acute central and peripheral ghrelin injections stimulate food intake in rats (Horvath et al., 2001; Naleid et al., 2005). Peripheral ghrelin injections induces appetite in healthy subjects (Wren et al., 2001). Ghrelin has also been implicated in the response to long term changes in body weight (Cummings, 2006). Taken together, there is strong evidence for ghrelin to be involved in initiating meals.

With regard to humans, plasma ghrelin levels have been found to be decreased in obesity and restored after weight loss (Hansen et al., 2002; Soriano-Guillen et al., 2004). On the contrary, during negative energy balance, plasma ghrelin levels are increased and decrease by weight gain (Soriano-Guillen et al., 2004; Otto et al., 2001; Janas-Kozik et al., 2007). In patients suffering from anorexia nervosa (AN), plasma ghrelin concentrations of underweight subjects are increased and tend to normalize with the recovery of body weight (Otto et al., 2005; Otto et al., 2001). It has been reported by Misra and colleagues that high plasma levels of ghrelin induced by fasting in humans are negatively associated with the percentage body fat and low levels of leptin and insulin levels, which might play an important role in the pathophysiology of AN (Misra et al., 2005).

Activity-based anorexia (ABA) is an animal model for AN, mimicking important characteristics of AN, in particular increased locomotion and reduced food consumption (Routtenberg and Kuznesof, 1967) and show similar endocrine abnormalities (de Rijke et al., 2005b). In this model, rats are given free access to a running wheel and fed once per day for a limited period of time (1-2 h). Exposure to the ABA model leads to a chronic catabolic state caused by a reduced food intake and increased running wheel activity. Increased activity, especially prior to food intake (when ghrelin levels are presumably high), is a hallmark feature of ABA and similar to hyperactivity observed in AN patients. Interestingly, peripheral and central ghrelin infusions in ad libitum fed rats stimulated locomotor activity (Jerlhag et al., 2006). Thus, increased ghrelin levels may underlie hyperactivity.

In the present study, we investigated whether changes in plasma ghrelin levels are associated with the development of hyperactivity and/or the reduced food intake

in rats exposed to the ABA model. Furthermore, we determined the direct effect of a ghrelin receptor antagonist on activity levels and food intake in running rats during ad libitum fed and food-restricted conditions.

Material and methods

Animals

Female outbred Wistar WU rats (n=77) (Harlan, Horst, The Netherlands) weighing 155-165 gram upon arrival were individually housed in a ambient temperature- and humidity-controlled room (21°C ± 2°C) under a 12-hour dark-light cycle, lights off at 2 pm. All described procedures were approved by the ethical committee on the use and care of animals of the University of Utrecht, The Netherlands. For ethical reasons, it was decided that rats were to be removed from the experiment when their body temperature was lower than 33°C before feeding, or when rats lost more than 25% of their initial body weight.

Experiment 1

In the first experiment, two experimental groups were used; 1) 1 h fed rats with access to running wheels (ABA, n=29), 2) pair-fed rats (pair-fed to the ABA rats, n=24) without access to running wheels. One week after arrival, ABA rats were individually housed in cages with running wheels for a training period of ten days (from day -10 to day 0). Running wheel activity (RWA) was continuously registered using a Cage Registration Program (Department Biomedical Engineering, UMC Utrecht, The Netherlands). Until the beginning of day 0, animals were fed *ad libitum* and water was continuously available. At the beginning of the dark phase at day 0, food was removed and animals were placed on the scheduled feeding of 1 hour per day (ABA) or on a pair-fed schedule (pair-fed sedentary). The experiment was staggered to allow pair-fed feeding: at dark onset pair-fed rats received the average amount of food eaten by the ABA rats the day before. Body weight of all animals was measured just prior the dark phase, and just before food access in both experimental groups. Every consecutive day of the ABA model (ABA day 0-5), 4-5 animals were sacrificed by decapitation at the end of the light phase (see also Figure 1). Trunk blood was collected into lithium-heparin (Sarstedt, Nümbrecht, Germany) containing tubes with 83 μmol EDTA and 1 mg aprotonin. Tubes were collected on ice until centrifugation (20 min. at 3000 rpm 4˚C), subsequently plasma was stored at -20˚C until assay. Brains were quickly removed, frozen and stored at -80˚C.

Experiment 2

One week after arrival, all rats (n=24) received transmitters (TA10TA-F40 Data Sciences International, St. Paul, Minnesota) in the abdominal cavity under fentanyl/fluanisone (0.1 ml/100 g body weight, IM; Hypnorm, Janssen Pharmaceutica, Beerse, Belgium) and midazolam (0.05 ml/100 gram body weight, IP; Dormicum, Hoffman-LaRoche, Mijdrecht, The Netherlands) anesthesia. After surgery, rats were treated with carprofen (0.01 ml/100 gram body weight, s.c.; Rimadyl, Pfizer Animal Health, Capelle a/d Ijssel, The Netherlands) and saline (3 ml, s.c.) and allowed to recover for two weeks. After recovery, animals were individually housed in cages with running wheels for a training period of ten days (from day -10 to day 0). RWA was continuously registered. In addition, transmitters were switched on by magnetic field induction to allow continuous assessment of (baseline) body temperature and locomotor activity. Until the beginning of day 0, animals were fed *ad libitum* and water was continuously available. Animals were divided into experimental groups, matched for body weight and baseline RWA. Baseline RWA was determined as average RWA during four days prior to the start of scheduled feeding (day -4 to day -1). Body weight of all animals was measured just prior food access at the beginning of the dark phase (see Figure 1). In order to investigate whether a single ghrelin receptor antagonist injection could suppress activity behavior and food intake, we administered ghrelin receptor antagonist acutely in the brain via an intracerebroventricular (ICV) cannula. At day -10 just before the start of training period in running wheel cages, animals were anesthetized (as indicated above) and provided with an ICV cannula placed into the lateral ventricle; coordinates were 1.0 mm posterior from bregma, 1.0 mm lateral from midline, 5 mm below the surface of the brain, and finally fixed in place with two small screws and dental cement. At the end of day -4 and day 4 of the experiment, just before animals normally display their daily hyperactivity, ghrelin receptor antagonist (concentration 4, 8 or 16 µg, 3 µl injection volume) or control (saline) was injected. The ghrelin receptor antagonist was kindly provided by Æterna Zentaris GmbH (ghrelin receptor ($GHS\text{-}R_{1A}$) antagonist, JMV2959, Frankfurt am Main, Germany). At the end of day 5, animals were sacrificed by decapitation as described in experiment 1.

Plasma analysis

Plasma levels of total ghrelin (Phoenix Pharmaceuticals, Belmont, CA, USA) were measured by commercially available radioimmuno assay (RIA) kits according to the manufacturer's protocol. From the plasma samples, 2× 50 µl was taken for measurement in duplo.

Statistical analysis

All data are expressed as mean ± standard error. In experiment 1, groups of rats were

decapitated at consecutive days. Therefore, average daily RWA and relative body weight values were based on varying number of individuals in the running group (varying from 29 till 4 rats) and pair-fed group (varying from 24 till 4 rats) over time. In experiment 2, each group consisted of 8 rats. All data were analyzed using SPSS 11.5 for Windows, using ANOVA with Bonferroni post hoc testing or Student's t-tests. On day 4 and 5 of experiment 1, association between physiological parameters and the development of hyperactivity in the light phase was investigated using Pearson's bivariate correlation analysis. We analyzed possible associations on day 4 and 5 while on these days hyperactivity developed in the light phase. Statistical significance was set at $p<0.05$.

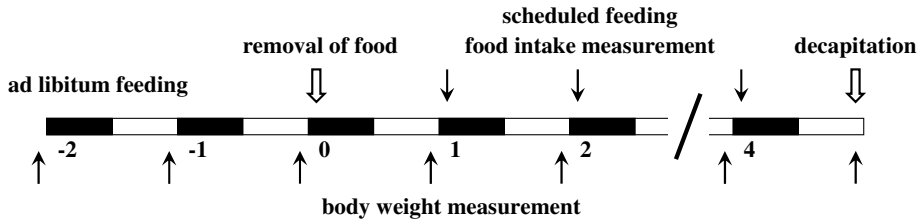

Figure 1.

Scheme of experimental set-up. Filled bars indicate the dark phase, open bars the light phase. At day 0, food was removed for the restricted experimental groups and placed on scheduled feeding of 1-1.5 hour food access at the beginning of the dark phase. Food intake and body weight were measured daily.

Results

Physiological parameters during the development of ABA

In order to determine whether changes in plasma ghrelin levels upon food restriction were dependent on the availability of a running wheel, we compared ghrelin levels in food-restricted rats exposed to running wheels with pair-fed sedentary rats.

Baseline body weight during *ad libitum* feeding was not significantly different between rats in running wheel cages and sedentary rats (running 213.7 ± 1.4 gram, sedentary 218.9 ± 2.8 gram, n.s.). However, running rats had a higher baseline food intake as compared to sedentary rats (running 19.7 ± 0.3 gram, sedentary 18.1 ± 0.4 gram, $p=0.001$), which is probably to compensate for increased physical activity.

During food restriction, body weight decreased in ABA rats and pair-fed sedentary rats. As depicted in Figure 2., ABA rats had a significantly lower relative body weight compared to pair-fed sedentary rats (day 1 to day 4, $p<0.001$; day 5, $p=0.008$).

Just prior to day 5, body weight of the ABA rats decreased remarkably.

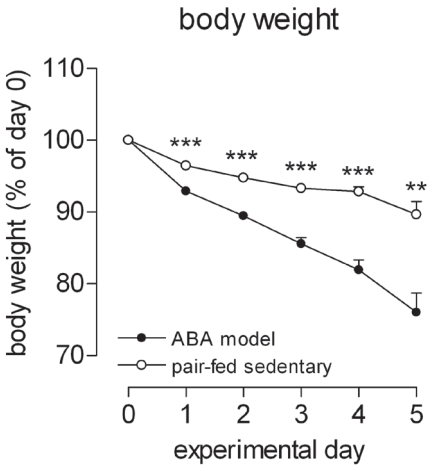

Figure 2.

Daily body weight (relative to ABA day 0, start of food restriction) in food-restricted running rats fed for 1 hour (black circle) and pair-fed sedentary rats (open circle). Significant differences between pair-fed sedentary and 1-hour fed running (ABA) rats are indicated by asterisks, **p<0.01, ***p<0.001.

Endocrine parameters during the development of ABA

Plasma ghrelin levels in 1-hour fed ABA rats and pair-fed sedentary rats are shown in Table 1. During exposure to the ABA model, plasma ghrelin leptin levels increased over time ($F(5,28)=4.50$, $p=0.01$) whereas no significant changes were observed for plasma ghrelin levels in pair-fed sedentary rats ($F(5,23)=2.12$, n.s.).

Association between plasma ghrelin levels and hyperactivity

Animals become more active during hours preceding food access. Based on a review by Mistlberger (Mistlberger, 1994), we defined food-anticipatory activity (FAA) as the running wheel activity (RWA) four hours prior to food access on days of scheduled food access. Since, under restriction conditions the food was given during the first hour of the dark phase, the RWA in the last four hours of the light phase was taken into consideration for the evaluation of FAA.

Increased activity, especially prior to food intake, is a hallmark feature of ABA and comparable to hyperactivity observed in AN patients (Hillebrand et al., 2008).

