Of dieting and leptin

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Colophon

Of dieting and leptin

Over afvallen en leptine
(met een samenvatting in het Nederlands)

Proefschrift

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Dedicated to my teachers

Contents

General Introduction

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The past decade has witnessed a steady surge in the incidence of obesity and obesity-related disorders [1, 2]. Although not considered as a disease itself, obesity is a risk factor for several, potentially life-threatening diseases including type II diabetes mellitus and ischemic heart diseases [3]. With its increasing incidence, the global costs for treating obesity-related disorders are also escalating [4]. For example, within the Netherlands, an estimated 41% and 13% of the population has been categorized as overweight and obese, respectively [5]. Such high numbers worldwide have led to the recognition of obesity as a global health hazard. Therefore, resources are been dispatched in order to get more insights into the underlying biology of overeating and obesity in hope of development of better tools, perhaps drugs, to treat obesity.

That said, decreasing one's caloric intake in an attempt to lose weight is popular amongst overweight individuals, but failure to maintain reduced body weight following dietary regimens is more the rule than the exception. Indeed, only a fraction of dieting individuals is able to maintain a 10% reduction in body weight for a year [6]. In fact, a substantial percentage of dieters even report weight gain following dietary interventions [7, 8]. It is known that dieting not only induces behavioral changes such as increased food cravings [9], but it also results in physiological adaptations such as decreased energy expenditure [10]. These behavioral and metabolic adaptations, that can be considered as normal physiological responses to decreased energy intake, ultimately make sustained weight loss a challenging matter. The current thesis describes studies that investigate the underlying neurobiology of a number of behavioral and physiological alterations that occur during dieting. The knowledge provided by the studies aims not only to provide a mechanistic explanation for the high failure rates amongst dieting individuals, but will also to open avenues for new drug targets.

Although the role of the brain in feeding behavior was reported as early as the first half of the 20th century, it was not until the discovery of leptin that people became aware of the importance of specific hypothalamic neuronal populations in body weight regulation [11] . Leptin is an adipokine secreted from white adipose tissue in amounts proportional to the amount of white adipose tissue [12]. Although peripheral effects of leptin have been reported, its central effects are studied to a greater extent [13]. Specifically, the discovery of leptin paved the way for identification of downstream neuronal targets of leptin that mediate the central actions of leptin on food intake and metabolism. The net effect of leptin on the brain principally involves suppression of food intake [12]. However, studies have also shown that leptin increases energy expenditure by influencing locomotor activity and thermogenesis [14-18]. Since the circulating levels of leptin are proportional to the amounts of white adipose tissue [19], this implies that conditions that affect fat depots i.e. either fasting or overeating would decrease and increase leptin levels respectively, which is indeed

observed in rodents and humans [20]. This would also mean that overweight individuals with higher leptin levels would have suppressed food intake, but this is not the case. Rodent studies have shown that increases in leptin levels are associated with a decrease in leptin receptor sensitivity, a term coined leptin resistance [21, 22].

Leptin resistance in diet-induced obesity is predominantly seen in the arcuate nucleus of the hypothalamus (ARC) [23], a structure lying close to the blood-brain-barrier and home to two principal types of neurons: the neuropeptide Y (NPY) neurons and the pro-opiomelanocortin (POMC) neurons [12]. The NPY and POMC neurons are often referred to as the first order neurons, under direct control of leptin [12]. The projections of these neurons form the second order neurons, and in turn, their projections form the third order. Interestingly, the effects of leptin on POMC and NPY neurons are antagonistic. Leptin decreases the firing of NPY neurons while increasing that of POMC neurons [12, 24]. Moreover, expression of the orexigenic peptide NPY is suppressed by leptin while that of anorexigenic peptide POMC is increased [13, 25, 26]. Thus, in a starved or underfed state, when circulating leptin levels are low, higher NPY and lower melanocortin levels promote hunger and the drive to eat [12, 19, 21]. In contrast, in a fed state leptin levels are increased and thus hunger is suppressed by decreasing NPY and increasing melanocortinergic peptide expression [27, 28]. This shows that shifts in energy status (hunger vs satiated state) have profound effects on circulating leptin levels and thereby on ARC neuropeptidergic expression.

Interestingly, dieting, i.e. decreasing one's caloric intake can also be considered to be a state of negative energy balance. Behaviorally, caloric deprivation in rodents is accompanied by increased food-seeking behavior and increased willingness to work for food [29-31]. The latter is considered as the 'intrinsic' motivation of an animal. In a laboratory setting, this is often measured by the amount of lever presses that an animal is willing to make in order to obtain food [32]. Researchers have also established a link between leptin and motivation. Situations of negative energy balance, such as food restriction that decreases fat levels and thus leptin levels, increases the motivation for food reward [33, 34]. This increased motivation can be blocked with leptin injections [35], indicating that alterations in leptin levels influence food motivation. Even though motivation in humans is a difficult parameter to quantify, studies in humans have also shown that decreased leptin levels are associated with increased positive perception of food rewards, suggesting that individuals with low leptin levels are more prone to overeat in order to feel satisfied [36]. Although these findings correlate leptin levels to food motivation, they do not identify the underlying biological mechanism. A logical question to ask is therefore whether the increased motivation during food restriction is mediated by the immediate downstream targets of leptin, i.e. the NPY or POMC peptides.

Next to behavioral adaptations, human studies have also shown that caloric restriction results in physiological adaptations that impede weight loss. Rosenbaum and Leibel, through their elegant weight-loss studies, have shown that attenuated leptin levels following weight loss, trigger adaptive responses that primarily decrease thermogenesis and basal metabolic rate to a level that is sufficient to arrest further weight loss [37, 38]. Interestingly, leptin injections in these subjects counteract the decreased thermogenesis and promote weight loss [18, 39, 40]. Furthermore, fMRI studies broadly connect decreased leptin signaling within the hypothalamus to decreased thermogenesis during dieting [38, 41]. These studies provided an excellent basis for our own research where we focused on leptin signaling within the rodent hypothalamus and its relation to thermogenesis.

Outline of the thesis

Food consumption and energy expenditure are the crucial two pillars of body weight regulation. To date, the majority of the studies on leptin has focused on its influence on food consumption. However, food consumption is a broad term and encompasses various facets such as intake, satiation, liking and motivation. The current thesis sheds light on the role of leptin and its immediate downstream targets, NPY and POMC peptides, on one important aspect of food intake i.e. motivation for food. The thesis also addresses the question on the role of leptin in energy expenditure by investigating its influence on thermogenesis. In short, the thesis focuses on the rather 'less discussed' aspects of leptin and its immediate downstream targets in metabolism i.e. its effects on motivation for palatable foods and its effects on thermogenesis.

The first goal of the thesis, addressed in section I, was to unravel how leptin and its immediate downstream signaling components affect food motivation, i.e. to unravel how POMC and NPY neuropeptides work in several brain areas to influence the motivation for food reward.

Chapter 1 reviews available literature on how different dietary manipulations that result in leptin resistance, affect the motivation of an animal for palatable rewards [42]. **Chapters 2 and 3** of the thesis review literature and give an overview how the leptin, NPY and melanocortins regulate hedonic food intake [43]. In summary, these chapters compile our existing knowledge on the hedonic aspects of food intake and the role of melanocortins and NPY in food reward [33, 44, 45]. The global message from the first three chapters is that both food restriction and obesity, besides influencing ARC neuropeptide levels (NPY up, POMC down), also increase motivation for palatable food rewards. This indicates that irrespective of the metabolic status (hunger or obesity), alterations in ARC NPY and POMC levels correlate with alterations in motivation. Thus, in order to investigate the causality of these correlational pieces of evidence, ad-libitum fed rats were centrally and locally treated

with NPY or POMC peptides and tested for motivation for palatable food rewards. The results from these experiments are presented in the subsequent three chapters. **Chapter 4** studies the role of NPY in three different brain areas, the lateral hypothalamus (LH), ventral tegmental area (VTA) and the nucleus accumbens (NAC), regions receiving projections from the first order ARC NPY neurons and being a part of the so called 'limbic reward circuitry', that regulates motivation and reward [46]. In this chapter we make a distinction between the effects of NPY on motivation and free consumption. Similarly, **chapter 5** and **chapter 6** focus on the motivational effects of feeding-related POMC peptides, i.e. the melanocortins. **Chapter 5** addresses the effects of central melanocortin 4 receptor stimulation on motivation and free consumption while in **chapter 6** the role of central melanocortin 3 receptor stimulation on motivation and free-consumption is investigated.

The goal of **section II** was to investigate leptin's involvement and to provide a mechanistic basis for the thermogenic adaptations as a response to decreased caloric intake in a animal model of dieting. **Chapter 7** reviews literature to summarize the contribution of leptin signaling within the various hypothalamic neuronal populations on thermogenesis. It gives a comprehensive insight on how leptin-sensitive hypothalamic neurons influence theremogenesis via the sympathetic nervous system through multiple parallel pathways projecting to the brainstem. Previous literature indicates that alterations in leptin levels during weight loss decreases thermogenesis and hinders further body weight loss [47]. Therefore, in **chapter 8** of the thesis, the dynamics of these thermogenic adaptations to body weight loss was first investigated in a rat model of dieting. We hypothesized that, similar to humans, a decrease in caloric load will be accompanied by reduced thermogenesis. Supported by evidence in literature (**chapter 7**), we further hypothesized that leptin signaling within the dorsomedial hypothalamus is affected during the dieting phase and therefore investigated the role of this nucleus in thermogenesis. Finally, the general discussion weaves together the findings from the experimental chapters and puts them in a broader perspective i.e. how leptin and its immediate downstream targets influence energy metabolism. In conclusion, the thesis addresses the role of leptin and its immediate downstream targets in food motivation and the effects of dieting. It also provides insights into the fact that not only food intake, but also the motivation for food and thermogenesis substantially contributes to the body weight of an organism.

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Section I

Chapter 1

Dietary factors affect food reward and motivation to eat

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Abstract

The propensity to indulge in unhealthy eating and overconsumption of palatable food is a crucial determinant in the rising prevalence of obesity in today's society. The tendency to consume palatable foods in quantities that exceed energy requirements has been linked to an addiction-like process. Although the existence of 'food addiction' has not been conclusively proven, evidence points to alterations in the brain reward circuitry induced by overconsumption of palatable foods and similar to those seen in drug addiction. The diet-induced obesity paradigm is a common procedure to replicate features of human obesity in rodents. Here we review data on the effect of various obesogenic diets (high-fat, Ensure, cafeteria type, sucrose) on the extent of leptin resistance, hypothalamic-neuropeptidergic adaptations and changes in feeding behavior. We also discuss to what extent such diets and properties such as macronutrient composition, physical structure, sensory stimuli, post-ingestive effects influence the brain-reward pathways. Understanding the interaction between individual components of diets, feeding patterns and brain reward pathways could facilitate the design of diets that limit overconsumption and prevent weight gain.

Introduction

In the past decade, increasing enthusiasm and debate has been generated on the concept of 'food addiction'. Even though the validity of such a process has so far not conclusively been proved [1], several researchers indicate similarities between over-eating and addiction [2, 3]. Functional brain imaging studies in obese and addicted individuals have shown similar activation patterns when subjects are exposed to food or drugs of abuse. For instance, involvement of the brain reward circuit, one of the principal neuronal pathways responding to natural (food, sex) or drug rewards, has been consistently associated with obesity and addictive states [4, 5]. Elevated dopamine levels in the nucleus accumbens noted in animals lever-pressing for food or receiving electrical stimulation of the lateral hypothalamus or systemic injections of cocaine or amphetamine [6] indicate that effects of both drugs and food converge on the midbrain dopaminergic circuit.

The role of genes regulating food intake and degree of adiposity is well-known [7]. Prominent examples include the leptin [8] and melanocortin systems [9]. Gene mutations in these neuropeptidergic systems are accompanied by obesity [10]. Even though human subjects with single-gene mutations are well suited for studying the function of a certain gene with respect to energy metabolism and reward sensitivity [11, 12], animals with monogenic forms of obesity are far from perfect tools to model the current obesity epidemic. Instead, the rising incidence of obesity can be studied as interplay between predisposing genetic factors and the consumption of obesogenic diets. A range of such diets are used in laboratories to induce obesity in rodents [13]. Despite the fact that these diets differ substantially in macronutrient composition, taste, form, and period of availability, a cardinal consequence is the development of obesity, hyperinsulinemia, and hyperleptinemia [14, 15].

High-calorie diets rich in sugars and fat are considered highly palatable and are readily consumed by the population [16]. But, what makes these food items more 'desirable' compared to other low-calorie foods? Several authors argue that such diets are more 'rewarding' and have an addictive potential [2, 17]. Thus, by identifying whether certain types of diets are more 'addictive' than others and the specific features (or components) of such diets that make them more 'rewarding', development of novel foods curbing overconsumption and weight gain can be attempted. Importantly, overconsumption of food can be viewed in the light of two mechanisms: i) disturbed homeostatic regulation of food intake or satiety mechanisms and ii) increased motivation to consume food. The mechanisms relating to disturbed homeostatic regulation of food intake or satiety are independent and far-reaching topics and are beyond the scope of the current review. Readers are referred to other relevant literature [18--20]). The current review is divided

into three sections and aims to understand how various obesogenic diets alter motivation for food reward. The first section gives an overview of the various laboratory diets used to model obesity in rodents. The second part delineates and compares behavioral and molecular data on these palatable diets with drugs of abuse in an attempt to elucidate how palatable diets modify the brain reward circuitry. In the final section, we try to focus on the individual (macronutrient composition, structure, sensory, biochemical and post-ingestive) properties of these diets and analyze their rewarding nature.