Table 1. Plasma ghrelin levels during scheduled feeding

	plasma ghrelin levels (ng/ml)	
day	1-hour fed running rats	pair-fed sedentary rats
0	0.80 ± 0.05	1.13 ± 0.15 [b]
1	2.10 ± 0.18	2.61 ± 0.40
2	2.28 ± 0.44	2.23 ± 0.57
3	3.64 ± 1.43	2.19 ± 0.54
4	2.74 ± 0.36	1.45 ± 0.19 [b]
5	5.10 ± 0.35 [a]	2.55 ± 0.28

Plasma total ghrelin levels in 1-hour fed running rats (ABA) and pair-fed sedentary rats. Significant differences within the experimental groups compared to day 0 are indicated by "a", significant different from ABA rats at the same day arte indicated by "b". ANOVA, Bonferroni, with significance set at $p<0.05$.

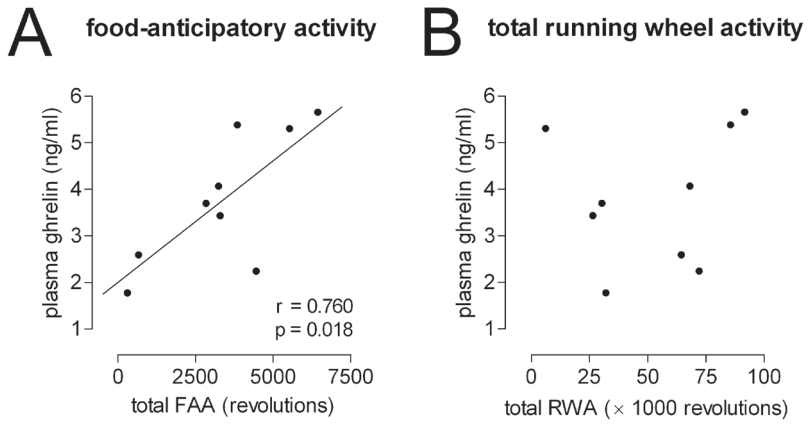

Figure 3.

Correlation analysis on day 4 and 5 between plasma ghrelin levels and (A) total food-anticipatory activity (FAA measured by wheel revolutions) or (B) total running wheel activity levels in running rats exposed to 1-hour feeding schedule (ABA, n=9). Statistical significance was set at $p<0.05$.

We therefore were interested whether high plasma ghrelin levels were associated with FAA. Data points from ABA day 4 and day 5 when FAA is usually fully expressed were combined. As depicted in Figure 3.A., correlation analysis on day 4 and 5 revealed that plasma ghrelin levels were significantly positively correlated with FAA in ABA rats

(r=0.760, p=0.018). There was no correlation between ghrelin levels and total RWA on the same days (Figure 3.B.).

Effect of central ghrelin antagonism on running wheel activity

To investigate whether ghrelin signaling contributes to the development of hyperactivity caused by caloric restriction, we injected a ghrelin receptor antagonist centrally into the brain during ad libitum fed and food restricted conditions. In *ad libitum* fed rats, acute ghrelin receptor antagonist injection (8 μg and 16 μg) in the lateral ventricle resulted in significantly decreased RWA (dose 8 μg, 66.24 ± 9.90%; dose 16μg, 43.37 ± 14.45%) as compared to RWA levels of the day before (Figure 4.A.). A similar reduction was observed for locomotor activity measured by telemetric devices (LMA; dose 8 μg, 71.18 ± 9.90%; dose 16μg, 63.27 ± 8.98%). Comparable to *ad libitum* fed conditions, ICV ghrelin receptor antagonist injection (8 μg and 16 μg) resulted in a significant reduction of RWA and LMA in food-restricted rats (RWA, dose 8 μg, 67.20 ± 7.86%, dose 16μg, 60.92 ± 11.99%; LMA, dose 8 μg, 86.04 ± 8.71%, dose 16μg, 74.30 ± 9.63%). No differences in activity levels, RWA and LMA, were found in running *ad libitum* fed as well as food-restricted rats treated with the lowest dose of ghrelin receptor antagonist (4μg/injection) or control injections.

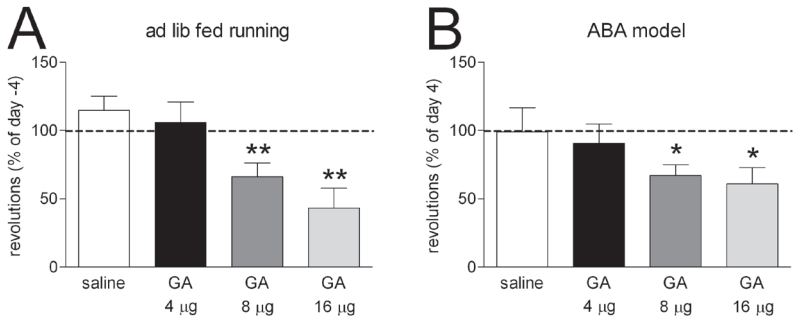

Figure 4.

Effect of acute ICV saline (n=6) or ghrelin antagonist (GA; 4 μg (*ad libitum* fed n=6; food-restricted n=10), 8 μg (*ad libitum* fed n=10; food-restricted n=6), 16 μg (*ad libitum* fed n=8; food-restricted n=6)) injection just prior to day 5 on running wheel activity in (A) *ad libitum* fed running rats and (B) rats exposed to the ABA model. Running wheel activity is presented as mean percentage compared to respectively ABA day -4 and ABA day 4, as shown by dashed line. Significant differences are indicated by asterisks, *p<0.05, **p<0.01, Student's t-test.

Effect of central ghrelin antagonism on food intake

In ad libitum fed rats, acute ICV ghrelin receptor antagonist injection (16 μg) reduced daily food intake as shown in Figure 5.A. A tendency towards decreased food intake was observed for the injection of 8 μg ghrelin receptor antagonist. Vehicle injection or injection with the lowest dose of 4 μg ghrelin receptor antagonist did not affect daily food intake.

In ABA rats, acute ICV ghrelin receptor antagonist injections had no effect on daily (restricted) food intake as shown in Figure 5.B. Thus, an acute icv injection of a ghrelin receptor antagonist reduces food intake in *ad libitum* fed running rats, but not ABA rats.

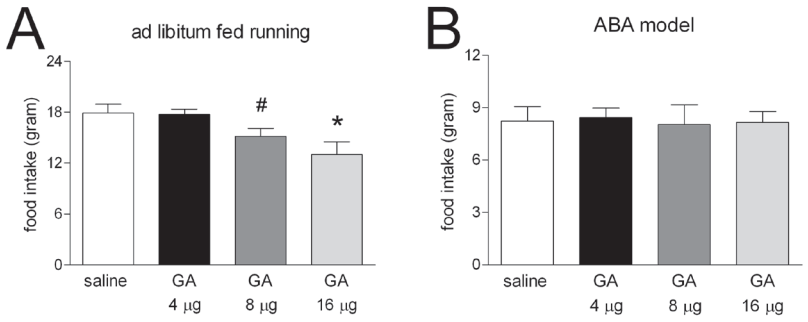

Figure 5.

Effect of acute ICV saline (n=6) or ghrelin antagonist (GA; 4 μg (*ad libitum* fed n=6; food-restricted n=10), 8 μg (*ad libitum* fed n=10; food-restricted n=6), 16 μg (*ad libitum* fed n=8; food-restricted n=6)) injection just prior to day 5 on 24-hour food intake in (A) *ad libitum* fed running rats and (B) rats exposed to the ABA model. Food intake is presented in absolute values. Significant differences are indicated by asterisks, *$p<0.05$, Student's t-test, whereas tendency to significant differences ($0.05<p<0.01$) is indicated with the symbol #.

Discussion

In this study, we investigated the involvement of plasma ghrelin levels in food anticipation in the activity-based anorexia (ABA) model. There was a strong positive correlation in ABA rats between the expression of food anticipation measured by activity levels (= food-anticipatory activity, FAA four hours prior to food access) and plasma ghrelin levels on day 4 and day 5 of the ABA model. Interestingly, plasma ghrelin levels were not correlated with total RWA. This finding supports a role of ghrelin signaling specifically in FAA. Furthermore, we here demonstrated that acute ICV ghrelin recep-

tor antagonist injections (8 μg and 16 μg) suppressed activity levels in *ad libitum* fed running rats as well as ABA rats. Injection with the highest dose (16 μg) of ghrelin receptor antagonist reduced food intake in *ad libitum* fed running rats, whereas no effect on food intake was observed in the ABA rats. Since plasma ghrelin levels are associated with FAA but not with total RWA and that a ghrelin receptor antagonist suppresses locomotor activity, including during FAA, provides strong evidence that ghrelin signaling is associated with the expression of food anticipation.

We report a strong correlation between plasma ghrelin levels and the expression of food anticipation. Similar results were reported in a study demonstrating a significant increase in plasma ghrelin levels during 2 hours prior to meal time in meal-trained rats, but no increase was found in ad libitum-fed rats (Drazen et al., 2006). The limited human data indicate that individuals in energy balance who consume three meals per day exhibit increased ghrelin concentrations around habitual mealtimes followed by a decrease 2 hours after food ingestion (Natalucci et al., 2005). This observation supports an involvement of ghrelin secretion during food anticipation rather than negative energy balance alone. Furthermore, Frecka and Mattes showed that the increase in ghrelin secretion is related to meal patterns in humans (Frecka and Mattes, 2008).

Recent data identified ghrelin receptors on dopaminergic neurons of the ventral tegmental area (VTA) in the mesolimbic midbrain (Howard et al., 1996; Guan et al., 1997), a brain area known to be involved in locomotion, reward and feeding behavior (Fields et al., 2007). The dopaminergic VTA neurons are part of the mesolimbic dopamine system, which has been implicated in locomotor activity and feeding. Abizaid and colleagues have demonstrated that ghrelin increases neuronal activity in dopaminergic VTA neuron (Abizaid et al., 2006). The present data support that ghrelin signaling increases locomotor activity which has been associated before with increased dopaminergic signaling (Quarta et al., 2008; Jerlhag, 2008; Jerlhag et al., 2007). We therefore propose that increased activity of the mesolimbic system by ghrelin underlies locomotor activity independent from the status of the energy balance. Furthermore, ghrelin antagonizes leptin action and, therefore, has an important role in the regulation of feeding behavior and energy metabolism (Shintani et al., 2001). We and others have shown before that leptin is able to suppress hyperactivity in the ABA model (Exner et al., 2000; Hillebrand et al., 2005b; Verhagen et al., 2009a). Taken together, both low levels of leptin and high levels of ghrelin are implicated in the hyperactivity observed in the ABA model. To better understand the mechanism underlying hyperactivity in AN, we propose to quantify both leptin and ghrelin levels and investigate its relation to hyperactivity in acutely ill AN patients.

Injection with the highest dose (16 μg) of ghrelin receptor antagonist reduced food intake in *ad libitum* fed running rats whereas no effect on food intake was ob-

served in the ABA rats. During food restriction in the ABA rats, we showed that plasma ghrelin levels increased over the time course of the ABA model. This suggests that injection with the highest dose of ghrelin receptor antagonist was still not sufficient to block the increased ghrelin signaling in the brain. Alternatively, the strong orexigenic drive of ghrelin-independent signals in ABA exposed rats may make these animals insensitive to the ghrelin antagonist during the limited period of food access.