Diet and Leptin Resistance

Diet-Induced Obesity

Extensive study of rodent models of obesity caused by either single gene loss-of-function mutation or targeted transgenic knock-out has identified a number of genes, the products of which are essential for normal regulation of body composition and body weight. These key regulatory genes are located on the energy intake rather than the energy expenditure side of energy balance, and a number of these are associated with the leptin-melanocortin hypothalamic pathway, thereby emphasizing the importance of this circuit. However, it is generally recognized that most obesity cases in the human population is not secondary to mutations in single genes essential to energy balance, but rather reflects a genetic predisposition to which many genes contribute. In this polygenic obesity, the obese genotype is exposed by the obesogenic environment of modern-day society, where there is free access to a variety of energy-dense, palatable foods and sedentary lifestyles are the norm. As a consequence, mechanistic studies of obesity have increasingly focused on rodent models where obesity is induced by dietary manipulation (diet-induced obesity), as a convenient model for the human situation. A range of rodent species, strain, and diet combinations have been employed in both rats and mice to generate diet-induced obesity [21], with outcomes recorded at the level of signaling from the periphery, such as leptin, insulin and gut peptides, and activity of brain signals in the hypothalamus and forebrain. Here, we will compare the impact of high-fat diets, cafeteria diets, the nutritionally complete liquid diet, Ensure™, and sucrose solutions on caloric intake, obesity, and the development of leptin resistance.

Leptin Resistance

The adipose tissue hormone, leptin, is secreted into the bloodstream in proportion to body adiposity and is elevated in common human obesity. This is despite the known catabolic role of leptin in the regulation of energy balance, i.e. reduction in food intake, elevation of energy expenditure, and overall drive towards negative energy balance. The failure of elevated leptin levels to control or reverse obesity suggests the existence of a leptin-resistant state [22]. Rodent studies have demonstrated two distinct components to leptin resistance -- a

resistance to peripherally administered leptin suggesting a failure of the hormone to access CNS target sites, and/or resistance to CNS leptin resulting from impaired responses in CNS neurons expressing the leptin receptor. Leptin resistance also accompanies the obesity associated with aging. Interestingly, there are a number of circumstances in which leptin resistance appears to be developmentally programmed to meet prevailing physiological requirements, including pregnancy, lactation, and seasonal body weight change (e.g. in the Siberian hamster [23]).

The Relationship between Diet-Induced Obesity and Leptin Resistance

There is evidence of both direct and indirect effects of diet, and more particularly high-fat diet, on leptin resistance. Thus, high-fat diets can induce leptin resistance either directly in the absence of obesity or indirectly as a consequence of obesity and resultant high leptin levels. The direct effect is presumed to be due to dietary components or their metabolites acting upon leptin-sensitive neurons. The evidence in support of this direct effect, which comes principally from assessment of early changes in sensitivity following dietary manipulation and/or the feeding of energy-restricted rations to prevent excess weight gain, has been reviewed by Morrison et al. [24]. In contrast to the putative direct effects, most leptin resistance is characterized under conditions of an existing hyperleptinemia that down-regulates leptin receptors and stimulates the negative regulators, suppressor of cytokine signaling 3 (SOCS3) and protein tyrosine phosphatase 1B (PTP1B). Thus, the picture emerging from high-fat diet-induced obesity is that of leptin resistance as a cause and consequence of obesity. There is evidence from outbred rat strains which exhibit within-population differences in susceptibility to diet-induced obesity that inherent differences in leptin sensitivity prior to dietary manipulation may be predictive of weight gain [25, 26], i.e. leptin resistance predisposes towards or promotes obesity, although the precise relationship and the underlying mechanisms remain to be resolved [for review see 22]. A particularly relevant observation is that all models of leptin resistance develop obesity on a high-fat diet, whereas only a few will show this effect on a stock diet [22]). This suggests a link between leptin resistance and overconsumption of palatable diets, most likely involving food reward.

High-Fat

A very wide range of high-fat diets have been used to induce obesity, with considerable variability in fat content and source, macronutrient composition, energy density, and physical formulation [21, 27, 28]. Most commonly, diets with either 45% or 60% fat by energy are employed, but variation in experimental specifics such as species, strain, duration of dietary manipulation, age at manipulation etc. all influence the precise phenotypic outcome. Whereas it may not be possible to identify an ideal high-fat diet, more standardization in dietary and experimental design would be advantageous. For diets with

fat contents of greater than 40% by energy, Buettner et al. [27] concluded that animal fats and Omega-6/Omega-9 fatty acid-containing plant oils will induce obesity whereas fish oil Omega-3-fatty acids will not.

Obesity and elevated leptin levels on high-fat feeding are accompanied first by resistance to leptin administered by peripheral injection and then by central leptin resistance, as outlined above. For example, in C57BL/6J mice fed a high-fat diet (45% fat; D12451, Research Diets, New Brunswick, NJ, USA), food intake responses indicated resistance to peripheral leptin within 16 days, although AKR mice subjected to the same dietary manipulation were still sensitive at this point [29]. By 56 days, both mouse strains were insensitive to peripheral leptin, but the AKR mice were still sensitive to intracerebroventricular (ICV) leptin. Using the same diet with C57BL/6J mice, a separate study showed no effect of high-fat diet on peripheral leptin-induced STAT3 signaling in the hypothalamus after 4 weeks, but leptin sensitivity was abolished after 15 weeks, although an attenuated response to ICV leptin remained [30]. In a similar study, also in C57BL/6J mice, but using a bespoke high-fat diet with 59% fat by energy [31], peripheral and central sensitivity to exogenous leptin was examined after 1, 8, or 19 weeks on diet. Temporal development of leptin resistance was described, with the three time points being characterized by full sensitivity to peripheral leptin, central but not peripheral leptin sensitivity and reduced central leptin sensitivity, respectively. In line with the conclusions of Buettner et al. [27], the fat component of D12451 is contributed by a mixture of approximately 7:1 lard: soybean oil, whereas the bespoke diet contains 3:1 safflower oil:tallow stearin, i.e. both diets contain a mixture of plant oils and animal fats. The composition and origin of fat in the diet may influence not only the progression towards obesity, but also leptin signaling. The direct effect of high-fat diet on leptin resistance may reflect the direct action of fatty acids, triglycerides, and lipid molecules at leptin-sensitive neurons, a possible component of hypothalamic nutrient sensing [32], or an effect on leptin transport across the blood-brain-barrier [33]. Importantly, several high-fat test diets also include a substantial amount of sucrose in its composition (for example, in D12451 17% of total calories comes from sucrose). This further complicates the interpretation of the effect of such diets on developing obesity and leptin resistance.

Cafeteria Diets

The high-fat diets referred to above are generally assumed to induce obesity as a result of palatability-driven over-consumption, but in most instances are fed as a single diet where the ratio of macronutrients effectively obliges the animal to consume what may be an unbalanced diet. This may not be palatability-driven overconsumption, but rather 'passive' overconsumption resulting from the obligatory consumption of the imposed macronutrient profile. It is interesting to observe how the composition of the diet changes when a

combination of different defined diets is offered and the animal is able to select a macronutrient profile that matches its 'requirements' [34]. The combination of variety and palatability in a so-called 'cafeteria diet' is almost certainly a better model for the human food environment.

Cafeteria diet is a general term used to describe an energy-dense diet composed of a variety of food items representative of the Western diet. These food items tend to be high in both fat and sugar and consequently are often energy-dense. Such a diet has the advantage of allowing novelty, choice, and variety, which are key characteristics of the modern human food environment, but the heterogeneous nature of the foodstuffs, and their means of presentation, can complicate the calculation of precisely how many calories are being ingested and what macronutrient profile is being selected [35]. Nevertheless, cafeteria diets typically drive hyperphagia and over periods of several weeks will induce obesity. Whereas direct assessment of leptin resistance through administration of exogenous leptin to cafeteria-obese rodents does not appear to have been performed, this obesity is accompanied by hyperleptinemia and hyperinsulinemia, and as with high-fat diets the downstream consequences of hyperleptinemia are indicative of leptin resistance. Following 10 weeks of cafeteria feeding, where 4 palatable human foods were changed every day on a weekly rotation, elevated body weight, body fat, and leptin levels were accompanied by a down-regulation of leptin receptor gene expression in the hypothalamus of male but not female rats [36], an indicator of reduced signaling through the receptor, at least in males. The availability of a wide choice of calorie-dense food is an important determinant for the overconsumption witnessed in humans. Apart from the cafeteria diet that incorporates the aspect of choice, the free-choice, high-fat, high-sucrose (fcHFHS) diet is another example where the connection between a high-energy choice diet and developing obesity can be studied. Animals given a choice between lard (high fat), 30% sugar (high sugar) solution and standard chow showed prolonged hyperphagia, progressive increase in body weight as well as increased fat mass and circulating leptin levels. Interestingly, in animals exposed to only lard or only sugar solution besides regular chow, the initial hyperphagic response and accelerated weight gain, was followed by normophagia and weight gain comparable to control chow-fed animals [37, 38]. Thus, the presence of palatable (sucrose) and energy-dense (lard) components in obesogenic diets may be crucial in the development of hyperphagia-induced obesity.

Ensure™

Ensure™ (Abbott Laboratories, Abbott Park, IL, USA) is a complete liquid diet that comes in a number of flavors, all of which increase total daily energy intake by approximately 15% when fed ad libitum as a supplement to stock (low-fat, low-sugar) diet pellets [39]. The most substantial body of work describing its use has come from the Sprague-Dawley rat model of

diet-induced obesity. Here, chocolate Ensure was deployed by Levin and co-workers [40] as a palatable supplement to stimulate obesity in rats with a low weight gain response to an obesogenic high-sugar, high-fat pellet diet. However, it is now clear that ad libitum Ensure supplementation stimulates sustained caloric overconsumption and increased weight and body fat gain irrespective of original body weight trajectory in Sprague-Dawley rats [15] and irrespective of the pellet diet background [41]. Ensure supplementation clearly provides an element of dietary choice but even within a single rat strain (Sprague-Dawley) there can be considerable variability in the proportion of calories taken from the liquid feed. Thus in 400 g+ rats, given a choice of Ensure and the Research Diets High Energy (HE) diet, 82% of calories were taken as the liquid [42], whereas in juvenile 100 g+ rats Ensure intake accounted for only 23% of calories with the same dietary combination, and only for 55% of calories when Ensure was fed as a supplement to a stock pellet [41]. Nevertheless, provision of the Ensure supplement ad libitum is generally accompanied by increases in caloric intake, weight gain, body adiposity, and plasma leptin [41, 42], with a doubling of plasma leptin concentration, for example, when deployed as a supplement to stock pellet [41] (Mercer et al., unpublished data). Although we have no evidence of an effect of Ensure on leptin receptor gene expression in any of the above studies, when Ensure was used as a supplement against a background of one of two pellet diets (HE or stock) or a combination of the two [41], a comparison between rats that did not have Ensure and those that did revealed that Ensure affects the association between adipose tissue and circulating leptin through an overall depressive effect on leptin concentrations. Whereas there is no firm evidence of induction of leptin resistance by Ensure, engagement with the reward system is indicated in studies in which scheduled (3 h daily) access to Ensure down-regulated striatal opioid gene expression [43], although these changes appeared not to be accompanied by changes in body weight or presumably blood leptin levels.

Sucrose

There is a multitude of publications on the effects of feeding sucrose and other sugars to laboratory rodents, and a range of outcomes have been reported. For example, with a 32% sucrose solution fed as an ad libitum supplement to stock pellet from weaning to 70 days of age, caloric intake was increased by 23% [44]. However, there was no difference in body weight, although percentage body fat was increased. A very similar outcome -- elevated caloric intake but no effect on growth -- was recorded in another study of weanling rats allowed access to 32% sucrose [45]. However, the variability in outcome between different studies is well illustrated by the outcome of feeding adult rats (up to 210 days of age) with the same concentration of sucrose solution, where no increase in total caloric intake was evident [46]. A direct comparison of sucrose, glucose, or fructose supplied as a 32% solution in addition to standard diet to 250--300 g rats resulted in the consumption of more calories, weight gain and increased body fat [47]. Using sucrose concentrations more akin to those

found in commercial soft drinks, we have determined that 12.5% or 25% solutions fed ad libitum to 250--300 g rats displace stock pellet in the diet and increase overall caloric intake by 20--25% but have no effect on body weight or leptin levels following a 4-week manipulation, although body fat tends to be elevated in the 12.5% group (Mercer et al., unpublished data). In addition, when rats were subjected to 30% sucrose in addition to stock pellets for 4 weeks, significant increases in caloric intake, fat mass, and leptin were observed [37, 38]; however, this did not result in changes in leptin sensitivity (la Fleur et al., unpublished data). In a recent study, 3 out of 4 mouse strains tested showed increased caloric intake (11-25%), weight gain, and body adiposity when fed a 34% sucrose solution for 40 days [48]. By contrast, 10% sucrose, although eliciting a powerful drinking response, did not elevate caloric intake to the same extent, and did not increase body weight or body fat significantly in any of 4 mouse strains, although there were trends towards excess weight gain in the three sensitive strains. Interestingly, the 'sucrose-resistant' FVB strain consumed more of each sucrose solution than the other three strains that went on to develop a relative obesity.