Anorectic patients often display abnormally high physical activity levels (Kron et al., 1978), which hinders the process of recovery (Holtkamp et al., 2004a; Kaye et al., 1988b). Thus, reducing hyperactivity in severely ill AN patients could be beneficial for therapeutic outcome. It has been shown that total ghrelin plasma level in AN patients was significantly higher than in a control group (Janas-Kozik et al., 2007). The present data demonstrating that ghrelin antagonism suppressed hyperactivity without influencing food intake in ABA rats suggest that the hyperactive behavior observed in AN patients may be targeted by ghrelin antagonism. In conclusion, these data suggest that ghrelin antagonism may be considered in the treatment of AN patients when reduction of hyperactive behavior is expected to accelerate recovery.

Acknowledgements

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Chapter 7

CHAPTER 7

Identification of a QTL on mouse chromosome 2 for food-anticipatory activity; a possible role for Ptpn1?

Linda A.W. Verhagen, Cigdem Gelegen, David A. Collier, Cathy Fernandes,
Hugo Oppelaar, Mieneke C.M. Luijendijk, Rea J. van Rozen,
Evangelos Vassos, Hein A. van Lith, Roger A.H. Adan, Martien J.H. Kas

Abstract

Feeding behavior and accompanied behaviors are tightly regulated by central and peripheral systems. By examining the response of different mouse strains to scheduled feeding, it has been shown that a genetic factor underlies anticipation to food. By testing a chromosome substitution panel derived from mouse strains that express (C57BL/6J) or lack (A/J) anticipation to scheduled feeding using the activity-based anorexia (ABA) model, we found a genetic locus on mouse chromosome 2 that contributes to food anticipatory activity. Correlation and behavioral analysis in CSS2 F_2 mice with a homozygous C57BL/6J or A/J genotype for markers chosen within the QTL-interval revealed a possible role for the gene protein tyrosine phosphatase, non-receptor type 1 (Ptpn1, PTP1B in humans) in the expression of food anticipation. In addition, no strong evidence was found for the specific involvement of Ptpn1 mRNA expression levels in FAA using radio-active in situ hybridization, whereas pharmacological intervention by peripheral injection of Ptpn1 inhibitor showed suppressed FAA and total RWA in C57BL/6J mice exposed to the ABA model. Further research is needed to confirm whether Ptpn1 plays a specific role in the generation of FAA. Conclusively, our data demonstrate that the genetic aspects of response to energy balance can be modeled in mice through the analysis of conserved behaviors and genetic variation.

Introduction

The ability to seek and find food is crucial for survival. Feeding behavior and accompanied behaviors therefore are tightly regulated by central and peripheral systems (review) (Schwartz et al., 2000; Woods and Seeley, 2000). During restricted feeding, animals show increased levels of locomotor activity during the hours preceding daily scheduled feeding (Bolles, 1963). This increase in activity levels, also called food-anticipatory activity (FAA), precedes daily food intake and has been suggested to reflect the natural search for food (review) (Mistlberger, 1994).

Activity-based anorexia (ABA) is an animal model in which FAA is expressed as increased running wheel activity (RWA) during food restriction (Epling, 1983) (Routtenberg and Kuznesof, 1967). In this model, animals are given free access to a running wheel and fed once per day at a fixed time during the 24-hr day. When exposed to the ABA model, animals become hyperactive and show high levels of FAA in the hours prior to scheduled food access. Previous studies using different inbred mouse strains, in particular A/J, C57BL/6J, and DBA/2J mice, showed strain-dependent differences in their behavioral and physiological response to the ABA model (Gelegen et al., 2006). For example, DBA/2J mice showed a more pronounced decrease in food intake than C57BL/6J mice upon exposure to the ABA model. On the contrary, A/J mice demonstrated low activity levels as compared to C57BL/6J and did not develop FAA. These findings show that genetic background affects the response of mice to ABA.

Chromosome substitution strains (CSS) have been used successfully to facilitate the mapping of genetic factors that control trait differences (quantitative trait loci, QTLs) in its progenitor strains (Nadeau et al., 2000). In these strains, a specific chromosome of the background strain (C57BL/6J) is substituted by the corresponding chromosome of the donor strain (A/J). Phenotypic differences between a CSS and the genetic background strain (e.g., C57BL/6J) indicate that at least one genetic locus on the particular chromosome contributes to the observed (behavioral) phenotype. Previous studies by Gelegen et al. using CSS panels with A/J and C57BL/6J as parental strains showed that mouse chromosome 2 is involved in FAA since in a C57BL/6J background, presence of the A/J chromosome 2 results in loss of FAA (Gelegen et al., in preparation). Using an F_2 population of the CSS chromosome 2 (CSS2 F_2) strain, we here identified a quantitative trait locus (QTL) contributing to FAA in the ABA model. Furthermore, we tested whether a candidate gene in this locus, in particular protein tyrosine phosphatase, non-receptor type 1 (Ptpn1), was responsible for FAA in C57BL/6J mice.

Material and methods

Animals

Initial breeding pairs for CS strains, their progenitors and additional strains were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). F_1 hybrids were generated by crossing CS-Strain 2 females and males to C57BL/6J males and females, respectively. An F_2 population (CSS2 F_2, n=125) was generated by intercrossing the F_1 hybrid males and females. In addition, C57BL/6J and A/J female were bred in the animal facility of the Rudolf Magnus Institute of Neuroscience (The Netherlands) and were 3-5 months old at the start of the experiment. Following weaning at 3-4 weeks, female mice were separately housed in cages in a room maintained on a 12/12 light-dark cycle (lights on at 2 a.m.), with an ambient temperature of 22 ± 2 °C. Animals were given *ad libitum* food and water. All described procedures were approved by the ethical committee on the use and care of animals of the University of Utrecht, The Netherlands. For ethical reasons, it was decided that mice were to be removed from the experiment when mice lost more than 20% of their initial body weight.

Experimental procedure

Female C57BL/6J (n=67), A/J (n=24), CSS2 (n=12), and CSS2 F_2 (n=125) mice were used for this experiment. To adapt the animals to running wheel cages, all mice were individually housed in cages with running wheels for one week before the start of the experiment. Running wheel activity (RWA) was registered by a small magnet and a counter that was activated by the magnet when it passed the counter during a revolution of the running wheel. After the adaptation period, all mice were placed on a restricted feeding schedule during five consecutive days. During this food restriction period, food access 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 daily just before the beginning of the dark phase and just after food access (see also Figure 1.). Individual RWA were continuously registered using Cage Registration Software (Department of Biomedical Engineering, UMC Utrecht, The Netherlands). Based on Mistleberger's review, food-anticipatory activity (FAA) was defined as the RWA 4 hours prior to food access during food restriction schedule. Since, under restriction conditions the food was given at the onset of the dark phase, the last four hours of the light phase were taken into consideration for the evaluation of FAA. Finally, animals were sacrificed by decapitation at the end of the light phase of day 3. Brains were quickly removed under RNAse free conditions, split into two hemispheres of which one was used for dissection of the hippocampus, frozen and stored at -80˚C. Interscapular brown adipose tissue (BAT), white adipose tissue (WAT) surrounding the oviducts (PGAT), retroperitoneal WAT (RPAT), subcutaneous WAT

(SCAT), liver, and spleen were collected, weighed, frozen and stored at -80˚C. Note that based upon previous experience, C57BL/6J mice and CSS2 F_2 exposed to the ABA model were excluded from further analysis when their body weight at the start of the ABA model was higher than 25 gram, and/or when their average RWA levels during dark phase were lower than 500 revolutions per hour.

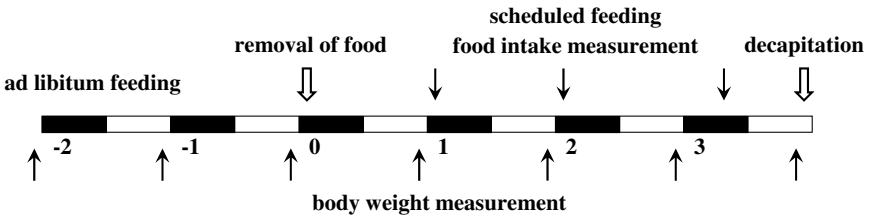

Figure 1.

Scheme of experimental set-up of the activity-based anorexia (ABA) model. Filled bars indicate the dark phase, open bars the light phase. At day 0, food was removed and animals were placed on scheduled feeding of 2 hours at the beginning of the dark phase. Food intake and body weight were measured daily. At the end of day 3, animals were sacrificed by decapitation.

SNP analysis

A total of 23 SNPs were selected across chromosome 2 using the Celera-database based on the presence of allelic differences between mouse strains A/J and C57BL/6 (see also Table 1.). For the selected SNPs Taqman Assay by Design were ordered (Applied Biosystems, Foster City, CA, USA). Genomic DNA was isolated from spleen from CSS2 F_2 intercross mice using a phenol/cholorphorm/iso-amylalcohol protocol (Laird et al., 1991). The Taqman assay was performed according to the Taqman protocol (Applied Biosystems, Foster City, CA, USA) on a total of 10 ng of genomic mouse DNA, in a total reaction volume of 5 µl. The assays were run on a sequence detection system (7900 HT) (Applied Biosystems, Foster City, CA, USA). The values obtained were corrected and normalized against controls. Genotype assessments were based on 95% confidence interval.

Map construction

Segregation ratio of the genotypes of individual markers was checked with the Chi-squared goodness-of-fit test. For all loci, the allele frequencies were not statistically different ($p>0.05$) from the expected ratio, 1:2:1 (BB:BA:AA). The genetic map distance for the markers was computed with the software package JoinMap, version 3.0 (Van Ooijen JW and Voorrips RE, 2001). The critical LOD scores used to establish

linkage groups and calculate map distance in JoinMap are called 'linklod' and 'maplod', respectively. Marker pairs with recombination LOD score above a critical 'linklod' are considered to be linked. Only information for markers pairs with a LOD score above 'maplod' was used in the calculation of map distances. To be sure that all markers were placed in the genetic map, a low value for 'maplod' was used. For the establishment of linkage groups, a critical minimal LOD score ('linklod') of 3.0 was used. For calculation of map distances and estimating most likely gene orders, a critical LOD score ('maplod') of 0.05 was used. Recombination frequencies were converted to map distances in centiMorgans using the Kosambi function.

Quantitative Trait Loci (QTL) analysis

For all days of measurements, the trait 'food-anticipatory activity corrected for individual total activity level (%FAA)' was normally distributed. The QTL location was determined using the interval-mapping module of the MapQTL software package (version 4.0) (Van Ooijen et al., 2002). Results were expressed as LOD scores. Based on Lander and Botstein (Lander and Botstein, 1989), taking into account that a genetic scan was performed across a single, complete chromosome rather the entire genome, an association was assumed significant when LOD score was ≥ 1.65. After grouping by genotype for the DNA marker flanking the peak of the QTL or at the peak of the QTL, trait comparison of the F_2 animals was performed. After grouping by genotype for the DNA marker flanking the peak of the QTL or 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. All data within genotype groups were normally distributed by using the Kolmogorov-Smirnov one-sample test. The co-segregation of phenotypes with alleles at the selected marker locus was evaluated with one-way ANOVA analysis with *post hoc* Student's t test. The mode of inheritance was chosen as free, additive, dominant or recessive according to the significance of differences in the mean values of the traits between mice that were homozygous C57BL/6J, heterozygous and homozygous A/J. In the ANOVA tests, homogeneity of variances was tested (Levene's test). When necessary, the variances were equalized by logarithmic transformation of the data. After transformation the within-group data were still normally distributed.