Taken together these studies raise doubts about sucrose feeding as the sole dietary manipulation as a reproducible model of diet-induced obesity, since outcomes appear to be heavily influenced by experimental details. The relatively small changes in body adiposity that do occur make it unlikely that there will be any effect on leptin sensitivity, which probably explains why this has not been systematically examined. However, interestingly, chronic fructose consumption will induce leptin resistance, and does so in the absence of obesity. Rats fed a 60% fructose diet for 6 months developed leptin resistance but had normal body adiposity and leptin levels [49]. These leptin resistant animals had increased susceptibility to diet-induced obesity on a high-fat diet. Table 1 gives an overview of various diets leading to obesity and their influence on leptin signaling.

Diet and the Brain Reward Circuitry

The previous section gives us a glimpse on the effects of diverse obesogenic diets culminating in obesity and leptin resistance. The current section takes a step forward and evaluates the influence of these diets (and diet components) on the brain reward circuitry. The brain reward circuit involves discrete nuclei: ventral tegmental area, nucleus accumbens, amygdalar complex, prefrontal cortex, and neurotransmitter systems, dopaminergic, GABAergic, opioid and serotonergic systems [50]. A majority of the studies conducted in rodents and humans focus on the mid-brain dopaminergic system. This system comprises a population of dopaminergic cells in the ventral tegmental area that project to the nucleus accumbens and the prefrontal cortex, among other brain areas [50]. The actions of dopamine in these target areas are mediated by two subsets of dopaminergic receptors: dopamine receptor type 1 and type 2 (D1 receptor and D2 receptor).

Sucrose

Sucrose is perhaps the most widely studied dietary component that has been discussed with respect to its influence on the brain reward circuitry. A naturally occurring plant sugar, this disaccharide, comprising of the monosugars fructose and glucose, is a well known natural reinforcer and has been extensively used in behavioral research (operant learning, sucrose preference test). The relation between sucrose consumption and psychiatric states like substance dependence and mood disorders (e.g. anhedonia in depression) has been documented [51]. Studies with human subjects undergoing methadone treatment for addiction showed enhanced sucrose consumption in these patients compared to healthy women [52], and propensity to overindulge in sweet foods was noted in a cohort of drug addicts in Norway [53].

Extensive work in the field of 'sugar addiction' has been conducted by Hoebel and colleagues [54, 55]. In their model of 'sugar addiction', schedule-fed rats that were repeatedly exposed to sugar solutions (10% sucrose or 25% glucose) in addition to standard laboratory chow engaged in binging [54, 55]. In these animals, enhanced dopamine release in the nucleus accumbens shell [56], increased D1 receptor binding in the dorsal striatum and decreased D2 receptor binding in the dorsal striatum and nucleus accumbens (core and shell) [54] were noted, a pattern of findings comparable to that observed in animals exposed to drugs of abuse [3]. Behavioral cross-sensitization experiments revealed that animals sensitized to amphetamine for 5 consecutive days, exhibit locomotor sensitization when exposed to 10% sucrose solution but not water [55]. Conversely, animals having intermittent access to 10% sucrose for 22 days showed heightened locomotor activity following low-dose amphetamine injection [56]. The paradigm used by Hoebel and colleagues [57, 58] involved food deprivation in the early dark phase (when animals are hungry and have a naturally high drive to eat), hence the effect of hunger (and low leptin levels) influencing sugar binging cannot be overlooked. Studies have consistently demonstrated a robust interaction between low leptin levels and heightened motivation. Food-restricted animals (i.e. low leptin levels) showed heightened sensitivity to drugs of abuse [59] and increased motivation to work for a palatable reward [60]. Contrarily, ICV administration of leptin decreased sucrose uptake in rats [60]. Thus leptin is implicated in modulating the reward system. For further details on the interaction between leptin and the brain reward circuitry, we refer the readers to Pandit et al., 2011 [18].

It is important to note that a combination of several factors (taste, caloric value, post-ingestive and sensory properties) act in concert when animals are exposed to sucrose solutions. But which of these individual factors actually play a role in making sucrose

'addictive' remains unknown. Cannon and Palmiter [61] concluded that sweet taste itself can be rewarding and the participation of dopaminergic system is not a prerequisite to experience the rewarding properties of sucrose. Dopamine-deficient mice given a choice between sweet solutions (sucrose and saccharin) and water preferred the former. However, the authors failed to dissociate the two facets of reward reinforcement: 'liking' and 'wanting' [62]. Preference for sweet solutions over water may simply indicate the enhanced 'liking' (an effect primarily mediated by the opioid system) in comparison to 'wanting' (involving the dopamine system). But, does sweet taste (rather than caloric value or nutrient type) influence the mesolimbic dopamine circuit? Animals sham-fed with varying concentrations (0.3 mol/l, 0.1 mol/l, and 0.03 mol/l) of sucrose showed a dose-dependent increase in intake accompanied by augmented dopamine levels in the nucleus accumbens, underpinning the fact that orosensory properties of sucrose are important in determining the rewarding aspects of sucrose [63]. Contrarily, Ren and colleagues [64] demonstrated that mice devoid of functional sweet and bitter taste receptors, when given a choice between sucrose and an isocaloric amino acid solution, preferred the former. Intragastric infusions of sucrose in these mice showed enhancement of dopamine levels in the nucleus accumbens, stating that besides the orosensory and caloric properties of sucrose [65], nutrient type itself influences the mesolimbic dopaminergic circuit.

Many experimenters use saccharin, an artificial sweetener without any caloric value, to dissociate the orosensory properties of sucrose (also provided by saccharin) from its post-ingestive metabolic properties. Animals trained to self-administer ethanol or saccharin in a fixed ratio schedule of reinforcement of operant training showed increased lever pressing for saccharin and ethanol compared to water [66]. Additionally, non-food-deprived naïve animals exposed to saccharin showed increase in nucleus accumbens dopamine levels [67]. But, when subjected for longer periods to saccharin, this effect wore off [66, 68], indicating that elevated dopamine levels in naïve animals exposed to saccharin may be a novelty effect and not a consequence of the rewarding properties of the compound. Likewise, sucrose, but not saccharin, conditioned a place preference in rodents while the amounts ingested were equal in both cases, underpinning that place conditioning for sucrose is a function of post-ingestive metabolism of sucrose [69].

Fat

Saturated fats make up of a substantial portion of cafeteria/fast food diets. The level of fats in a diet correlates positively with palatability and negatively with satiety [70]. Lean and obese human subjects given a carbohydrate or a fat-rich diet showed increased hunger ratings and more caloric consumption in subsequent meals after a fat-rich breakfast [71]. Analogous findings with fat-rich diets being associated with a relatively low sense of fullness and increased sense of hunger have been documented [70, 72, 73]. This low satiating effect

of fat-rich diets may be due to the slower digestion of fats that delays the post-absorbtive phase of satiety [71], thus augmenting the possibility of overindulgence in such diets. The amount of data focusing on the effect of an exclusively high-fat diet on the brain reward circuitry is limited. Furthermore, the variability in the type of diet used in animal studies makes comparison of results across laboratories difficult [13, 74]. The fat content in a high-fat diet [74], the type of fat (animal vs. plant) [37, 75], the flavor [76], the inclusion of other diet components besides fat (chow, sugar) [14, 37], and the duration of access to the diet [77] all have an effect on the behavior and neurobiological alterations.

Using the conditioned place preference test, Figlewicz and colleagues [78] demonstrated that ad-libitum fed animals spent substantially more time in a high-fat paired compartment. Animals given restricted access to either vegetable shortening or a high-fat diet exhibited binge eating-like behavior [75, 79] reminiscent of the binge eating phenotype observed in rodents with limited access to sucrose [54, 55] or alcohol [80] solutions. Changes in the mesolimbic dopaminergic circuit include an initial increase in nucleus accumbens dopamine levels following exposure to a high-fat diet [81], followed by attenuated dopamine levels after more prolonged access [82]. Decrease in tyrosine hydroxylase (a rate-limiting enzyme in dopamine synthesis) messenger RNA was also observed in mice exposed to a high-fat diet for 6 weeks, which was not reversed when obese-prone animals were switched to and maintained on standard chow for 6 weeks [83]. Moreover, reduced dopamine levels were noted in obese-prone animals which were initially on a 5-day high-fat diet and later switched to a 2-week diet of standard laboratory chow, suggesting long lasting neurobiological modifications in response to high-fat diets [84]. Finally, reduced striatal D2 receptor levels were noted in response to prolonged exposure to a high-fat diet [3, 85]. Collectively, these findings are in agreement with the neurobiological alterations that have been observed in rodent models of drug addition. Prolonged indulgence in drugs or high-fat diet leads to alterations in the mesolimbic dopaminergic circuitry, which may not be immediately reversible after discontinuation of the abused drug or high-fat food [82, 83, 86].

High-Fat, High-Sucrose Choice Diet

Bearing in mind the availability of different high-calorie food choices in the market, the free-choice high-fat, high-sucrose diet is a relatively novel paradigm employed to study diet-induced obesity. Animals kept on this diet for 1 week demonstrated hyperphagia, hyperinsulinemia and leptinemia [14, 37]. Exposure to this diet delayed the breakpoints in a progressive ratio scheme of reinforcement for a palatable reward [14]. Besides, the arcuate nucleus of these animals shows an increase in neuropeptide Y expression [37], a peptide well known for augmenting motivation in a progressive ratio paradigm [87]. Thus, augmented motivation in response to a high-fat, high-sucrose diet may be mediated by this neuropeptide. A recent paper evaluates the effect of this diet on the mesolimbic

dopaminergic circuitry. Prolonged but restricted access to the high-fat, high-sucrose diet decreased D1 and D2 receptor levels in the nucleus accumbens, an effect unseen when animals are given unlimited access to this diet. Thus the observed alterations in D1 and D2 receptors are a direct consequence of the exposure to the diet rather than a result of the hyperphagia or obesity associated with this diet [88].

Cafeteria Diet

The cafeteria diet is another example where animals are given a choice of several high-fat, high-sugar food items besides standard rodent chow to induce obesity [89-- 91]. Even though the compositions of such diets vary widely, a central feature includes the availability of high-caloric food components (rich in sugars and fat). Like most other palatable foods [73, 76], withdrawal from extended access to cafeteria diet leads to pronounced hypophagia [90, 92]. The basal (low) levels of dopamine in the nucleus accumbens after ad-libitum feeding on a cafeteria diet were augmented when animals were re-exposed to a cafeteria diet but not when exposed to standard chow [89]. These data echo the findings where animals given prolonged access to amphetamine, self-administer the drug to maintain a stable tonic level of dopamine in the nucleus accumbens [93]. Similar to drugs of abuse, decreased amounts of striatal D2 receptors were seen in rats exposed to a cafeteria diet [90]. This reduction in D2 receptors was not only accompanied by deficits in reward sensitivity but also by compulsive food-seeking behavior in the light of adversity [90], a crucial criterion for the diagnosis of substance abuse [94]. Compulsive overconsumption of cafeteria food was also noted by Heyene and colleagues [95]. In their model, animals exposed to ad-libitum cafeteria diet for 11 weeks showed inflexibility in increasing their chow intake coupled with depression of motor activity during limited access to cafeteria diet. In addition, cafeteria diet adulterated with a bitter taste was consumed by a substantial proportion of animals which were chronically exposed to this diet [95]. Thus, chronic exposure to 'addictive' substances (drugs or palatable foods) lowers the extracellular dopamine and D2 receptor levels in the nucleus accumbens, and animals respond to this neurochemical alteration by self-administration in excessive amounts of drugs or food to experience the same hedonic level (of subjective pleasure).

Opioids and Palatability

Molecular cross-talk between the mesolimbic dopaminergic system and other neuropeptides at the level of the brain reward centers appears to regulate reward processing [96--98]. The endogenous opioid system, comprising of the opioid receptors (Δ-, -, μ-opioid receptor) and their peptide ligands (endorphins, enkephalins, dynorphins) is distributed throughout the brain reward centers and has been implicated in drug addiction [98, 99]. Opioids increase dopamine release in the nucleus accumbens by either increasing μ- and δ-receptor activation in this area [100] or by diminishing GABA inhibition of the

dopaminergic neurons in the ventral tegmental area [101]. Neurobiological correlates to the tolerance and dependence triggered by chronic opioid abuse includes the subdued sensitivity of the μ-opioid receptor [98] and attenuated levels of endogenous opioids [102]. Analogous to their effects on the mesolimbic dopaminergic circuitry, obesogenic diets interact with the endogenous opioid circuitry. Down-regulation of enkephalin gene expression was noted after repeated ingestion of a highly palatable nutritionally complete liquid diet Ensure [43], reminiscent of the effects of chronic morphine exposure on striatal gene expression [102]. Since Ensure has a heterogeneous macronutrient composition (including carbohydrates, fats, proteins, and added flavors), the interdependence of the endogenous opioid system and individual macronutrient composition of diets remains undetermined.

Morphine-injected animals increased the consumption of fat [103], whereas treating animals with naloxone (a competitive μ-opioid receptor antagonist that precipitates withdrawal symptoms in opioid addicts) attenuated fat intake [104]. Intra-accumbens injection of D-Ala2 N-Me-Phe4 Gly-ol5-enkephalin (DAMGO), a selective μ-opioid receptor agonist, enhanced fat consumption in satiated rats [105]. This suggests that, similar to the effects of morphine [106], intake of high-fat food is mediated by the μ-opioid receptor and involves the nucleus accumbens [105, 107], but the debate whether opioids specifically favor fat intake or promote the consumption of a preferred diet (fat vs. sugar) is ongoing [108].

Interactions between palatable foods other than fat and the endogenous opioid system have been reported [109, 110]. For example, animals binging on sugar [55] exhibited signs of withdrawal when administered naloxone [110], and naloxone-treated animals decreased the amount of time spent in a sucrose-paired chamber in a conditioned place preference paradigm [111]. Likewise, withdrawal symptoms in morphine-dependent animals after naloxone treatment were attenuated when animals were given prior access to a 30% sucrose solution [112]. Despite the behavioral observations, underlying mechanisms dictating the interaction between the endogenous opioid system and palatable food intake remain undetermined. Evidence suggests that the morphine-induced preference for sucrose solutions [113] may be taste-dependent. ICV injection of DAMGO showed a dose-dependent enhancement in saccharin intake [114], and animals sham-fed with sucrose solutions exhibited a dose-dependent decrease in sucrose consumption when pre-treated with naloxone [115], excluding the possibility of post-ingestive effects of sucrose on the endogenous opioid system (readers are directed to an excellent review on this topic by Kelly et al. [116]). An overview of the interaction between various diets and the brain reward circuitry has been provided in Table 2.

Most of the current knowledge on the pathogenesis of obesity and the motivational aspects

of palatable diet comes from rodent models. Hence, the current review is limited to experimental data on rodent species. Despite the relevance of the transition from rodent models to humans, studying the onset and development of obesity in humans is a challenging task bound by ethical and technical constraints. Research conducted on humans can be narrowed down to comparisons between obese subjects, obese subjects on decreased body weight and normal-weight controls [117], thus impeding the study of factors responsible for the development of obesity.

Nonetheless, the rapid development in the field of imaging techniques and psychological testing has enabled us to study and verify the similarities between neuroanatomical/ behavioral processes in humans as encountered in rodent models. Wang and colleagues [118] have shown an inverse relationship between D2 receptor availability and BMI in obese individuals, echoing the findings of several rodent studies. In addition, it was shown that, similar to processes observed in drug users, lower striatal D2 receptor availability in obese humans correlated with decreased pre-frontal cortical metabolism, an area implicated in inhibitory control [3, 119]. Finally, genome-wide association studies are yet another approach where human data are used to study a particular medical condition. It entails an analysis of differences in gene polymorphisms between diseased and healthy populations [120]. Although this approach enables us to identify candidate genes implicated in obesity, the use of animal models remain compelling for the verification of the role of these genes.

Exploring the Role of Food Composition and Structure

Initial hedonic ratings and long-term motivation to eat foods are weakly correlated [121]. Apparently, food reward is shaped not just by organoleptic stimuli such as aroma, taste and texture, but also by processes based on post-ingestive physiological signals that are registered as more or less rewarding by the CNS. The relationship between food properties and their sensory and physiological effects will be described next.

Sensory Effects of Food

Aroma, taste, and texture govern the decision to ingest or reject foods. Not surprisingly, the food industry puts major investment into product optimization to meet consumer preferences and motivate repeat purchase of foods [122]. This is well illustrated by the history of chocolate design. When introduced to Europe in the early 16th century, chocolate was only available as a relatively unprocessed, unpalatable, and gritty cocoa beverage with an oily surface layer. Centuries of technological innovation shaped today's highly rewarding chocolate bars. Nowadays, chocolate's attributes include a pleasant bite with just the right amount of snap (German: 'Knack'), fat crystals purposely designed to have an attractive gloss and melting point slightly below body temperature, cocoa particles ground to a size smaller than 20 μm for a silky-smooth texture, and levels of sugar, fat and e.g. vanilla flavoring that are hedonically optimized to local taste [123]. Importantly, although the

immediate appeal of chocolate is predominantly sensory, it is highly likely that repeated experience with its energy-delivering macronutrients (fat, sugar and -- if present -- milk protein) and neurochemical impact of its bioactive constituents (biogenic amines, methylxanthines and cannabinoid-like fatty acids) contribute to chocolate reward as well [124, 125].

Food sensing can trigger acute physiological effects even before major nutrient metabolism sets in. Oral exposure to sucrose and fat stimulates dopamine release in the nucleus accumbens in rodents [126] and releases triglycerides from intestinally stored lipids and glucoregulatory hormones in humans [127]. Anticipatory physiological responses to scheduled meals can be learned, as shown for the hormone ghrelin [128] which activates CNS reward pathways and increases the motivation to eat [129, 130]. Sensory effects of foods may influence energy intake via diverse learned and innate mechanisms [98, 131]. During food consumption, aromas are released retronasally in patterns that stimulate brain reward areas involved in motivation and increase satiation in humans [132, 133]. Sugar and fat have synergistic effects on hedonic value and intake, but inhibitory effects on taste sensitivity [134].

Energy Density, Stomach Fill, and Eating Rate

The combination of high energy density (ED; number of calories per unit of food weight) and low market prices for snack foods has been blamed for today's overweight epidemic [135]. Relevantly for body weight management, ED is strongly increased by fat, while at the same time fat is poorly detected and relatively weakly satiating, facilitating energy overconsumption. To cope with this, a 'volumetrics' approach has been proposed as a tool to reduce caloric intake of diets that are still filling and satisfying [136]. This can be accomplished by increasing water or indigestible fiber content. One of the assumptions behind this approach is that noncaloric increases in food volume may stimulate oral and gastric signals that contribute to satiation, and possibly reward. An added benefit of ED reduction is that the lowered rate of energy intake may allow post-gastric (i.e., intestinal and post-absorptive) satiation signals sufficient time to limit meal size [137].

Gastrointestinal and Post-Absorptive Signals

De Araujo et al. [138] have shown that rodent strains with genetically ablated sweet-taste transduction still develop preferences for sucrose and release brain dopamine after sugar consumption. This reveals a potent role of (nonsensory) gastrointestinal or post-absorptive signals in food reward.

The small intestine is a major source of post-meal humoral, neural, and metabolic signals with relevance for food reward [139]. Intestinal presence and absorption of digested food

components is associated with local and systemic release of gastrointestinal satiation peptides, like CCK, GLP-1(7-36), PYY(3-36) and dozens of others, while blood levels of the orexigenic stomach hormone, ghrelin, are suppressed [140--142]. Of further relevance is the nutrient-related release of leptin and insulin, both of which can access the CNS and potentially influence the activity of pathways related to homeostatic and reward feeding [143]. Because the intensity and time course of the above mentioned gastrointestinal signals depend on food digestion, modifying food composition and structure could provide a practical approach to tune food reward signals in the CNS.

Food properties that modify post-meal physiological signals and -- potentially -- reward value include macronutrient composition [139, 140, 144], choice of macronutrient subtypes (e.g. fructose vs. glucose;[145]), energy density, stomach acid-induced lumping and retention leading to prolonged bioavailability of proteins and carbohydrates [146, 147], and nutrient embedding or modification to control digestive rate [148, 149]. Ingredients also interact post-ingestively. For example, in mixed meals, fat content may lower gastric emptying rate of carbohydrates and moderate the rise in blood glucose and plasma insulin [150], and glycemic index of diet appears to be relevant for weight control [151]. Following absorption from the intestinal tract, circulating fats, monosaccharides and amino acids may stimulate extra-intestinal hormone release (insulin, amylin, leptin) and/or enter the CNS to either activate or inhibit CNS pathways controlling homeostatic and reward feeding [152].

Conclusion

Regulation of food intake can be investigated as the function of two systems: homeostatic and hedonic regulation. The former is primarily achieved via hindbrain and hypothalamic control of food intake, while hedonic control of food intake occurs primarily by CNS reward circuitry. Despite this theoretical classification, the anatomical and functional distinction between these two systems is rather obscure. Firstly, connections exist between the hypothalamic nuclei maintaining homeostatic control of food intake and reward-related brain structures (lateral hypothalamus and nucleus accumbens), indicating a cross-talk between the two systems [153]. Secondly, receptors of various feeding peptides and peripherally produced satiety signals (e.g. leptin) are present in brain reward centers [154]. This interaction between the two systems is also seen in animals under food deprivation that increase their motivation to obtain a reward [60]. Moreover, humans have evolved in an environment where food availability was uncertain. Therefore, the concept of overeating as a survival mechanism to increase storage of energy as fat cannot entirely be dismissed. Based on this consideration, it seems likely that the homeostatic and hedonic regulation of feeding cannot simply be disentangled. Since past research in the field of 'food addiction' has been primarily directed at understanding the mechanisms by which the three macronutrients fat, protein, and carbohydrate influence brain reward circuitry, major

advances should be expected from future research directed at i) improving our understanding of neurobiology of hedonic versus homeostatic consumption and ii) researching diets that would have a strong homeostatic component, but without generating changes in CNS as encountered after chronic drug use [3] or obesogenic diets [14, 37].

Perspective

Triggered by the increasing incidence of obesity due to overconsumption of high-caloric palatable foods, research on feeding behavior in the past decade has expanded its focus from strictly hypothalamic circuitries to include reward-related brain pathways.

The current review demonstrates that indeed certain high-caloric diets, mimicking the ones in today's western society influence brain reward centers and ,upon chronic exposure, generate molecular and behavioral changes similar to drugs of abuse. Importantly, in this research the role of specific components of these diets on the brain reward circuitry has received relatively little attention. Disintegrating complex diets into their individual components and studying the way the components modulate the brain reward circuitry would mean an essential next step towards improving our understanding on the interaction between food and brain reward pathways. Such research could inform the design of novel foods which are palatable yet fail to produce obesogenic consequences.

However, in pursuit of such an approach, assessing effects of single food components on reward and motivation to (over)eat seems a daunting task. Natural and processed foods alike are complex structures that may contain hundreds of components [155]. Therefore, at this moment a concept- rather than ingredient-driven experimental strategy may be most suitable. With this approach, validated sensory and physiological concepts guide food modifications of common model foods used in reward studies (i.e., sucrose solutions, nutritionally complete liquid meals) with the aim to modulate the reward value of food. Starting from these model foods, ingredients are added, modified, or replaced to alter food properties that are theoretically relevant to reward. These properties include aroma release, taste intensity, mouth feel, macronutrient composition, energy density, nutrient absorption rate, food digestibility, and concentration of bioactive substances.

Upon addition of these food variants to regular diet, measurements will be made to evaluate the energy intake, preference as well as anticipatory and motivational response to such novel diets. Subsequent studies will then evaluate how the food modifications tune peripheral physiological signals of appetite and satiation and affect the CNS. From an obesity management perspective, identifying foods with reduced energy density that are palatable yet do not evoke overconsumption could provide important rationales for food design while demonstrating the potential of combining insights from food technology and the neurobiology of feeding.

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Tables

Table 1: Gives an overview of various diets leading to obesity and their influence on leptin signaling.

Table 2. Evidence of interactions between various diet components and the brain reward circuitry (additional recommended references [159, 160])

Chapter 2

Neurobiology of overeating and obesity: the role of melanocortins and beyond

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Abstract

The alarming increase in the incidence of obesity and obesity-associated disorders makes the etiology of obesity a widely studied topic today. As opposed to 'homeostatic feeding', where food intake is restricted to satisfy one's biological needs, the term 'non-homeostatic' feeding refers to eating for pleasure or the trend to over-consume (palatable) food. Overconsumption is considered a crucial factor in the development of obesity. Exaggerated consumption of (palatable) food, coupled to a loss of control over food intake despite awareness of its negative consequences, suggests that overeating may be a form of addiction. At a molecular level, insulin and leptin resistance are hallmarks of obesity. In this review, we specifically address the question how leptin resistance contributes to enhanced craving for (palatable) food. Since dopamine is a key player in the motivation for food reward, the interconnection between dopamine, leptin and neuropeptides related to feeding will be discussed. Understanding the mechanisms by which these neuropeptidergic systems hijack the homeostatic feeding mechanisms, thus leading to overeating and obesity is the primary aim of this review. The melanocortin system, one of the crucial neuropeptidergic systems modulating feeding behavior will be extensively discussed. The inter-relationship between neuronal populations in the arcuate nucleus and other areas regulating energy homeostasis (lateral hypothalamus, paraventricular nucleus, ventromedial hypothalamus etc.) and reward circuitry (the ventral tegmental area, nucleus accumbens) will be evaluated and scrutinized.