Localization and quantification of candidate gene Ptpn1 by radioactive in situ hybridization

Frozen brains of CSS2 F_2 mice with the locus homozygous for C57BL/6J or A/J were sliced using a cryostat (Leica, Rijswijk, The Netherlands). Series of 16 μm coronal sections were thaw-mounted onto RNAse free Superfrost slides (Menzel, Germany) and

stored at -80 °C until processing. In brain sections, expression of Ptpn1 was localized and quantified by radioactive in situ hybridization with a ^{33}P-labeled antisense RNA probe against a Ptpn1 sequence fragment amplified by PCR using specific primers (forward primer: GCACTTCTGGGAGATGGTGT; reverse primer: GTAAGAGGCAG-GTGTCA GCC; PCR product 422 bp). Brain sections were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 min, washed in PBS, pretreated with 0.25% acetic anhydride in 0.1 M triethanolamine, washed again in PBS, and dehydrated in graded ethanol followed by 100% chloroform and 100% ethanol. The sections were hybridized overnight at 72 °C with RNA probe (106 cpm) in buffer containing 50% deionized formamide, 2× standard saline citrate (SSC), 10% dextrane sulphate, 1× Denhardt's solution, 5 mM EDTA and 10 mM phosphate buffer, after 5 min heating at 80 °C. After hybridization, section were rinsed in 2× SSC (short, 72 °C) and 0.2× SSC (2 hrs, 72 °C) and dehydrated in graded ethanol with 3 M ammonium acetate. Sections were exposed to X-ray film (Kodak Bio-Max MR) for 20 days. The films were developed and expression of Ptpn1 was semi-quantitatively analyzed using a calibration curve. Further analysis was performed using Image J Software (National Institute of Health, Bethesda, Maryland, USA).

Pharmacological intervention, candidate gene Ptpn1

To investigate the involvement of Ptpn1, single intraperitoneal (IP) injections of Ptpn1 inhibitor (Ptpn1 inhibitor, # 539741, Calbiochem, Germany) in female C57BL/6J were performed. After adaptation to the running wheel during one week, C57BL/6J mice (n=24) were divided into 3 experimental groups, matched for body weight and baseline RWA. Baseline RWA was determined as average RWA during four days prior to the start of food restriction (day -4 to day -1). After the adaptation period, all mice were placed on a restricted feeding schedule during four consecutive days (day 0-3). During this food restriction period, food access was given during the first two hours of the dark phase. Body weight and food intake were measured daily just before the beginning of the dark phase and just after the food access. At the middle of day 2, 2-3 hours before C57BL/6J mice normally express FAA during daily scheduled food access, the Ptpn1 inhibitor (concentration 10 or 20 mg/kg Ptpn1 inhibitor, 250 µl injection volume) or control (vehicle, 10%DMSO) was injected. Finally, animals were sacrificed by decapitation (as described before) at the end of the dark phase on day 3.

Statistical analysis

All data are presented as mean ± standard error unless otherwise indicated. Data were analyzed using SPSS 11.5 for Windows and were controlled for normality and homogeneity. For the behavioral measurements in body weight, food intake and RWA, baseline levels were not significantly different between all experimental groups. Dif-

ferences in body weight, food intake, and RWA were assessed by general linear model (GLM) repeated measurements procedure, using a between subject factor (STRAIN) and within subject factor (DAYS). In case of significant difference between strains, one-way ANOVA was performed on each individual day. The effect of genotype on FAA was assessed with one-way ANOVA. The GLM or ANOVA procedure was followed by Bonferroni multiple comparison. Furthermore, association between %FAA and physiological parameters in CSS2 F2 mice exposed to the ABA model was investigated using Pearson's bivariate correlation analysis. Statistical significance was set at $p<0.05$.

Results

C57BL/6J, A/J, and CSS2 mice exposed to the ABA model

The three inbred strains, C57BL/6J, A/J, and CSS2 mice, were exposed to the ABA model. Whereas C57BL/6J mice showed increased activity prior to food access (=FAA), A/J and CSS2 mice did not display FAA (see Figure 2.). Furthermore, total RWA during scheduled feeding was significantly different. Compared to A/J mice, total RWA was highly significantly increased in C57BL/6J and in CSS2 mice (A/J, 23818 ± 3028 revolutions; C57BL/6J, 68056 ± 3338 revolutions; CSS2, 51083 ± 5327 revolutions) ($p<0.001$ for both comparisons). Moreover, total RWA in C57BL/6J mice was significant higher than in CSS2 mice ($p=0.04$) but to a much lesser extent as compared to A/J mice ($p=0.04$). Note that the amplitude of RWA during the dark phase was lower in A/J mice as compared to C57BL/6J and CSS2 mice. While expressing the same levels of activity during dark phase, the lack of FAA prior to food access in the CSS2 strain supports that chromosome 2 is linked to expression of FAA during scheduled feeding.

Food-anticipatory activity and QTL analysis in CSS2 F_2 population

To identify QTLs underlying FAA, a CSS2 F_2 population was generated and all mice (n=125) were exposed to the ABA model. Due to selection criteria (see Materials and Methods section), 20 CSS2 F_2 mice exposed to the ABA model were excluded from further analysis.

As depicted in Figure 3.A., percentage FAA (%FAA=total FAA as percentage of total RWA) in C57BL/6J and CSS2 mice was significantly higher as compared to A/J mice (respectively, $p=0.002$, and $p=0.02$). In the CSS2 F_2 population, the distribution of %FAA could be defined by three separate groups expressing less than 5% FAA, between 5-10% FAA and higher than 10% FAA similar to the expression of food anticipation seen in A/J mice, CSS2 mice and C57BL/6J mice respectively (see Figure 3.B.).

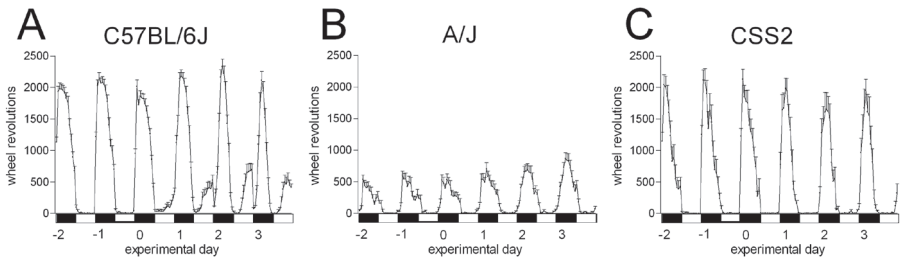

Figure 2.

Hourly running wheel activity pattern of (A) C57BL/6J mice (n=67), (B) A/J mice (n=24), and (C) CSS2 mice (n=12). Filled bars indicate the dark phase, open bars the light phase. At day 0, food was removed and animals were placed on scheduled feeding of 2 hours at the beginning of the dark phase. C57BL/6J strain displayed an increase in RWA during the hours prior to food access (=FAA) in contrast to A/J and CSS2 mice. Note that the total RWA is higher in C57BL/6J and CSS2 mice than in A/J mice.

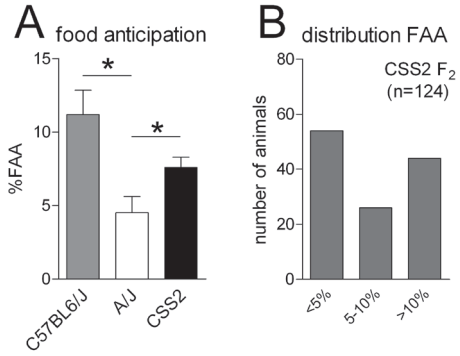

Figure 3.

Percentage food-anticipatory activity (%FAA=total FAA as percentage of total RWA) in C57BL/6J mice (n=67), A/J mice (n=24), and CSS2 mice (n=12) exposed to the ABA model (A). %FAA was significantly increased in C57BL/6J mice (n=67) and CSS2 F_2 mice (n=105) compared to A/J mice (n=24). As plotted by a histogram (B), the distribution of %FAA in CSS2 F_2 mice could be defined by three separate groups expressing less than 5% FAA, between 5-10% and higher than 10% FAA similar to the expression of food anticipation seen in A/J mice, CSS2 mice and C57BL/6J mice, respectively.

To map the QTL on chromosome 2, 23 SNPs were selected and each individual was genotyped for all SNPs. Interval mapping showed significant LOD scores for %FAA,

with maximum LOD score of 4.85 (see Figure 4). QTL-interval on chromosome 2 is located at 164.9 – 169.9 Mb (based on the 1-LOD-score interval taken from the peak of the QTL (with a current LOD-score of 4.85) (Lynch & Walsh, 1998). While the 5 Mb remaining QTL interval contains approximately 45 genes, genetic mapping data of %FAA showed two small peaks on the top of the LOD-score peak delimited by two markers, respectively marker A (rs27292002, located on chromosome 2 at 167658209bp) and marker B (rs27289000, located on chromosome 2 at 168262109bp). Further genotyping efforts with the current F_2 population size will likely not provide us any further information. Instead, we have done some further analysis to select and study one very good candidate gene within the region.

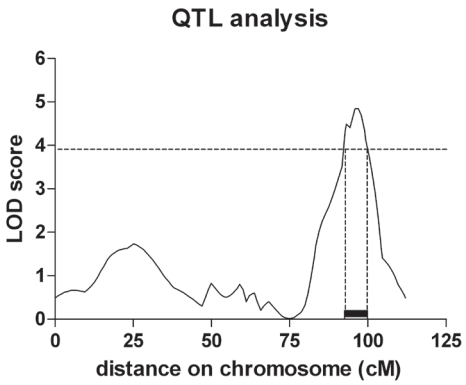

Figure 4.

LOD score distribution of the quantitative trait loci (QTL) for food-anticipatory activity (%FAA) on mouse chromosome 2. The corresponding location (in cM) on mouse chromosome 2 is depicted on the x-axis. Interval mapping was based on %FAA levels in CSS2 F_2 mice (n=105) and genotypes from all these individuals using 23 SNPs across mouse chromosome 2. LOD score threshold for significance was 1.6. Dotted lines delimit the 1-LOD support interval (black box on x-axis).

Correlation and behavioral analysis in CSS2 F_2 mice with the locus homozygous for C57BL/6J or A/J

In order to determine whether measures of energy balance (body weight loss, food intake, total activity and fat stores) affected the variance in %FAA dependent on genotype within the identified QTL, correlation analysis was performed in CSS2 F_2 mice exposed to the ABA model. We thereby made a distinction between CSS2 F_2 mice with a homozygous C57BL/6J or A/J genotype for the markers chosen within the QTL-interval.

In CSS2 F_2 mice with a homozygous C57BL/6J genotype for the markers within the QTL-interval, %FAA was strongly negatively associated with relative body weight and the relative amount of white adipose tissue at the end of the ABA experiment (respectively, $p<0.001$ and $p<0.001$) (see Figure 5.A and 5.B.). No correlation was found for total food intake and total RWA. Interestingly, in CSS2 F_2 mice with a homozygous A/J genotype for the markers chosen within the locus, a much less strong negative correlation was found for %FAA with the relative amount of white adipose tissue ($p=0.03$). Furthermore, association with any of the other behavioral and physiological parameters measured during and after exposure to the ABA model including relative body weight, total food intake, and total RWA was not found. These results support the hypothesis that the C57BL/6J genotype in the QTL makes mice more sensitive for displaying FAA during negative energy balance.

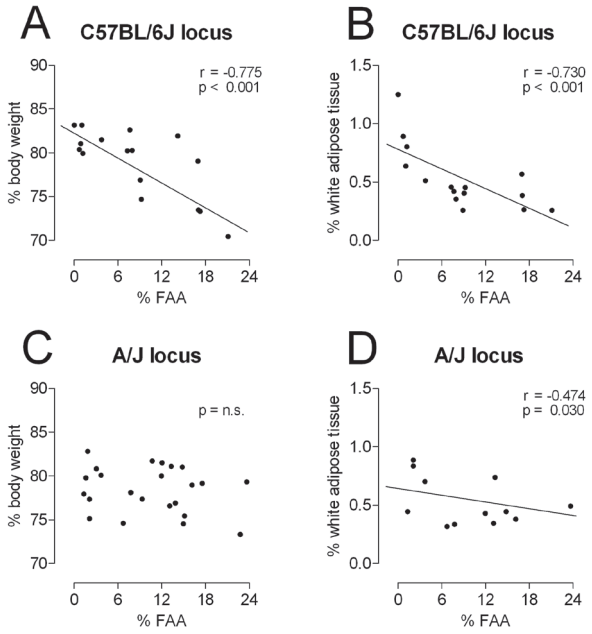

Figure 5.

Correlation analysis between percentage food-anticipatory activity (%FAA) and percentage body weight or percentage white adipose tissue in CSS2 F_2 mice with a homozygous C57BL/6J (n=29) or A/J (n=39) genotype for the markers (A and B) chosen within the QTL-interval. Statistical significance was set at $p<0.05$.

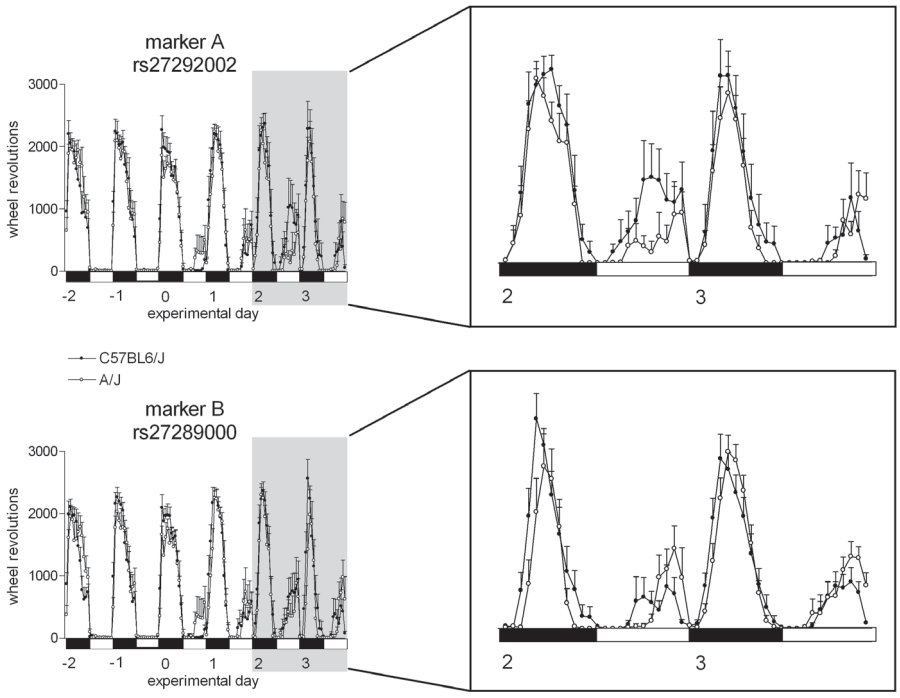

Figure 6.

Hourly running wheel pattern in CSS2 F_2 mice with the homozygous C57BL/6J (n=14) or A/J (n=22) genotype for the marker A or homozygous C57BL/6J (n=19) or A/J (n=21) genotype for the marker B. Filled bars indicate the dark phase, open bars the light phase. At day 0, food was removed and animals were placed on scheduled feeding of 2 hours at the beginning of the dark phase. As observed in the right panels, mice that had a homozygous C57BL/6J genotype for the marker A displayed an increase in RWA during the hours prior to food access (=FAA) in contrast to mice with a homozygous C57BL/6J genotype for the marker B. Note that the mice were sacrificed during the expression of FAA at the end of day 3.

Genetic mapping data of %FAA showed a QTL with two small peaks at the position of two markers. In order to determine the possible involvement of these genes, we performed behavioral analysis in CSS2 F_2 mice that had a homozygous C57BL/6J or A/J genotype for the marker A and the opposite homozygous C57BL/6J or A/J genotype for the marker B. In Figure 6, hourly RWA patterns are plotted. Although FAA show similar patterns on ABA day 0 and ABA day 1, a difference in FAA can be observed on ABA day 2. Interestingly, marker A is located on the gene Ptpn1 on mouse chromosome 2. Taken together, these data support a possible involvement of

the gene Ptpn1 in FAA.

Ptpn1 is a cellular protein implicated in the regulation of insulin and leptin signaling. More specifically, Ptpn1 dephosphorylates Janus Kinase 2, the initial tyrosine kinase mediating leptin signaling (Zabolotny et al., 2002), and mice with whole body Ptpn1 deletion are hypersensitive to leptin (Elchebly et al., 1999; Klaman et al., 2000). Besides, chronic leptin treatment reduced FAA in rats exposed to the ABA model (Hillebrand et al., 2005b; Exner et al., 2000; Verhagen et al., 2008). We further explored the role of Ptpn1 during FAA by two distinct approaches; 1) localization and quantification of Ptpn1 mRNA expression levels by radio-active in situ hybridization, and 2) direct pharmacological intervention of Ptpn1 signaling in C57BL/6J mice exposed to the ABA model.

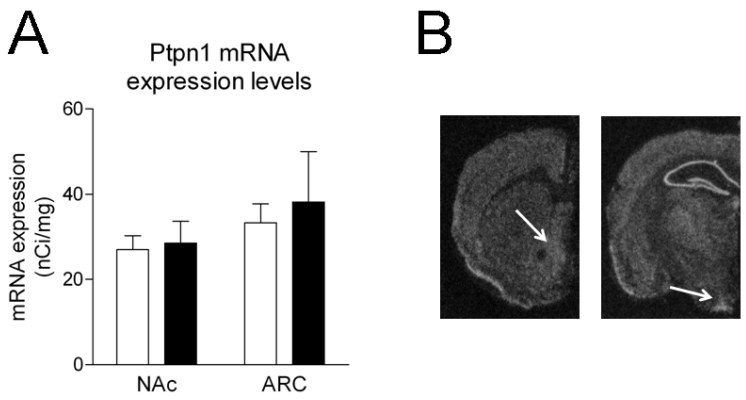

Figure 7.

Quantification of Ptpn1 mRNA expression levels in CSS2 F_2 mice with the locus homozygous for C57BL/6J or A/J. (A) Ptpn1 mRNA expression levels in the NAc and ARC of CSS2 F_2 mice with the locus homozygous for C57BL/6J (n=6) or A/J (n=6) measured by radio-active *in situ* hybridization. (B) Example of Ptpn1 mRNA expression in the NAc and ARC. NAc, nucleus accumbens; ARC, arcuate nucleus. No significant differences were detected.

Ptpn1 mRNA expression levels in the brain of CSS2 F_2 mice with the locus homozygous for C57BL/6J or A/J

Radio-active in situ hybridization on brain slices of CSS2 F_2 mice with the locus homozygous for C57BL/6J or A/J demonstrated Ptpn1 mRNA expression levels in the following brain areas; nucleus accumbens shell (NAc), arcuate nucleus (ARC), ventromedial hypothalamus (VMH), hippocampus, ventral tegmental area (VTA, and

substantia nigra (SN) (see Figure 7.B.). Unfortunately, expression levels in the VMH, VTA, and SN were too low for any further quantification. Whereas the NAc and ARC are known to regulate feeding behavior, we analyzed Ptpn1 mRNA expression in these brain areas. No significant differences in Ptpn1 mRNA expression levels in the NAc and ARC could be observed (see Figure 7.A.) suggesting that the level of Ptpn1 mRNA expression in the NAc and ARC does not explain the differences in FAA.

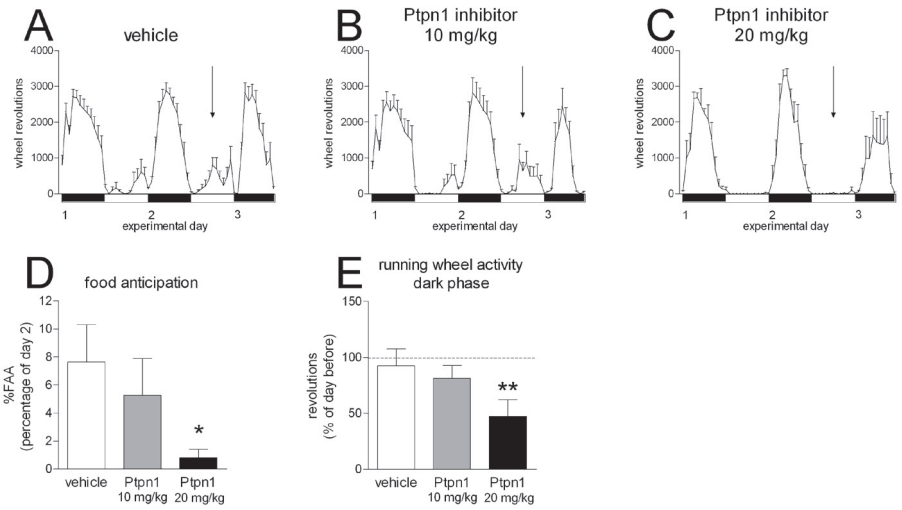

Figure 8.

Pharmacological intervention with a Ptpn1 inhibitor. (A-C) Effect of acute peripheral injection with vehicle (10% DMSO, n=10) or Ptpn1 inhibitor (10 mg/kg, n=10; 20 mg/kg, n=5) during the middle of the light phase on day 2, just prior to food-anticipatory activity in C57BL/6J mice exposed to the ABA model. The arrow illustrates the time point of peripheral injection. (D) Food-anticipatory activity is presented as percentage of total running wheel activity of day 2 (%FAA). Significant differences between vehicle and Ptpn1 inhibitor injection is indicated by the asterisks, *$p<0.05$, Student's t-test. (E) Running wheel activity during the subsequent dark phase on day 3 is presented as mean percentage compared to the dark phase on the day before, as indicated by dashed line. Significant differences are indicated by asterisks, **$p<0.01$, Student's t-test.

Acute Ptpn1 inhibitor in C57BL/6J mice exposed to the ABA model

We hypothesized that increased activity of Ptpn1 resulting in decreased leptin sensitivity underlies FAA. Inhibition of Ptpn1 activity upon ABA exposure would render mice to become more sensitive to leptin and thus result in decreased FAA. Therefore, prior to FAA, we injected a Ptpn1 inhibitor (concentration 10 or 20 mg/kg) or vehicle

solution peripherally in C57BL/6J mice exposed to the ABA model. The injection was performed during the middle of the light phase at day 2 prior to anticipation.

Hourly RWA of C57BL/6J mice injected with vehicle or Ptpn1 (10 mg/kg and 20 mg/kg) are plotted in Figure 7.A-C. No significant changes on %FAA (4 hours before dark phase) were observed after injection with 10 mg/kg Ptpn1 inhibitor (see Figure 7.D.). Injection with 20 mg/kg Ptpn1 inhibitor significantly suppressed %FAA ($p=0.012$). Moreover, food intake on day 3 (after the injection) was not different between mice injected with vehicle or Ptpn1 inhibitor (vehicle, 0.96 ± 0.26 gram; 10 mg/kg Ptpn1 inhibitor, 0.85 ± 0.18 gram; 20 mg/kg Ptpn1 inhibitor, 0.94 ± 0.26 gram). As depicted in Figure 8.E., injection with vehicle or Ptpn1 10 mg/kg did not affect RWA during the following dark phase as compared to the dark phase of the day before. On the contrary, the dose of 20 mg/kg Ptpn1 inhibitor showed a reduction in RWA during the dark phase subsequent to the injection of Ptpn1 inhibitor.