Introduction

The rapid urbanization throughout the globe in the past few decades, marks the rise in the incidence of many chronic illnesses, including obesity and diabetes [2]. Recent data from Europe and The United States shows a high incidence of obesity in the general population, 20% and 34%, respectively [2, 3]. Interestingly, only in an insignificant subset, obesity is a result of single mutations in genes involving energy homeostasis. A majority of cases of obesity results from a combination of genetic, behavioral and environmental factors. The improvements in food availability and alterations in dietary patterns with a prevalence of energy-dense fat and sweet foods are crucial environmental factors in today's obesity epidemic. Distinguished from 'homeostatic feeding', where food intake is restricted to satisfy one's biological needs, this kind of 'non-homeostatic feeding' or 'feeding for pleasure' has gained a special place in our society and overeating, food craving and compulsive eating are important deleterious factors culminating to obesity.

The increased attraction towards pleasurable feeding and the loss over food intake has been compared to addictive behavior. Studies on animals and humans have demonstrated activation of the brain reward system when subjects are exposed to palatable food. Thus, homeostatic control of feeding, where the brain maintains a temporal control on the amounts of food ingested involves the hypothalamus and the brainstem. The reward circuit, encompassing brain areas such as the ventral striatum, prefrontal cortex and amygdala is sensitive to the hedonic aspects of food. Interestingly, the systems involved in homeostatic and non-homeostatic feeding are not entirely separated, as multiple connections between these two systems exist [4]). Additionally, in a situation of hunger even non-palatable foods will be rewarding. This suggests the existence of a distributed neural network that controls different aspects of feeding behavior, such as rostral brain areas being more important for pleasure feeling and caudal parts for controlling meal size [5].

Overconsumption in this paper will be studied in the light of two contributing factors: (a) increase in meal size, i.e. animals consume bigger amounts of food due to defective satiation and/or augmented desire for certain foods [6] (b) increase in meal frequency. We aim to understand the neuronal mechanisms by which the hedonic signals interact and hijack the homeostatic regulation of food intake. Although at first glance, hijacking of the homeostatic regulatory mechanisms by its hedonic counterpart may seem conflicting, it should be borne in mind that during evolution, humans have lived in an environment where food availability was restricted and uncertain (e.g. hunter-gatherers) and the biological system has been 'hard-wired' to maximize energy stores [7].

Homeostatic control of food intake

The question concerning the regulation of food intake has intrigued scientists for several decades. The actual shift from the earlier 'peripheral' theories, where hunger and satiation were considered to be a unique property of the stomach, to the more 'central' theories, involving the brain in feeding control, did not occur until the 1950's. Correspondingly, the glucostat theory of Mayer and the lipostat theory of Kennedy suggested the role of carbohydrate and fat as major components regulating energy balance [9, 10]. Lesion experiments during this period also identified the ventromedial hypothalamus and the lateral hypothalamus as the brain 'satiety' and 'feeding' center, respectively [11]. These observations, although somewhat preliminary, laid the foundation for further elucidation of the complex neuronal networks influencing feeding and satiation. A major breakthrough in obesity research came through studying spontaneously obese mice; the ob/ob (obese) and the db/db (diabetic) mice being the forerunners [12]. Using surgical vascular-anastomosis between these strains and normal mice, it was shown that the ob/ob and the db/db mice had a dysfunctional 'satiety factor' and 'satiety center', respectively [13]. Later, with the advent of better molecular cloning techniques, this satiety factor was identified in 1994 as circulating leptin, which appeared to be absent in the ob/ob mouse, while a dysfunctional long form of leptin receptor (see below) was identified as the cause of obesity in the db/db mouse [14, 15].

Leptin is perhaps the most widely studied biological factor controlling food intake. Secreted primarily from the adipose tissue, leptin is a 146 amino acid protein circulating in the blood. It accomplishes a biochemical communication between adipose tissue and the brain areas involved in energy homeostasis, updating the latter on the degree of peripheral adiposity [16]. The action of leptin in the brain is mediated by the leptin receptor, which belongs to the class-I cytokine receptor family [15]. Three principle forms of leptin-receptor have been found in mammals: the secreted (leptin receptor-c), the long (leptin receptor -b) and short intracellular domain (leptin receptor-a) leptin receptor. Each carries the same extracellular domain but differs in the length of the cytoplasmic domain [17, 18]. The leptin receptor-b is vital for the physiological action of leptin in the hypothalamus: arcuate nucleus, dorsomedial hypothalamus, ventromedial hypothalamus and lateral hypothalamus express this form of leptin receptor [19]. Similar to other cytokine receptors, the leptin receptor lacks an intrinsic enzymatic activity and is dependent on the Jak-2 kinases for signal transduction.

Leptin Resistance

The amount of circulating leptin is proportional to the degree of peripheral adiposity [33]. Intriguingly, enhanced (and prolonged) increase in the circulating levels of leptin, do not further enhance the leptin receptor-b signaling cascade. Analogous to the concept of insulin resistance, where augmented amounts of insulin fail to decrease plasma glucose levels,

leptin resistance implies a clinical condition associated with obesity, where the anorectic action of leptin is blunted despite its high circulating amounts in the periphery[34, 35].

Being one of the central issues in understanding obesity, several explanations have been put forward to explain the phenomenon of leptin resistance. First, studies comparing the db/db mouse, which lacks only leptin receptor-b and mouse mutants devoid all isoforms of leptin receptor, showed that the soluble and short isoforms of leptin receptor may be responsible for leptin's transport across the blood brain barrier [30]. Deficiencies in the peripheral levels of these isoforms in obese conditions indicates their potential role in leptin resistance [36]. Second, the inability of leptin to reach its target can be also due to other factors, like the high levels of circulating triglycerides in obesity, which hinder leptin transport across the blood brain barrier [37]. Third, intracellular mechanisms activated by the leptin signaling cascade also modulate the action of leptin receptor by negatively regulating its own receptor activity. One such mechanism is suppressor of cytokine signaling-3 (SOCS-3) activation. Signal transducer activator of transcription-3 (STAT-3) protein activated upon phosphorylation of leptin receptor-b, further activates SOCS-3 protein, which in turn suppresses the activity of the leptin receptor by acting at the level of Jak-2 kinase and Tyr 985 residue of the leptin receptor. Neuronal SOCS-3 deficient mice show enhanced STAT-3 protein phosphorylation together with a leaner phenotype [38]. Focusing on the dose-related effect of leptin on its receptor to explain leptin resistance, Munzberg and colleagues hypothesized that under baseline levels, small increments in leptin concentrations would result in enhancement of leptin signaling, whereas elevated amounts of leptin as encountered in obesity, would lead to higher expression of SOCS-3, thereby dampening leptin signaling [31]. The increase of SOCS-3 expression in obesity may also occur irrespective of blunted STAT3 activation, indicating the involvement of an alternative pathway [39]. Last, the tyrosine phosphatase protein 1B has shown to negatively regulate the activity of the Jak-2 kinase [40, 41]. Single nucleotide polymorphisms (SNP) in the gene have been associated with obesity and diabetes mellitus type II [42, 43]. Thus, current evidence indicates the involvement of multiple simultaneous mechanisms in leptin resistance. Therefore, targeting a single process to combat this phenomenon is not likely to be successful. Additionally, leptin resistance itself and the presence of a feedback loop within the leptin signaling cascade draws our attention to its evolutionary perspective where increased adiposity may be beneficial (e.g. seasonal animals, pregnancy) [39]. Keeping this evolutionary bias towards weight gain in mind, Leibel and colleagues in their studies on obese and non-obese human subjects reported that a 10 % decrease in body weight in either group, resulted in the decrease in non-resting energy expenditure of up to 20%, enough for recidivating obesity [44]. This effect may be partially due to the dose-response curve of leptin. When leptin levels are below a particular 'threshold', a decrease in catabolic expenditure with simultaneous enhancement in feeding occurs. Conversely, high-

circulating levels of leptin (as seen in obesity) have no pronounced effect on metabolism and feeding. This 'threshold' is in turn defined as a neurobiological correlate that is influenced by genetic, internal and external environmental factors. Interestingly, chronically elevated levels of leptin can also alter this 'threshold'. It has been hypothesized that a rightward-shift in the 'threshold' can be encountered in obese subjects, i.e. even small dips in leptin levels will lead to increased activation of anabolic mechanisms culminating in pronounced weight gain [44] Fig. 2 summarizes the above-mentioned mechanisms of leptin resistance.

Hypothalamic control of feeding

The hypothalamus is a prime relay station controlling feeding behavior and the distinct roles of the various hypothalamic sub-nuclei with respect to food intake have been studied in great detail. After crossing the blood brain barrier, circulating leptin reaches the arcuate nucleus, which contains two principle neuronal populations: the neuropeptide Y /agouti related peptide (AGRP) and the pro-opio melanocortin (POMC) / cocaine and amphetamine related transcript neurons (CART) [49]. Both populations express the leptin receptor, project to other hypothalamic nuclei and have an opposing action on energy balance [49, 50]. The action of leptin on these neurons is also contrasting. Intracerebroventricular (i.c.v.) administration of leptin, decreases neuropeptide Y / AGRP [51] while enhancing the activity of the POMC neurons [52]. A sub-population of the neuropeptide Y neurons contains γaminobutyric acid (GABA) and sends inhibitory projections to the POMC neurons within the arcuate nucleus [52, 53]. Furthermore, i.c.v. administration of neuropeptide Y has been shown to increase feeding [54]. Likewise, states of negative energy balance such as fasting result in increased neuropeptide Y levels in the arcuate nucleus, indicating the anabolic effect of this neuropeptide [55, 56]. The POMC neurons, on the other hand, secrete αmelanocyte stimulating hormone (αMSH) and promote anorexia, an effect mediated by second order neurons primarily in the paraventricular nucleus and ventromedial hypothalamus expressing the melanocortin 3 or 4 receptor (MC3 receptor and MC4 receptor). In contrast, AGRP, a protein co-secreted by the neuropeptide Y neurons of the arcuate nucleus, is a potent and long lasting orexinergic agent that has an inverse agonistic action on the MC4 receptor [5, 49]. Both neuropeptide Y and POMC neurons project to different hypothalamic second order neurons in various hypothalamic areas (paraventricular nucleus, lateral hypothalamus, ventromedial hypothalamus etc.) that regulate further anabolic or catabolic events. Within the paraventricular nucleus, several target neurons of the POMC / CART and neuropeptide Y / agouti related peptide neurons have been identified: thyrotropin releasing hormone, oxytocin and corticotropin releasing hormone neurons, all well-known catabolic modulators [49, 57, 58].

Another hypothalamic nucleus, the lateral hypothalamus, contains neurons that produce two orexinergic neuropeptides, i.e. orexin and melanin concentrating hormone. i.c.v.

infusion of both elicits a robust feeding response [59, 60]. The orexins (orexin A and B) are alternative splice forms of the orexin precursor protein. From the lateral hypothalamus, the orexin-containing neurons project to various brain areas regulating feeding and arousal. Furthermore, orexinergic fibers also project to the neuropeptide Y / AGRP and POMC / CART neurons of the arcuate nucleus, thus activating the former and inhibiting the latter cell-populations via orexin 1 and 2 receptors (OX1 receptor, OX2 receptor), respectively [61]. The underlying mechanisms by which orexins and melanin concentrating hormone control feeding are poorly understood, but the projections to the brain reward centers may play an important role [60, 62]. Finally, the ventromedial hypothalamus, an important hypothalamic site containing brain derived neurotropic factor (BDNF) neurons, receives connections from neuropeptide Y / AGRP and POMC / CART neurons. Recent studies have shown BDNF to be a downstream target of MC4 receptor, and BDNF also acts as a direct effector for leptin-mediated anorexia [29, 63]. The downstream targets of BDNF mediating feeding remain to be elucidated. The importance of the MC4 receptor and BDNF for human body weight regulation has been recently shown in genome wide association studies, where allelic variants in these loci contribute to the variance of body mass index observed in human population (See Fig. 3) [64].