Discussion

Genetic factors underlie food-anticipatory activity (FAA), as it has been shown that on a C57BL/6J background, presence of the A/J chromosome 2 results in loss of FAA (Gelegen et al., in preparation). In the present study, we mapped FAA during food restriction in a F_2 population of the mouse chromosome 2 using a mouse panel for chromosome 2 with A/J and C57BL/6J as parental strains. We found linkage for the development of FAA at mouse chromosome 2, located at 164.9 – 169.9 Mb. Interestingly, it was found that relative body weight and percentage white adipose tissue was significantly correlated with FAA when the identified locus had a C57BL/6J genetic make-up. This supported that in the C57BL/6J genotype, expression of FAA is dependent on the status of energy balance, whereas on a A/J genetic background in this region looses this dependency. Since leptin is known to suppress RWA in rats exposed to the ABA model, we determined whether a possible candidate gene within the QTL-interval, namely Ptpn1, underlies FAA. Since, Ptpn1 modulate leptin sensitivity (Bence et al., 2006), we hypothesized that increased expression of Ptpn1 resulting in decreased leptin sensitivity triggering an animal to anticipate for food. We did not observe gene expression differences in various brain areas between CSS2 F_2 mice with the locus homozygous for C57BL/6J or A/J. Pharmacological inhibition of Ptpn1 activity showed suppression of FAA in C57BL/6J mice anticipating for food at a dose that suppressed also dark phase running. Thus, we did not find convincing evident that FAA was selectively suppressed by inhibition of Ptp1n activity.

Prior to this study, we already argued about the possible involvement of leptin during FAA in animals exposed to the ABA model (Verhagen et al., 2009a). Although

treatment with leptin suppresses locomotor activity in ABA rats (Hillebrand et al., 2005b) similar to treatment with a dopaminergic antagonist and antipsychotic, leptin specifically decreases FAA to a greater extent (Verhagen et al., 2009a). This finding suggest that leptin is involved in FAA. However, in this study, we found no evidence by in situ hybridization to conclude that Ptpn1 underlies FAA, while the pharmacological experiment was inconclusive. Despite a decrease in total RWA, peripherally injected Ptpn1 inhibitor suppressed %FAA in C57BL/6J mice exposed to the ABA model. However, this does not support the specific involvement of Ptpn1 in FAA. Therefore, involvement of Ptpn1 in FAA should be further investigated, for example, using RNA interference to locally block Ptpn1 signaling in specific brain areas or by exposing neuronal specific Ptpn1 knockout mice (Bence et al., 2006) to the ABA model.
In this study, Ptpn1 gene expression levels were found in the NAc, ARC, VMH, VTA, SN and hippocampus of the brain. Ptpn1 gene expression levels in the ARC, VMH and hippocampus were already described by Zabolotny and researchers (Zabolotny et al., 2002), but not for the NAc, VTA and SN. Recent data identified leptin and insulin receptors on dopaminergic neurons in the VTA and SN (Figlewicz et al., 2003). The expression of leptin receptors in the VTA have been implicated in feeding behavior and locomotor activity (Hommel et al., 2006) (Verhagen et al, in preparation, chapter 5). Therefore, the expression of Ptpn1 in the mesolimbic dopaminergic system might be implicated in the expression of food anticipation. Since, to our knowledge leptin receptors nor insulin receptors are expressed in the NAc, it remains to be determined what the exact role of Ptpn1 in the NAc is.

Taken together, the involvement of Ptpn1 in FAA should be further investigated. The QTL interval contains approximately 45 genes and at this stage we can certainly not exclude the involvement of the other 44 genes in FAA. In conclusion, we found a QTL on mouse chromosome 2 for the expression of food anticipation measured by activity levels due to variation in response to a negative energy balance. Although some evidence was found for the involvement of Ptpn1 in FAA, further studies are needed to uncover the role of Ptpn1 in food anticipation.

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Chapter 8

GENERAL DISCUSSION

The neural mechanisms underlying anorexia nervosa (AN) remain obscure. Disturbances in dopamine and serotonin neurotransmission and to a limited extent in some peripheral hormones (i.e. leptin and PYY) have been described in AN. Studies on the frequently used animal model for AN, the activity-based anorexia model, suggest a starvation response at the level of the hypothalamus in the brain (de Rijke et al., 2005b; Hillebrand et al., 2005a; Hillebrand et al., 2006c; Hillebrand et al., 2006b). Therefore, in my research project, I focused on the role of leptin, ghrelin and dopamine and their possible link in the ABA model.

The regulation of energy balance involves complex interactions between peripheral signals and neural circuits that influence metabolism and behavior. Dysfunction of this energy balance regulatory system may underlie eating disorders, such as AN. In the present thesis I focused on regulatory neuronal pathways underlying anorectic behavior and the possible involvement of endocrine signaling with special emphasis on the involvement of the mesolimbic dopaminergic system. I concentrated on starvation-induced hyperactivity, since this trait appears prominent in patients suffering from AN. Hence, the overall aim of this thesis was to identify the neural mechanism(s) underlying starvation-induced hyperactivity in order to better understand the pathophysiology seen in AN patients.

Activity-based anorexia model

As discussed in the introduction (chapter 1), the activity-based anorexia (ABA) model mimics important features of AN and in this thesis I focused on one of those, hyperactivity. Earlier studies indicated that leptin as well as olanzapine were able to reduce hyperactivity in the ABA model (Hillebrand et al., 2005c; Hillebrand et al., 2005b). In order to investigate the mechanisms underlying ABA, I focused on dopamine, leptin and ghrelin and performed a linkage study for food-anticipatory activity (FAA).

Food-anticipatory activity

Upon exposure to the ABA model locomotor activity in the dark phase increases, but increased locomotor activity also develops prior to food access (Mistlberger, 1994). This is called food-anticipatory activity (FAA). FAA strongly contributes to the total hyperactivity observed in animals exposed to the ABA model.

An increasing number of researchers have been studying which brain nuclei are involved in anticipation to food. Specific brain lesion studies showed that destruction of hypothalamic brain areas induced major changes in homeostasis. Until now, the

neural substrate underlying food anticipation is not precisely known. In ABA rats, FAA can already be observed after two days of exposure to the ABA model (see hourly RWA pattern in chapter 3, figure 4). In chapter 2, it was aimed to identify hypothalamic and mesolimbic brain areas involved in FAA. The ARC, DMH and LH in ABA rats showed increased neuronal activity as compared to rats exposed to the same restraint in food intake. In addition, the neuronal activity in the DMH showed a strong positive correlation with FAA measured by activity levels, whereas no correlation was found for neuronal activity in the ARC and LH. This supports for an involvement of the ARC and LH in food anticipation, but not in the expression of FAA by locomotor activity. I propose that the ARC, which is considered a gateway between the periphery and the central nervous system, senses peripheral (endocrine) signals and communicates with the DMH and LH to induce anticipation to food. NPY neurons in the ARC are good candidates to be involved in FAA, since NPY mRNA levels in the ARC are high in FAA and these neurons project to the DMH and LH.

As discussed in chapter 3 and 4, dopamine signaling is not involved in the expression of FAA in rats exposed to the ABA model. Dopamine antagonism by chronic treatment of rats with *cis*-flupenthixol resulted in reduced locomotor activity and increased food intake in ABA rats compared to vehicle-treated ABA rats, but FAA still persisted. These results with *cis*-flupenthixol in the ABA model were remarkably similar to those reported earlier for olanzapine (Hillebrand et al., 2005c) suggesting that dopamine antagonism, rather than serotonin antagonism, results in decreased locomotor activity given the fact that *cis*-flupenthixol is a more specific dopamine receptor antagonist. Furthermore, the lack of involvement for dopaminergic signaling in FAA was confirmed by microdialysis measuring extracellular levels of dopamine release in the nucleus accumbens (NAc) (chapter 4). Moreover, the neuronal activity in the NAc was not associated with FAA (chapter 2). Thus, no direct evidence was found for the involvement of dopamine signaling in the NAc during FAA, at least not during the development of it which was the time course investigated in chapter 2.

An argument in favor of the involvement of dopamine signaling was determined by the measurement of quantitative mRNA expression levels of Tyrosine Hydroxylase, the rate-limiting enzyme in dopamine synthesis, in the VTA of ABA rats sacrificed during the expression of FAA. Although no changes were found in total TH mRNA expression levels between ABA rats and sedentary food-restricted rats, a significant positive correlation was found with total FAA in ABA rats (see Figure 1.). This suggests that, when taking TH mRNA expression levels as measure for dopamine neuronal activity, dopamine neuronal activity is increased during FAA. The capacity to increase dopamine synthesis may thus have been increased whereas this was not reflected in increased dopamine overflow in the NAc. Although part of the mesolimbic neural circuitry, I could not find evidence that the NAc is involved in FAA when quantify-

ing c-fos expression levels. I cannot exclude that dopamine signaling from the VTA to other brain areas such as the amygdala, hippocampus and prefrontal cortex (see chapter 1, Figure 1.) might be involved in the expression of FAA. Further studies are needed to unravel whether the dopaminergic signaling pathway(s) is(are) implicated in FAA.

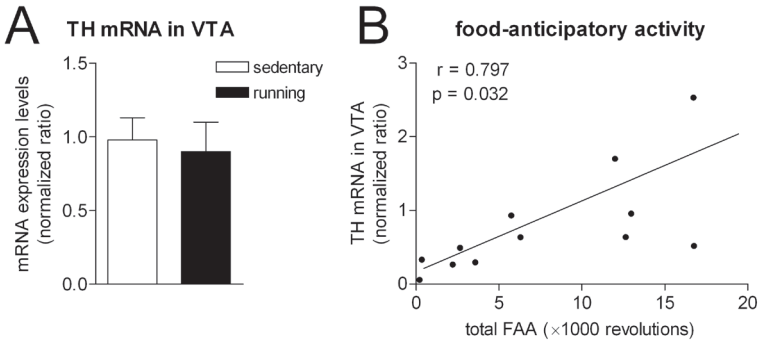

Figure 1.

(A) Expression of tyrosine hydroxylase (TH) mRNA levels in the ventral tegmental area (VTA) in food-restricted sedentary rats (white bar) and food-restricted running (ABA) rats (black bar). (B) Correlation analysis between food-anticipatory activity and TH mRNA levels in the VTA of ABA rats revealed a positive association. Statistical significance was set at $p<0.05$.

Leptin signaling

Apart from energy homeostasis, leptin has been shown to be involved in neuronal networks regulating activity (Exner et al., 2000; Hommel et al., 2006). Chronic leptin treatment significantly affected the ABA model by reducing food intake and inhibiting running wheel activity whereas leptin treatment in *ad libitum* fed rats only reduced food intake without influencing activity levels (Hillebrand et al., 2005b). In chapter 5, we demonstrated that local injections of leptin in the VTA suppressed locomotor activity in the ABA model. Furthermore, leptin receptor knockdown in the VTA of ABA rats using RNA interference exaggerated the increase in locomotor activity and central leptin injections were ineffective in suppressing locomotor activity when leptin receptors in the VTA were knocked down. Thus, lack of leptin signaling in the VTA accelerates starvation-induced hyperactivity in rats exposed to the ABA model.

In *ad libitum* fed animals, leptin acutely applied to the brain suppresses food intake (see overview in Table 1.). During starvation induced by food restriction, leptin no longer inhibits food intake. While it has been shown that leptin signaling in

the VTA decreases food intake during *ad libitum* feeding (Hommel et al., 2006), I could not found evidence for leptin's role in the VTA regulating food intake under food-restricted conditions (chapter 5). These opposite effects of leptin on food intake during different feeding conditions might be explained by the difference in the duration of food access. In the ABA model food access is limited to 1-2 hours per day and it is observed that rats immediately start to eat when food is presented. The strong orexigenic drive of leptin-independent signals in food-restricted rats may make these animals insensitive to the leptin injection during the limited period of food access.

Table 1. Acute central and intra-VTA effects of leptin

	ad libitum feeding		food restriction	
	food intake	activity	food intake	activity
Central leptin	↓	↑	=	↓
Intra-VTA leptin	↓	=	=	↓

VTA, ventral tegmental area.

Both central and intra-VTA leptin reduces activity levels during food restriction (chapter 5). However, under *ad libitum* fed conditions, central leptin increases activity whereas intra-VTA leptin had no effect on activity levels (Hommel et al., 2006). This supports the hypothesis that leptin acts on other brain areas besides the VTA to induce activity in *ad libitum* fed animals. The hypothalamus, in particular the ARC, might be a good candidate for leptin's effect on activity during *ad libitum* feeding. Mesaros and researchers already showed that activation of the most potent intracellular mediator of leptin signaling, the signal transducer and activator of transcription 3 (STAT3), in AgRP neurons in the ARC promotes locomotor activity (Mesaros et al., 2008). Future studies should therefore be performed to investigate the underlying neuronal pathway stimulated or inhibited by leptin in the ARC with regards to activity and FAA.

Ghrelin signaling

Ghrelin has been associated with meal initiation (Cummings et al., 2001). Plasma ghrelin levels rise before mealtime, and decrease after food ingestion. Peripheral and central administration of ghrelin in *ad libitum* fed rats stimulated locomotor activity (Jerlhag et al., 2006). We and others have shown that leptin is able to suppress hyperactivity in the ABA model (Exner et al., 2000; Hillebrand et al., 2005b; Verhagen et al., 2008). Thus with regards to feeding behavior and locomotor activity (at least during exposure to the ABA model) leptin and ghrelin antagonize one another. Taken

together, we suggest that low levels of leptin and high levels of ghrelin are implicated in hyperactivity.

We found a strong correlation between plasma ghrelin levels and FAA to food in rats exposed to the ABA model (chapter 6). Furthermore, central ghrelin antagonist injections suppressed locomotor activity in *ad libitum* fed rats and ABA rats whereas food intake was only reduced during *ad libitum* feeding. In chapter 2, increased neuronal activation of the ARC, DMH and LH was found to be associated with food anticipation. Ghrelin selectively increases c-fos expression in NPY-synthesizing neurons in the ARC (Guan et al., 2003; Hahn et al., 1998; Wang et al., 2002) and increases c-fos expression in DMH neurons surrounded by AgRP fibers (Kobelt et al., 2008) supporting for an involvement of the co-localized AgRP/NPY projection from the ARC to the DMH in FAA. Interestingly, leptin antagonizes ghrelin action; leptin suppresses ghrelin-induced activation of NPY neurons in the ARC (Kohno et al., 2007). This together with the fact that leptin signaling via activation of STAT3 in AgRP neurons in the ARC promotes locomotor activity (Mesaros et al., 2008) suggests that ghrelin and leptin signaling in the ARC converge and that low leptin and high ghrelin levels contribute to development of FAA.

Ptpn1 signaling

Based upon family and twin studies, it has been acknowledged that genetic factors strongly contribute to the susceptibility of AN. Likewise, studies in different inbred mouse strains showed that the genetic background affects the response of mice to ABA. In order to determine the genetic factor underlying FAA, we conducted a linkage study using a chromosome substitution strain derived from mouse strains that express (C57BL/6J mice) or lack (A/J mice) anticipation to scheduled feeding using the ABA model (chapter 7). Using an F2 population of the mouse chromosome 2 (CSS2 F_2), QTL analysis identified a genetic locus on mouse chromosome 2 that contribute to FAA. In CSS2 F_2 mice with a homozygous C57BL/6J genotype for the markers within the QTL interval, correlation analysis revealed a strong association between FAA and percentage body weight or percentage white adipose tissue. This suggests that, in the C57BL/6J genotype, expression of FAA is dependent on the status of energy balance. Further behavioral analysis in CSS2 F_2 mice with a homozygous C57BL/6J or A/J genotype for the markers chosen within the locus strongly supports the involvement of a candidate gene Ptpn1 (PTP1B in humans). Ptpn1 is known to inhibit leptin signaling. Therefore, we hypothesized that increased Ptpn1 activity results in decreased leptin sensitivity and that this underlies FAA. So far, we could not find strong evidence by quantification of Ptpn1 mRNA levels in the ARC and NAc using *in situ* hybridization or by pharmacological intervention with peripheral injections of a Ptpn1 inhibitor to conclude whether or not Ptpn1 underlies FAA.

Quantification of Ptpn1 mRNA levels was conducted in small group sizes. Additional analysis of CSS2 F_2 mice with a homozygous C57BL/6J or A/J genotype for the markers within the QTL interval is required. Although peripheral injections with the highest dose of Ptpn1 inhibitor showed a reduction in %FAA in C57BL/6J exposed to the ABA model, these animals did not anticipate to food on the day before injection and the group size was relatively small. Thus, replication studies with the Ptpn1 inhibitor are certainly needed. Another alternative to determine the role of Ptpn1 in FAA is the application of RNA interference. By downregulation of Ptpn1 expression levels using adeno-associated viral vectors against Ptpn1 in specific brain areas (most likely in the hypothalamic region) the precise role of Ptpn1 in FAA might be determined. Thus, follow-up studies are needed to elucidate whether Ptpn1 is actually involved in FAA.

Serotonin involvement

Disturbances of serotonergic activity have been implicated in AN. During the acute phase of AN low levels of serotonin have been found whereas, after recovery, AN patients have high levels of serotonin metabolites. It has been hypothesized by Kaye and colleagues that individuals who develop AN may have high levels of serotonin and that food restriction serves to decrease serotonin levels (Kaye et al., 2005). Indeed, we found that in the ABA model rats have low serotonin levels in the NAc and that circadian activity of serotonin was blunted in ABA rats as compared to ad libitum fed running rats (chapter 4). The low levels of serotonin may be caused by malnutrition resulting in reduced protein intake containing the essential amino acid tryptophan, the precursor of serotonin. Therefore, it would be of interest whether tryptophan supplements are beneficial in the treatment of AN.

Clinical implications

Until now, no drugs have been effective in treating AN patients. Although not part of the DSM IV criteria, hyperactivity seems to be an important trait in AN and might hinder the process of recovery. Thus, reducing hyperactivity in severely ill AN patients could be beneficial for therapeutic outcome. Treatment with the antipsychotic olanzapine has been shown to reduce hyperactivity in ABA rats as well as AN patients (Hillebrand et al., 2005c). Although it is not clear via which receptors olanzapine reduces hyperactivity, the dopaminergic receptors are good candidates. In chapter 3, we showed that blocking dopamine receptors by treatment with the non-selective dopamine antagonist cis-flupenthixol in ABA rats resulted in decreased hyperactive behavior and increased food intake. Together with the comparison with olanzapine treatment in ABA rats from earlier studies suggest that hyperactive behavior and reduced food intake observed in AN patients may be targeted by dopamine receptor

antagonism. Therefore, I recommend to treat AN patients with antipsychotics with dopamine receptor selectivity to treat hyperactivity.

Not only dopamine antagonism resulted in reduced hyperactivity in ABA rats, also acute ghrelin antagonism suppressed hyperactivity in rats exposed to the ABA model. Further research is needed to elucidate whether chronic treatment with a ghrelin antagonist suppresses hyperactivity and affects food intake in a similar manner as chronic leptin treatment. In conclusion, these data suggest that dopamine and ghrelin antagonism may be considered in the treatment of AN patients when reduction of hyperactive behavior is expected to accelerate recovery.

Previous experiments showed that exercising before mealtime resulted in decreased appetite and reduced food reinforcement (Scheurink et al., 1999; Sherwin, 1998). In the ABA model it is observed that rats start to eat immediately as soon as food is presented, but these rats show a reduced duration of feeding resulting eventually in decreased food intake in ABA rats (Routtenberg and Kuznesof, 1967). AN patients were found to be highly anhedonic to food as compared to bulimia nervosa patients (Davis and Woodside, 2002). Moreover, AN patients demonstrating excessive exercise tended to be more anhedonic than those who did not exercise. This supports the hypothesis that hyperactivity before mealtime can override the "hunger" signal. Therefore, I recommend to include measurements of hyperactivity specifically before mealtime in the phenotypic analysis of AN patients in order to directly investigate whether hyperactivity itself reduces food intake.

Future perspectives

Regulation of food intake and locomotor activity is complex and involves many brain circuits and signaling molecules. In this thesis, I focused on leptin and ghrelin signaling and on the involvement of the hypothalamus and the dopaminergic mesolimbic system. Nevertheless, other signaling pathways might be involved in the control of energy balance as well. For example, the endocannabinoid system has been suggested to be involved in appetite regulation in rodents (Berry and Mechoulam, 2002) and humans (Siegfried et al., 2004). Endocannabinoid levels are increased after short periods of food restriction. Interestingly, higher levels of endocannabinoids were found in db/db mice lacking the leptin receptor (Di, V et al., 2001). Furthermore, CB1 receptor-deficient mice have less circulating leptin as compared to wild-type mice and have enhanced leptin sensitivity (Ravinet et al., 2004). During food restriction, endocannabinoids are high in the hypothalamus as well as in the NAc (Kirkham et al., 2002). Thus, the endocannabinoid system might be involved in AN as well.

A majority of the endocrine changes that occur in AN represent physiological adaptation to starvation, except for PYY levels. PYY is an intestinal peptide known to reduce appetite in humans. So far, not much is known about plasma levels of PYY in

response to changes in energy balance. Interestingly, as high levels of PYY have been shown to reduce food intake, elevated PYY levels has been observed in AN patients (Misra et al., 2006). Furthermore, in AN patients, a strong inverse correlation was found between PYY levels and BMI (Utz et al., 2008; Misra et al., 2006). Further studies are needed to determine whether PYY plays a role in initiation or perpetuation of food restriction in AN. Taken together, further studies might be taken into account to investigate the role of endocannabinoids and PYY in feeding behavior and their relation to anorectic behavior.

As a final point, this thesis provides novel data on the role of ghrelin, leptin and dopamine in particular on the hyperactivity observed in the ABA model. These data give new insights into the mechanisms underlying starvation-induced hyperactivity that may also underlie hyperactivity as observed in AN.