Hedonic control of food intake

It is important to note that not only hunger elicits feeding. Humans, for example, tend to 'finish their plate' and cue-induced feeding in a satiated state has been shown in both rats and humans [21, 65]. This phenomenon of enhanced food consumption beyond one's nutritional need points to the fact that food is a natural reward. Food has been shown to be reinforcing in the similar manner as drugs are, although this does not necessarily mean that food is addictive, as animals will work for a variety of natural rewards that benefit survival, such as water and sex [66-68]. When trying to relate drug addiction to overeating, it is important that we define terms like: 'liking', 'wanting' and 'compulsion', because they are often used to describe components of the addiction syndrome [69-71]. 'Liking' refers to the pleasurable feeling associated with the receipt of a reward, while 'wanting' is considered as a subjective desire that induces a goal directed behavior to obtain a reward [66, 72]. The neural substrates of 'wanting' and 'liking' have been widely discussed. Berridge has described that the neural substrate of 'liking' is a combination of several brain nuclei. Starting from deep brainstem structures which act as an initial gateway for sensory perception, it includes higher order centers like the nucleus accumbens, ventral pallidum and the orbitofrontal cortex, involving GABAergic, opioid, and endocannabinoid neurotransmission [70]. 'Wanting', which is often considered to be closely related to motivational influences on behavior, has been associated with dopamine signaling in the mesolimbic system, as well as its inputs from the prefrontal cortex and amygdala [70, 73-75]. 'Compulsion' refers to behavior that is continued or repeated, whilst being dissociated

from an apparent goal, or in the light of adverse consequences. It is important to realize that 'compulsion' (not 'wanting' or 'liking') is a key point in the definition of addiction [71]. In fact, it has been observed that certain addicts no longer 'like' but rather 'need' drugs of abuse, although the reductions in the hedonic properties of drugs ('liking') in addiction strongly depend on the type of drug used [76]. The behavior of drug addicts is no longer primarily mediated by the outcome of their actions (action – outcome behavior), as the neural circuits underlying reward and motivation have been altered by prolonged drug abuse, so that exposure to drug-associated stimuli leads to automatic, habitual patterns of drug seeking, that are no longer voluntary or goal-directed (stimulus – response behavior) [69]. The neural substrates of compulsive drug seeking constitute involvement of dorsal striatal regions that mediate habitual behavior, together with a breakdown of cognitive control over behavior, mediated by the prefrontal cortex [69, 77]. In addition, prolonged drug abuse engages brain stress systems, and dysregulates neural substrates of motivation [71, 78]. The ability of food to induce compulsive behavior, like drugs of abuse do, has (in our view) not convincingly been shown. It has, however, been shown that food has a very high motivational value and may sometimes even be preferred over drugs [79]. In fact, although obesity may or may not be explained as a 'food addiction', certain similarities in overconsumption and addiction (e.g. both relate to a 'loss of control' over intake) are worth mentioning. The following sections will elaborate more on this and the interaction of the dopaminergic system with leptin and other (feeding) neuropeptides (melanocortin system, orexins, BDNF, opioids) with respect to overeating will be discussed in detail.

Dopamine and overeating

Drugs of abuse increase dopamine levels in the nucleus accumbens, activating postsynaptic dopamine 1 and 2 receptors (D1receptor, D2receptor) on the target neurons [67, 80]. These elevated dopamine levels may enhance the association of primary rewards with environmental cues and initiate goal-directed behavior [74, 81]. The mechanism by which this elevation of dopamine is achieved differs for the various substances of abuse (See Fig. 4) [81-84].

Similarities between drug addiction and obesity come from multiple studies. First, involvement of the brain reward circuitries during feeding comes from in-vivo micro dialysis studies in rodents, where increased levels of dopamine were detected in the brain reward regions in response to eating and drinking [85], analogous to receipt of other rewards [86] although it should be borne in mind that the magnitude of the dopamine response to food is much smaller than the dopamine response to drugs. In addition, activation of comparable brain areas (hippocampus, insula, caudate nucleus, and the ventral striatum) in response to food and drug cravings has also been shown [87-90]. The mesolimbic dopaminergic system, projecting from the ventral tegmental area to the nucleus accumbens and the frontal cortex,

is one of the major pathways implicated in addictive behavior, and is therefore a focus in this review.

It has been shown that drugs of abuse increase extra-synaptic levels of dopamine in the nucleus accumbens, either directly or indirectly [71]. The repeated increase in dopamine in response to prolonged abuse of addictive substances, in the long run leads to a reduced D2 receptor density in the striatum [71]. Interestingly, D2 receptor levels in the striatum of obese subjects are also decreased [91], possibly affecting the cortico-striatal top-down inhibitory mechanisms [92]. Conversely, D2 receptor levels are up-regulated in previously obese subjects who underwent gastric bypass surgery to combat excessive adiposity [93]. Decreased D2R levels have been simultaneously noted in some rodent models of obesity [94], and this reduction in D2 receptor levels in these animals is reversed if animals are placed on a restricted feeding schedule. This data suggests that overeating may be a compensatory mechanism to adjust for decreased dopamine activity. This process, namely the 'reward deficiency syndrome', is often used as a model to explain compulsive behavior in an addictive state. It hypothesizes that the initial use of drugs of abuse leads to exaggerated amounts of synaptic dopamine in the nucleus accumbens leading to excessive stimulation of the post-synaptic dopaminergic receptors [95]. This chronic receptor stimulation ultimately results in decreasing post-synaptic receptor density. Due to this de sensitization to increased dopaminergic stimulation, some of the effects of a fixed drug dose wane over time, resulting in tolerance to these effects. This may be reflected by the decreased thresholds for electrical self-stimulation in rats given extended access to cocaine. These rats become insensitive to low electrical currents and only self-stimulate when the reward (stimulation intensity) is substantially increased [96]. Consistent with this notion, Wang and colleagues have proposed the dopaminergic hypofunction theory of overeating, where overeating is an adjustment of the obese brain to compensate for low extracellular dopamine levels [97, 98]. Indeed, it was recently shown that obese rats fed on high-fat diet for a span of 40 days display an increase in threshold for rewarding self-stimulation [99]. In addition, using a lenti-viral approach, Johnson and Kenny (2010) demonstrated that knocking down striatal D2 receptors resulted in compulsive eating in rats exposed to high caloric diet [99]. However, whether the decreased D2 receptors levels are a cause or a consequence of addiction is not clear. Studies have shown that humans with the Taq1A allele near the D2 receptor gene have lower number of these receptors and are also more prone to addiction [100, 101]. In addition, as the dopaminergic system is involved in the motivational aspects of reward, decreased dopamine and D2 receptor levels in various addictive states and obesity have been hypothesized to lead to an increased motivation for drugs and palatable food [98]. Consistent, animals exposed to high fat diet show an increased motivation for a sucrose reward when tested under a progressive ratio schedule of renforcement [102]. However, it still needs to be elucidated how chronically reduced dopaminergic neurotransmission in the nucleus accumbens induce increased motivation for rewards in addiction and obesity, as

reduced dopaminergic transmission in the nucleus accumbens is well known to reduce the motivation for food and drugs [70, 73, 75].

The role of opioids with respect to intake of drugs is well established [84]. Opioid infusions in the nucleus accumbens result in enhanced intake of palatable solutions (including ethanol and saccharin solutions) and increased preference for high-fat food [103, 104]. It has been suggested that opioids do so by increasing the hedonic appreciation of a reward, in other words: by increasing 'liking' [73], (but see:[105]), rather than through modulation of the motivation for rewards (which, as mentioned, depends upon dopaminergic activity). Interestingly, this also works the other way around, as consumption of highly palatable food results in changes in opioid expression [106]. Most importantly, when access to a highly palatable diet is discontinued in rats, decreased levels of endogenous opioids are encountered in these animals when compared to animals that were never (or only briefly) exposed to palatable chow [107]. This, of course, is also reminiscent of the reward deficiency theory. Furthermore, important interactions between the opioid system and cannabinoids in both addiction and feeding have been identified. Examples include the permissive function that opioids and cannabinoids have on alcohol intake: increased alcohol intake by treatment with morphine is blocked by administration of a cannabinoid receptor antagonist (Rimonabant) and stimulation of alcohol consumption using a cannabinoid receptor 1 agonist is blocked by naloxone [108, 109].

Although the compulsive aspects of procurement and intake have not been as convincingly shown for food [99], as they have been for drugs (e.g. [110, 111, 111-113]), the evidence described above indicated that there definitely is some overlap between overeating and addiction. However, these similarities between overeating/obesity and addiction, still fail to answer the fundamental question as to how the homeostatic mechanisms controlling body weight are overpowered by its hedonic counterpart. One of the answers might lie in the extended role of leptin beyond the hypothalamus and its interaction with the dopaminergic system.

Leptin, reward circuitry and overeating

The earliest evidence of leptin-induced modulation of the brain reward pathways comes from intracranial self-stimulation studies in rodents [114]. The presence of a metabolically active leptin receptor in the dopaminergic neurons of the ventral tegmental area suggests an interaction between brain reward mechanisms and leptin [115]. Food deprivation decreases circulating leptin levels, and this has been used for many years to study the effect of leptin on the brain reward centers. Food restriction in rodents has been shown to reduce the threshold for lateral hypothalamus self-stimulation [8] and central administration of leptin attenuates the effects of food restriction on self-stimulation [114]. Moreover, the rewarding

properties of heroin are enhanced under food-restricted conditions [116]. As food restriction lowers the amount of circulating leptin, Figlewicz and colleagues have argued that reduced amounts of leptin are thus associated with higher reward sensitivity, whereas increased leptin signaling will dampen this heightened reward sensitivity [117]. Indeed, an functional magnetic resonance imaging study by Farooqi and colleagues on two congenital leptin deficient human subjects showed that 7 days of leptin replacement therapy modulated the activity of the ventral striatum together with decrease in total calorie intake and attenuated liking ratings to food images [118]. In line with this finding, it was reported that food-restricted animals demonstrate conditioned place preference for low calorie sucrose pellets that was reversed by peripheral leptin administration [119]. i.c.v. administration of leptin or insulin blocked the high fat diet-induced conditioned place preference in rats [120]. Likewise, sucrose self-administration in rats on a progressive ratio schedule of reinforcement was also attenuated by i.c.v. administration of insulin or leptin [121]. Supported by their studies on food-deprived animals, Figlewicz and colleagues suggest that the rewarding value of food is determined by the nutritional state of the individual at that time [117, 121]. Hence, a hungry individual (with lower leptin levels) will assign a higher rewarding value to food compared to an individual that is satiated. However, does that imply that the obese brain is always 'hungry' and thus assigns a higher value to food?

Based on the concept of cross-sensitization between food and drugs of abuse, Carr and colleagues showed that animals that are maintained at 80% of their initial body weight exhibit an augmentation of the rewarding effects of cocaine and amphetamine in an intracranial self-stimulation paradigm [122]. Consistently, prior studies have shown that food-restricted animals consume higher amounts of drugs of abuse [123]. An important question addressed by Carr was whether the increased sensitivity for drug reward in food-deprived conditions is a form of sensitization and persisted after cessation of food deprivation [122]. In fact, the change to an ad-libitum diet simultaneously reversed the effect of food restriction on amphetamine reward. This indicates that the enhancement of reward sensitivity to drugs during food-restriction might differ from the sensitization due to repeated drug exposure [122], as drug induced behavioural sensitization is known to be persistent [78, 124]. However, it is important to note that in the food-restriction paradigm animals are non-satiated. Therefore it is difficult to distinguish whether the witnessed alterations in behavior are due to the homeostatic or hedonic aspects of low leptin levels. Stress is yet another confounding element in these studies, because food-restriction may cause stress and stressed animals are more sensitive to the motivational and rewarding properties of food and drugs [125-128]. Interestingly, when comparing studies in food-restricted rodents with leptin-resistance/obesity, we notice certain striking similarities. In both situations, attenuated leptin signalling either due to low circulating amounts or due to

alterations in the leptin signal transduction pathway, leads to heightened motivation for food rewards. Low leptin levels, occurring during obesity/leptin resistance, may trigger a pathological situation where the body 'thinks' it is in a hungry state and simultaneously enhances motivation for obtaining rewards. This would imply, that normalizing the disrupted leptin signalling cascade in the obese brain may be sufficient to decrease motivation for food reward.

At least two studies have shown activation of the leptin signaling pathway in the dopaminergic neurons of the ventral tegmental area [129, 130], but the proposed mechanisms by which leptin regulates the dopaminergic neurons are contrasting. Fulton et al., based on their studies of the ob/ob mice, showed that decreased dopamine content in the ventral tegmental area and nucleus accumbens encountered in these mice, is reversed by 3-days of leptin administration [130]. In contrast, Hommel and colleagues demonstrated that direct administration of leptin into the ventral tegmental area inhibited the firing of dopaminergic neurons and attenuated feeding response. Furthermore, a virus mediated knockdown of the leptin receptor-b lead to enhanced feeding and sensitivity to palatable food [129]. This discrepancy between the two studies (i.e. opposing effects of leptin on the ventral tegmental area neurons) can be attributed to the fact that total deficiency of leptin during development can result in morphological alterations in the neuronal circuits and synapses resulting in a different behavioral response [131, 132]. Interestingly, the decrease in feeding in response to i.c.v. leptin-treatment was also accompanied by lower dopamine levels in the nucleus accumbens [133]. An attenuated feeding response was also noted when leptin was directly injected into the ventral tegmental area [134]. Taken together, it is evident that leptin action is not limited to the homeostatic centers for food intake, but extends to the brain reward circuits. Therefore, conducting further studies as to how leptin resistance contributes to preference for palatable food will be central to the understanding of leptin's role in overeating.

Prolonged exposure to a high fat diet in rodents has shown to induce diet-induced obesity together with leptin resistance [135]. In a study by Munzberg and colleagues, it was reported that exposure of animals to a high fat diet results in the development of leptin resistance selectively in the arcuate nucleus (measured by STAT-3 phosphorylation levels), while other brain areas expressing the leptin receptor were spared [34]. Since knocking down the leptin receptor would mimic a leptin-resistant state, the study by Hommel and colleagues provides a novel view on the effects of leptin on brain reward circuitry [129]. In addition, Munzberg et al. detected altered STAT-3 protein levels after 16 weeks of high fat diet consumption [34]. Hence, it is possible that a leptin-resistant condition can develop in brain areas other than the arcuate nucleus, but at a different time-point after exposure to a high fat diet. Again, animals exposed to diet-induced obesity show decreased dopamine

levels in the nucleus accumbens combined with an attenuated response to sucrose and amphetamine rewards [136]. Thus, analogous to chronic substance abuse [137], prolonged exposure to high far diet can lead to hypofunction of the dopaminergic system [136]. Likewise, nucleus accumbens dopamine levels are decreased in obese-prone animals but not obese-resistant animals, further underscoring the fact that mesolimbic dopaminergic signaling may be dampened in leptin-resistant conditions. Central leptin administration in lean animals decreases basal and food-invoked dopamine levels in the nucleus accumbens [133] together with a decrease in food intake [138]. However, leptin-resistant conditions (as in diet-induced obesity) also dampen mesolimbic dopaminergic signaling [136] but simultaneously enhance feeding [139]. One of the answers to this paradox might be that leptin possibly activates alternative intracellular pathways in the ventral tegmental area and the hypothalamus, exerting differential effects on food intake [134]. It is also possible that under lean conditions, leptin reduces dopamine levels without altering the dopamine receptor densities, whereas, overeating and obese conditions decrease of dopamine and D2 receptor levels [140]. Interestingly, it is not only leptin that influences the dopaminergic system; recent findings suggest that the leptin-dopamine interaction is bi-directional and dopamine has been shown to negatively influence leptin action in the hypothalamus [141].

Melanocortin system in feeding

The melanocortin system, comprising of melanocortin receptors, natural agonists and inverse agonists, plays a critical role in the regulation of body weight. Of the five melanocortin receptors, the MC3 receptor and MC4 receptor are widely expressed in the brain, and these have been extensively studied with respect to energy balance [142]. Studies on C57/BL/6J MC4 receptor knock-out animals have shown that these animals exhibit late-onset obesity, accompanied by enhanced longitudinal growth, hyperphagia, hyperinsulinemia and hyperleptinemia. Male MC4 receptor knock-out mice additionally show a reduction in nocturnal locomotion [143]. Thus, weight gain in the MC4 receptor knock-out animals has been conceived as being the result of increased food consumption and low locomotor activity [144]. The MC3 receptor knock-out animals, on the other hand, display obesity, hyperleptinemia and decreased locomotion without hyperphagia. Weight gain in these animals occurs as a result of increased feeding efficiency (weight gain to food intake ratio)[145, 146]. Heterozygous MC4 receptor mice show an intermediate phenotype compared to wild type and MC4 receptor knock-out animals, whereas the heterozygous MC3 receptor animals do not differ significantly from their wild type littermates [145, 146].

αMSH, the endogenous ligand of the MC3 receptor and MC4 receptor is the product of the precursor pro-opio melanocortin protein. As indicated in the preceding section, the POMC neurons of the arcuate nucleus synthesize αMSH that acts on MC3 receptor and MC4 receptor expressing neurons. AGRP, co-synthesized in the neuropeptide Y neurons is an

inverse agonist at the MC4 receptor. Downstream targets of the melanocortin system include several neuronal populations in diverse brain areas implicated in food intake, meal choice and satiety [144]. Immunohistochemical studies show that MC3 receptors and MC4 receptors are expressed in the hypothalamus, cortex, amygdala and parts of the brainstem [147]. Interestingly, MC3 receptors are also expressed in the POMC neurons of the arcuate nucleus, establishing a feedback regulatory control over the melanocortin system. Both peripheral and central administration of an MC3 receptor agonist stimulates feeding, whereas treatment with a low dose of an MC3 receptor antagonist has an opposite effect [148]. The expression of POMC messenger RNA in response to MC3 receptor agonist treatment has been also shown to decrease, underscoring the auto receptor role of MC3 receptor in these neurons [52]. Comparable to MC4 receptor knock-out animals, mutations in the MC4 receptor in humans have been associated with obesity, hyperphagia, tall-stature and hyperinsulinemia, suggesting a similar role of the melanocortin pathway in humans and rodents [149-151]. Indeed, there is converging evidence to support the association between human MC4 receptor mutation and morbid obesity [149, 152, 153]. Mutations in the MC3 receptor have been also reported in humans [154, 155]. These mutations have been associated with obesity, hyperleptinemia and relative hypophagia, features reminiscent of the MC3 receptor knock-out animals [145].

The connection between leptin and the melanocortin system has been well established. Low leptinemic conditions such as fasting increase the amount of AGRP/neuropeptide Y messenger RNA. The levels of POMC messenger RNA correspondingly decrease [156]. Furthermore, animals with defective leptin signaling, such as the ob/ob and db/db mice, show increased AGRP and attenuated POMC expression, mimicking conditions of fasting [157]. Both POMC and AGRP neurons express the leptin receptor-b and leptin has been shown to increase the firing of the former neurons while inhibiting the latter [158]. Thus, there is convincing evidence to suggest that the melanocortin system is crucial in body weight regulation, and a number of mechanisms have been hypothesized by which this regulation is achieved. Compared to wild type mice, peripheral injections of the MC3 receptor / MC4 receptor agonist Melatonan II in MC4 receptor knock-out mice failed to decrease food consumption or enhance metabolism. This confirms the notion that the melanocortin system acts by either decreasing the amount of food consumed or by increasing metabolism [145, 146]. However, leptin does not mediate its anorexic effects exclusively via the melanocortin system; simultaneous catabolic pathways other than melanocortin system exist. Evidence from humans with MC4 receptor deficiency or leptin deficiency shows that ad-libitum feeding in the former group is less, suggesting the presence of alternative anorexic pathway mediated by leptin [150]. By influencing downstream catabolic modulating neurons, the melanocortin system promotes energy expenditure, presumably via the paraventricular nucleus. Indeed, microinjection of an αMSH analogue:

Melatonan II into the paraventricular nucleus has been shown to result in reduced feeding. This inhibition of feeding was blocked by a pre-injection of a MC3 receptor / MC4 receptor antagonist [159]. In contrast, over-expression of the MC4 receptor inverse agonist agouti in the paraventricular nucleus resulted in hyperphagia and weight gain [160]. Among the second order neurons populating the paraventricular nucleus, thyrotropin releasing hormone and corticotropin releasing hormone-containing neurons are important targets of the melanocortin system. It has been shown in vitro that both leptin and αMSH enhances the promoter activity of thyrotropin releasing hormone gene, an integral neuropeptide in the hypothalamus-pituitary-thyroid axis that regulates energy expenditure. i.c.v. administration of agouti related peptide suppresses circulating thyrotropin releasing hormone levels in male rats whilst injection of αMSH analogue has an opposing effect [141]. Similar to fasting levels of leptin and α- melanocyte stimulating hormone, thyrotropin releasing hormone levels are also decreased during periods of fasting, where energy conservation is the primary goal [161, 162]. In an analogous fashion, Melatonan II potently increases corticotropin releasing hormone gene transcription in the paraventricular nucleus and subsequently enhances plasma corticosterone levels in rats, thus modulating activity of the hypothalamus-pituitary-adrenal axis [58]. The exact mechanism by which these second order neurons in the paraventricular nucleus regulate energy balance is unclear. Recent data suggest that the brain derived neurotropic factor system may be a downstream target of melanocortin system that mediates anorexia. One of the following sections in the current review specifically focuses on this neuropeptide. We have already mentioned that leptin-resistant conditions like obesity or high fat diet exposure are accompanied by blunted leptin receptor signaling. Interestingly, although decreased leptin signaling in the arcuate nucleus leads to lower αMSH secretion, the functionality of the melanocortin system downstream of the pro-opio melanocortin neurons remains intact. Enriori and colleagues demonstrated that when Melatonan II was injected intraperitoneally in diet-induced obesity animals, they showed up to 90% decrease in food intake [163].

Another mechanism by which the melanocortin system might regulate feeding is by influencing the amount of food consumed during a meal i.e. the meal size. Meal size is determined by several parameters including gut-associated satiation signals (cholecystokinin, amylin, glucagon, peptideYY 3-36), metabolic signals (leptin, glucose), meal composition and palatability [164]. Administration of Melatonan II in the third or fourth ventricle reduces total caloric intake in terms of meal size although the meal frequency and inter-meal intervals remain unaltered [165, 166]. Evidence suggests that during meal consumption, gut-associated peptides relay satiety signals to the brain, either by the afferent fibers of the vagus nerve or through area postrema, which ultimately converge on the nucleus of solitary tract [164, 167]. The nucleus of solitary tract, in turn, is reciprocally connected to brain areas involved in feeding, meal choice and motivation [168].

This nucleus serves as a sensory gateway for various visceral signals, and it sends projections to a range of brain areas, including hypothalamic and extra-hypothalamic sites monitoring food intake [169]. Importantly, the brain- stem melanocortin system comprises a separate population of pro-opio melanocortin neurons in the nucleus of solitary tract [170], the pro-opio melanocortin neuronal projections from the arcuate nucleus that extend to the dorsal vagal complex and the melanocortin receptors in the brainstem [142, 171]. This suggests that the melanocortin system might play a role in the control of feeding by altering meal size. In fact, it was shown that MC3 receptor/MC4 receptor agonist or antagonist (SHU9119) administration either into the fourth ventricle or directly to the dorsal vagal complex, elicited a suppression and enhancement in feeding response, respectively [171, 172]. Through a series of experiments, Berthoud and colleagues have stressed the importance of the connection between gastric satiety signal cholecystokinin and the brainstem melanocortin system to induce meal termination. It was shown that brainstem specific Melatonan II infusion decreases feeding by enhancing the satiating capacity of a given meal, independent of diet type (regular chow Vs high fat diet) [166]. Furthermore, Melatonan II and SHU9119 can both modulate ERK 1/2 activity within the cholecystokinin signal transduction pathway, which mediates the cholecystokinin-induced suppression in food intake [166, 173, 174]. In addition MC4 receptor have been identified to modulate presynaptic vagal and non-vagal glutamergic inputs into the nucleus of solitary tract, which is consistent with the fact that peripheral administration of cholecystokinin decreases food consumption in MC3 receptor knock-out but not MC4 receptor knock-out animals [175]. Thus, the nucleus of the solitary tract is considered to be a neural hub where the peripheral satiety signal cholecystokinin (via dorsal vagal afferents) and melanocortin signaling (via hypothalamic projections or native population) interact to influence downstream second order neurons necessary for meal termination [63, 142, 147, 171-173]. Regardless of the evidence of the involvement of the melanocortin system to influence feeding via a brainstem mechanism, further research is necessary to fully elucidate whether the hyperphagia observed in MC4 receptor knock-out animals is a consequence of defective satiation.

The third mechanism by which the melanocortin system may influence feeding is through reward-related brain structures. A close relationship exists between the MC3 receptor, MC4 receptor and dopaminergic neurotransmission in the ventral tegmental area and nucleus accumbens [176, 177]. Thus, infusion of Melatonan II into the ventral tegmental area increases dopamine release in the nucleus accumbens [178]. Other evidence on the influence of melanocortin system on the reward circuitry comes from studies where animals exposed to various addictive drugs show alterations in hypothalamic pro-opio melanocortin transcripts [179, 180]. Also, central administration of Melatonan II facilitates the threshold lowering effect of amphetamine in a lateral hypothalamic self-stimulation paradigm [181], i.e. melanocortin receptor stimulation increased the rewarding properties of amphetamine.

Consistent results were also reported by Hsu et al. where the rewarding and psychomotor stimulant effects of cocaine were blocked by intra-nucleus accumbens injection of SHU9119 [182]. Furthermore, up and down-regulation of the MC4 receptor in the striatum has been shown respectively after chronic cocaine or morphine treatment [182, 183]. It was also demonstrated that the locomotor activation after cocaine administration was abolished in the MC4 receptor knock-out mice and reduced in heterozygous MC4 receptor animals [182]. The up-regulation of striatal MC4 receptor in animals in response to repeated cocaine administration suggests that the melanocortin system plays a role in drug induced behavioral sensitization. Collectively, these studies suggest that increased melanocortin signaling via the αMSH pathway enhances the sensitivity to drugs while reduced αMSH signaling will lead to the opposite. Thus, if we compare drug and food reward, an opposite situation would be expected. As the melanocortin system mediates anorexia and satiation, the reward-enhancing effect of melanocortins appears paradoxical. This discrepancy may be due to the fact that several of the above studies used Melatonan II, which binds to both MC3 receptor and MC4 receptor, making it impossible to distinguish the individual roles of these receptors. Second, it is possible that the melanocortin system interacts with other neuropeptidergic systems to influence homeostatic and reward mechanisms, but future studies need to clarify this. Cabeza de Vaca and colleagues also suggest that a differential αMSH tone exists in brain areas responding to food or drug rewards [181]. This results in a differential role of melanocortins in regulating food-intake as opposed to rewarding effects of drugs. Even though several studies have been conducted to study the interrelationship between melanocortins, dopamine and drugs of abuse, there are almost no data on the role of melanocortin signaling in the mesolimbic dopaminergic neurons with respect to food intake.