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NEDERLANDSE SAMENVATTING

Anorexia nervosa is een psychiatrische aandoening en wordt gekenmerkt door een sterke drang naar vermagering. Het belangrijkste kenmerk van personen met de diagnose is de weigering om het lichaamsgewicht op een minimaal aanvaard lichaamsgewicht (body mass index (BMI) ≤ 17,5 kg/m^2) te houden. Een ander essentieel kenmerk van anorexia nervosa omvat de intense angst om in lichaamsgewicht toe te nemen en is er ook sprake van een vertekend lichaamsbeeld. Naast een verlaagde voedselinname en een extreem laag lichaamsgewicht, wordt hyperactiviteit vaak gerapporteerd in anorexia patiënten. Anorexia nervosa komt voor bij 3 op de 1000 jonge vrouwen en omvat een hoog sterftecijfer (>10%). Tot op heden zijn er geen doeltreffende geneesmiddelen voor de behandeling wat pleit tegen een straight-forward gemeenschappelijke betrokkenheid van neurotransmitters in de etiologie van anorexia nervosa. De ontwikkeling van atypische antipsychotica zoals Olanzapine en Risperidone heeft het onderzoek van deze geneesmiddelen bij de behandeling van anorexia nervosa aangemoedigd. Naast een toename in lichaamsgewicht wordt hyperactiviteit onderdrukt in anorexia patiënten bij behandeling met atypische antipsychotica. Toch is het exacte werkingsmechanisme nog niet bekend. Het dopaminerge en serotonerge systeem zijn goede kandidaten. Genetische en associatie studies in patiënten hebben de betrokkenheid van dopaminerge en serotonerge receptoren in anorexia nervosa aangetoond. Bovendien is de interactie tussen hersengebieden essentieel voor een normaal eetgedrag en de regulatie van lichaamsgewicht. De centrale regulatie van energiehomeostasis omvat een complex neuronaal circuit, opgebouwd uit verscheidene hersenkernen en neuropeptiden die weer op hun beurt communiceren met (hormonale) signalen vanuit de periferie. Het is daarom van groot belang om meer inzicht te krijgen in de regulatie van energiebalans.

Fysiologische aspecten van anorexia nervosa kunnen worden nagebootst in een diermodel genaamd activity-based anorexia (ABA). Het ABA model is een uniek diermodel dat voedselrestrictie in combinatie met vrijwillig rennen in een loopwiel omvat en resulteert bij ratten in hyperactiviteit, verdere verlaging van de voedselinname en een daling van het lichaamsgewicht en de lichaamstemperatuur, net als bij anorexia patiënten. Daarnaast vertonen ABA ratten structureel een verhoogde activiteit voor een maaltijd (=anticipatie op voedsel). Eerdere studies in het ABA model wijzen op een normale hongerreactie op het niveau van de hypothalamus in de hersenen. Het onderzoek beschreven in dit proefschrift richt zich op de neuronale routes onderliggend aan anorectisch gedrag en de mogelijke betrokkenheid van endocriene hormonale signalering met speciale nadruk op de betrokkenheid van het mesolimbische

dopaminerge systeem in de hersenen. Daarbij hebben we gebruik gemaakt van het ABA model.

Eetgedrag wordt aangestuurd door de hypothalamus in de hersenen. Om meer inzicht te krijgen welke specifieke hersengebieden een rol spelen bij verhoogde (fysieke) activiteit gedurende voedselrestrictie is onderzocht welke hersengebieden geactiveerd zijn tijdens anticipatie op voedsel (hoofdstuk 2). Resultaten laten zien dat er een verhoogde activatie van neuronen in hypothalame kernen plaatsvindt, te weten de arcuate nucleus (ARC), dorsomediale nucleus (DMH) en de laterale hypothalamus (LH). De verhoogde neuronale activatie in de DMH blijkt sterk gecorreleerd te zijn met de (fysieke) activiteit tijdens anticipatie op voedsel.

Eerder studies in anorexia patienten hebben aangetoond dat er veranderingen in het dopaminerge en serotonerge systeem van de hersenen plaatsvinden. Behandeling van anorexia patiënten met een atypisch antipsychoticum zorgt voor een toename in lichaamsgewicht maar ook in een afname van hyperactiviteit. Deze antipsychotica grijpen aan op zowel het serotonerge als het dopaminerge systeem in de hersenen aan. In hoofdstuk 3 is onderzocht of signalering via dopaminerge receptoren bijdraagt aan anorectisch gedrag. Hierbij is gebruik gemaakt van een dopaminerge antagonist genaamd cis-flupenthixol. Chronische behandeling met cis-flupenthixol verminderde fysieke activiteitniveaus onafhankelijk van het eetgedrag van de rat. Ondanks de vermindering in activiteit, anticipatie op voedsel werd niet beïnvloed. Het gebrek aan betrokkenheid van dopaminerge signalering gedurende anticipatie op voedsel werd bevestigd door het meten van extracellulaire niveaus van dopamine afgifte in de nucleus accumbens (NAc) (hoofdstuk 4). Wel werd er in ABA ratten behandeld met cis-flupenthixol een verminderde afname in lichaamsgewicht en een verhoogde voedselinname geconstateerd. Deze gegevens ondersteunen de rol van dopamine in hyperactiviteit bij anorexia patiënten.

De regulatie van voedselinname in de hersenen wordt vanuit de periferie aangestuurd door o.a. het hormoon leptine. Leptine, afkomstig uit vetweefsel, grijpt aan op neuronen in de ARC en zorgt voor een remming van de voedselinname. Ratten die blootgesteld worden aan het ABA model worden hyperactief en hebben erg lage leptine niveaus in het bloed, overeenkomstig met anorexia patiënten. Wanneer ABA ratten chronisch behandeld worden met leptine wordt de hyperactiviteit tegen gegaan, terwijl de activiteit van normaal gevoede leptine-behandelde ratten onveranderd blijft. Het hersengebied genaamd het ventrale tegmentale gebied (VTA) ligt in de middenhersenen en bevat dopaminerge neuronen, die mogelijk een belangrijke rol spelen bij (hyper)activiteit. Recentelijk is gebleken dat deze dopaminerge VTA neuronen leptine receptoren bevatten. Acute centrale leptine injecties en leptine injecties direct in de VTA verlagen de hyperactiviteit in ABA ratten (hoofdstuk 5). Bovendien, lokale blokkade van leptine receptoren in de VTA met behulp van een virale vector resulteert

in een verhoogde activiteit in normaal gevoede ratten en draagt bij aan een opmerkelijk hoger niveau van honger-geïnduceerde hyperactiviteit vergeleken met controle ratten bij blootstelling aan het ABA-model. Deze gegevens ondersteunen het feit dat dalende leptine niveaus gedurende voedselrestrictie resulteren in een verhoogde activiteit als gevolg van een verminderde leptine signalering in de VTA.

Plasma leptine concentraties zijn laag tijdens vasten en verhoogd in een normaal gevoede staat, terwijl plasma concentraties van ghrelin (een belangrijk hormoon afkomstig uit de maag) hoog zijn na vasten en net voor de maaltijd pieken. Leptine remt de voedselinname, terwijl ghrelin de voedselinname stimuleert. Plasma ghrelin niveaus in anorexia patiënten zijn vele malen hoger dan in controle patiënten. Gedurende anticipatie op voedsel is gebleken dat plasma ghrelin niveaus sterk correleren met de hyperactiviteit in ABA ratten (hoofdstuk 6). Daarnaast is aangetoond dat centrale toediening van een ghrelin receptor antagonist (stof die de ghrelin receptoren blokkeren) in de hersenen van ABA ratten leidt tot een verlaging van hyperactiviteit terwijl de voedselinname onveranderd blijft. Dit in tegenstelling tot de toediening van een ghrelin receptor antagonist in normaal gevoede ratten, dat gekenmerkt werd door een verlaging in activiteit als wel een verlaging van de voedselinname. Toekomstig onderzoek zal moeten uitwijzen via welke mechanismen ghrelin de activiteit beïnvloedt.

Familieleden van anorexia patiënten hebben een hoger risico anorectisch te worden. Dit risico is ongeveer tien keer groter dan normaal. Familie- en tweelingonderzoek duidt op een genetische component in anorexia nervosa. Doel van het project beschreven in hoofdstuk 7 is het identificeren van genen die bijdragen tot een verhoogde gevoeligheid voor anorexia nervosa. Uit eerdere studies met verschillende inteelt (muis)stammen, met name A/J en C57BL/6J muizen, bleek dat er stam-afhankelijke verschillen in gedrags- en fysiologische reactie op de ABA-model bestaan. In dit project is er gebruik gemaakt van een panel van inteelt muizenstammen, waarin telkens één chromosoom van de donorstam is ingekruist (zogenaamde chromosoom substitutiestammen). Voor elke stam wordt bepaald in welke mate hyperactiviteit optreedt gedurende blootstelling aan het ABA model. Zo zijn de chromosomen te identificeren waarop de 'door voedselrestrictie geïnduceerde activiteitsgevoeligheidsgenen' liggen. Door een combinatie van slim doorkruisen van de stammen en moderne technieken werden vervolgens de betreffende kandidaatgenen geïdentificeerd. Verder onderzoek zal moeten uitwijzen of de kandidaatgenen bijdragen tot een verhoogde gevoeligheid voor anorexia nervosa.

In conclusie, het huidige proefschrift toont aan dat verhoogde hyperactiviteit bij verminderde voedselinname veroorzaakt wordt door een complexe interactie tussen verschillende perifere en centrale regelsystemen op het gebied van eetgedrag en (mogelijk) beloning. De hypothalamus in het brein en perifere (hormonale) signalen

naar de hypothalamus spelen daarbij een grote rol. Verder onderzoek zal een beter inzicht geven in de regulatie van energiebalans met betrekking tot hyperactiviteit. Dit biedt hopelijk nieuwe perspectieven voor verder onderzoek en behandeling van patiënten lijdend aan anorexia nervosa, dat grotendeels gekenmerkt wordt door hyperactiviteit.

CURRICULUM VITAE

Linda Adriana Wilhelmina Verhagen werd geboren op 13 november 1979 te Sint-Oedenrode. In 1998 behaalde zij het VWO diploma aan het Mgr. Zwijsen College te Veghel, waarna zij aan haar studie Biologie en Medisch Laboratoriumonderzoek begon aan de Hogeschool van Utrecht te Utrecht. In 2001 volgde zij een afstudeerstage bij het Nederlands Instituut voor Hersenonderzoek te Amsterdam. Onder begeleiding van professor dr. R.M. Buijs werd neuro-endocrinologisch onderzoek verricht naar de invloed van de biologische klok op de voedselopname. Na haar afstuderen in 2002 werkte ze in datzelfde jaar gedurende zes maanden in Strasbourg (Frankrijk), onder begeleiding van dr. A. Kalsbeek (Nederlands Instituut voor Hersenonderzoek) en dr. P. Pévet (CNRS-Université Louis Pasteur). Het onderzoek richtte zich op de regulatie van de biologische klok op de hormoonafgifte van corticosterone in een dagdier genaamd *Arvicanthis ansorgei* Thomas 1910. In 2002 begon zij aan de specialistische master opleiding Neuroscience aan de Vrije Universiteit te Amsterdam. Daarop volgde een afstudeerstage bij Beth Israel Deaconess Medical Center en Harvard Medical School te Boston in de Verenigde Staten. Onder begeleiding van professor dr. J.S. Flier en dr. E.E. Kershaw werd gewerkt aan de rol van β_3-adrenerge receptoren in de regulatie van 11β-Hydroxysteroid Dehydrogenase type 1 in vetweefsel. In oktober 2004, nog voor haar afstuderen, begon zij aan het promotieonderzoek onder begeleiding van professor dr. R.A.H. Adan. De resultaten van het onderzoek naar de moleculaire mechanismen bij eetstoornissen zijn in dit proefschrift beschreven.

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