Orexins and overeating

The lateral hypothalamus is one of the fundamental sites bridging the gap between the homeostatic and hedonic aspects of feeding. Lesions of the lateral hypothalamus induce anorexia [184], underscoring its role in feeding. Evidence of its influence on the brain reward circuit comes from electrical stimulation studies, as electric stimulation of the lateral hypothalamus is highly reinforcing [99, 185]. Studies in rodents demonstrate connections between the lateral hypothalamus and brain reward areas (the ventral tegmental area and nucleus accumbens) [186-188], and lateral hypothalamic neuronal populations of orexinergic [189], GABAergic [186] and melanin concentrating hormone-containing neurons [190], have been shown to modulate dopaminergic signaling in the mesolimbic dopamine circuit.

The role of orexins with respect to feeding and addiction has been widely studied. Acute central administration of orexin leads to a robust hyperphagic response in rodents and other

vertebrates [175]. Analogous to leptin, orexin also plays a dual role in regulating both homeostatic and hedonic aspects of food intake. It has been suggested that orexin mediates its homeostatic aspect of feeding through its connection with the arcuate nucleus where it has been shown to regulate the neuropeptide Y and POMC neurons [191]. Leptin hyperpolarizes orexinergic neurons [192] and decreases orexin messenger RNA expression [23]. However, administration of an OX1 receptor antagonist only partially reverses the obese phenotype in ob/ob mice [193]. Likewise, leptin only partly ameliorates the orexinergic effect of orexin in rats [194], suggestive of simultaneous neuronal pathways being involved in body weight regulation. A single i.c.v. orexin A administration induced enhanced food intake in satiated animals [195], indicating that orexin A induced hyperphagia may be mediated by orexin's effect on the brain reward mechanisms. Additionally, similar to drugs of abuse, orexin administration in the nucleus accumbens increases locomotor activity with a simultaneous increase in feeding [196]. Likewise, increased fos activation in the lateral hypothalamus orexinergic cells was shown in animals that demonstrated a conditioned place preference for cocaine, morphine or food [197]. In the same paper, it was also shown that systemic injection of an OX1 receptor antagonist reverses the conditioned place preference for morphine. Furthermore, chemical activation of orexinergic neurons in the lateral hypothalamus re-instated extinguished morphine place preference [197]. All these data indicate an important role for orexins in the modulation of the reward function and that activation of these neurons is important to couple drug rewards with environmental cues [198].

Multiple lines of research also connect orexins to hedonic feeding. Central administration of orexin A increased free feeding of sucrose pellets and also responding for sucrose pellets under fixed ratio and progressive ratio schedules of reinforcement [196]. The increased self-administration of sucrose pellets was decreased by a systemic injection of an OX1 receptor antagonist [62]. I.c.v. administration of orexin also augments high fat diet preference [199] and treatment with an OX1 receptor antagonist decreased self-administration of high fat diet [200], indicating that the approach behavior towards a reward is possibly mediated through OX1 receptor. Analogously, animals given i.c.v. orexin show reduced breakpoints for high fat diet reward under a progressive ratio schedule, and this increase in breakpoints is reversed when an OX1 receptor antagonist is systemically administered [201]. In a recent publication, Choi et al. also showed that systemic administration of an OX1 receptor antagonist decreased high fat diet consumption in satiated animals [201], an observation similar to that of Nair and colleagues [200]. Hence, it may be concluded that orexin signaling is important in overriding homeostatic mechanisms regulating food intake to drive animals towards hedonically determined food consumption. Orexinergic fibers from the lateral hypothalamus project both to the ventral tegmental area and the nucleus accumbens; and in the former area, they make extensive connections with dopaminergic cell bodies [202] that express OX1

receptor and OX2 receptor [203]. Indeed, orexin A injections into the ventral tegmental area cause elevated dopamine levels in the nucleus accumbens [203] and orexins have been found to increase the firing of dopaminergic neurons in vitro [204]. Since orexins modulate dopaminergic signaling in the nucleus accumbens, it is likely that the effects of orexin on reward processes discussed in the preceding paragraph are mediated via the mesolimbic dopaminergic circuit. Direct effects of orexin A in the nucleus accumbens were also reported by Thrope and Kotz, where infusion of orexin A into the nucleus accumbens -shell was accompanied by heightened locomotion and feeding [196]. It is interesting to note that dopaminergic neurons of the ventral tegmental area project both to the nucleus accumbens -shell and neurons from the nucleus accumbens -shell area project back to the lateral hypothalamus orexin neurons, establishing a feedback loop in this circuitry [198].

Recently, it was shown that appetite, meal frequency and length of a meal were also increased with central administration of orexin A [205]. This suggests that hindbrain satiation mechanisms may be involved in orexin A mediated feeding. In fact, orexin A administration in animals with hindbrain (area postrema, nucleus of solitary tract) lesions, resulted in a decrease in meal size without altering the meal frequency [205]. In support of this, orexin-immunoreactive fibers and orexin receptors are present in the dorsal vagal complex, a neural hub where peripheral satiety signals interact with neuropeptidergic systems to control satiety [206]. Thus, we can conclude that orexin-mediated hyperphagia may result due to enhanced hedonic feeding or altered satiation. However, the exact mechanism of action of orexin demands further investigation.

Brain derived neurotropic factor and implications in overeating

The ventromedial hypothalamus is yet another hypothalamic area participating in energy balance. Lesions of this area are associated with enhanced feeding while electrolytic stimulation results in suppression of feeding [207, 208]. Brain derived neurotropic factor, a neuronal growth factor, belonging to the neurotropin family [209], is expressed in high levels in the ventromedial hypothalamus [210]. More than a decade ago, the importance of brain derived neurotropic factor in feeding was established [210, 211]. It was shown that i.c.v. infusion of BDNF led to weight loss in rodents [211]. Mice heterozygous in the BDNF locus showed enhanced adiposity accompanied with increased locomotor activity and leptin resistance [210]. Expression of the BDNF receptor, tyrosine kinase B (TrkB receptor) leads to weight gain and hyperphagia, while stimulation of the receptor is accompanied by weight loss in animals exposed to a diet-induced obesity paradigm [212]. Food deprivation has been also shown to reduce BDNF expression in the ventromedial hypothalamus [213], highlighting the anorexic role of brain derived neurotropic factor. Studies in humans support this anorexic property of this neuropeptide, as mutations in the BDNF gene were

accompanied by hyperphagia, obesity and hyperactivity [214]. Similarly, Yeo and colleagues reported a de-novo missense mutation in the TrkB receptor gene that resulted in overt hyperphagia and obesity [215]. Genome wide association studies conducted on a European population reported a BDNF locus with a genome wide significance (p≤1.6×10−7) for obesity [216].

Since BDNF is widely expressed in the ventromedial hypothalamus [213], the next step was to understand if the anorexic effects of BDNF were region specific. Indeed, ventromedial hypothalamus specific depletion of BDNF has been shown to enhance weight gain and hyperphagia [217], but the underlying molecular mechanisms governing brain derived neurotropic factor effect on feeding remain unclear. One of the hypotheses is that BDNF is a downstream target of the melanocortin system, as MC4 receptor stimulation enhances BDNF secretion [63]. In keeping with this hypothesis, reduction of MC4 receptor signaling in the ventromedial hypothalamus is characterized by decreased BDNF messenger RNA, and melanocortin receptor agonist treatment reverses the food deprivation-induced reduction in BDNF messenger RNA in the ventromedial hypothalamus [213]. Whilst exogenous leptin injection does not revert hyperphagia in diet-induced obesity models, it was shown that BDNF injection in these animals successfully decreased food intake [218]. This suggests that despite disrupted leptin receptor signaling in obesity, downstream effector pathways (melanocortins, BDNF) are still functional and modulation of these pathways may be helpful in devising new tools to treat obesity. Indeed, it was demonstrated in the same paper that chronic brain derived neurotropic factor infusion for 6 days not only decreased food intake and body weight, but also decreased serum leptin concentrations in animals exposed to high fat diet for 4 months. Likewise, fasting insulin levels were also reduced in diet-induced obesity animals repeatedly treated with BDNF [218]. However, there are also data to suggest that BDNF is directly regulated by leptin on BDNF secreting neurons, since after leptin administration, co-localization of phosphorylated STAT-3 protein positive neurons and brain derived neurotropic factor messenger RNA was observed [29].

Apart from being a downstream target of the melanocortin system, BDNF can also modulate feeding by influencing the mesolimbic dopaminergic circuit. Alterations BDNF and TrkB receptor messenger RNA levels in the ventral tegmental area are found in animals exposed to a high fat diet paradigm [219]. The connection between BDNF, drugs of abuse and mesolimbic dopaminergic system has been studied to some extent. BDNF knockdown in the ventral tegmental area and nucleus accumbens reduced cocaine place conditioning [220]. Furthermore, nucleus accumbens -specific TrkB receptor or BDNF deletion decreased cocaine self-administration [221]. Likewise, cocaine injections elevate BDNF protein and TrkB receptors in the nucleus accumbens. There is evidence to indicate that the ventral tegmental area has its own sub-population of BDNF secreting neurons and recently, it was

shown that BDNF knock-out animals, were not only hyperphagic, as previously reported [217], but also showed increased high fat diet consumption in a restricted access paradigm, simulating binge eating behavior [219].

Converging evidence further indicates that BDNF increases dopaminergic signaling in the mesolimbic circuitry. Central BDNF depletion was accompanied by blunted dopamine release and diminished D2 receptor in the nucleus accumbens-shell and the dorsal striatum. Furthermore, knocking down the BDNF gene in the ventral tegmental area increased the desire for high fat diet compared to standard chow [219]. Thus, Cordeira and colleagues suggest: (1) differential functions of hypothalamic and ventral tegmental area BDNF, the former modulating homeostatic control of feeding and the latter governing its hedonic aspects; and (2) since electrically evoked dopamine release in the dorsal striatum is reduced in BDNF knock-out mice, it may be possible that the hyperphagia found in ventral tegmental area brain derived neurotropic factor knock-out animals is a way to compensate for low dopamine levels.

It is known, that hypothalamic BDNF levels are decreased in response to high fat diet consumption in obesity-sensitive mice [222], which is in line with the finding that high fat diet induces leptin resistance and directly or indirectly (via the melanocortin system) decreases BDNF secretion. Animals fed on palatable food, show an increase in the TrkB receptor transcript and a decrease in BDNF in the ventral tegmental area 30 and 60 minutes post high fat diet exposure [219]. However, the effects mentioned above were either in the hypothalamus or transient. Thus, the consequence of leptin resistance for BDNF expression in the ventral tegmental area/ nucleus accumbens is yet to be determined. Comparing BDNF effects in the hypothalamus and in the ventral tegmental area, in the former, leptin resistance might induce low levels of BDNF and thus minimize its anorectic effect, while in the latter the situation is more complex. Based on the dopamine hypo functioning theory of addiction [71, 223], one might expect that decreased leptin receptor signaling, as encountered in obesity, will also decrease BDNF levels, further dampening dopamine release and thereby elevating craving for rewards in order to compensate for lower striatal dopamine levels. However, a detailed analysis of the state of the BDNF system in the mesolimbic circuitry needs to be performed with respect to diet-induced obesity and different stages of leptin resistance, to assess the possible similarities of this system in obesity and addiction.

It has been reported that BDNF might interact with satiety signals in the brainstem to reduce food intake [224]. BDNF immunoreactive fibers and TrkB receptors are found in the dorsal vagal complex [225]. Infusions of BDNF into the dorsal vagal complex have been shown to attenuate food consumption and promote weight loss. Moreover, the amounts of

endogenous BDNF in this site were diminished in response to low leptinemic condition as in food restriction whereas they were correspondingly increased after peripheral injections of leptin or satiety hormone cholecystokinin [224]. However, whether this anorexic action of BDNF is mediated by leptin itself or via the melanocortin pathway is uncertain. Therefore, further studies looking into the effect of BDNF on meal size would be helpful.

Conclusions

The emerging similarities between obesity and substance abuse disorders, makes us think about the possibility that obesity is a form of addiction where the brain reward system, which responds to natural rewards like food and sex, has been biologically re-programmed to enhance 'liking' or 'wanting' for food. Several similarities between overeating and drug addiction have been shown. These include highly increased motivation to seek food and lasting neurobiological changes in reward-associated brain regions. However, whereas there is an enormous body of evidence to document the changes in brain and behavior that result from repeated and prolonged exposure to drugs of abuse which may contribute to the development of drug addiction [71, 81, 124] this kind of research on the neurobiology of obesity is only emerging [5].

Therefore, future research must be directed at studying the most pertinent aspects of addiction in the context of obesity, e.g. to compare food and drug seeking behavior under aversive consequences, to elucidate whether overeating indeed has a compulsive element [99, 110, 111]. The link between the hypothalamic sites which integrate metabolic information and the brain reward system becomes increasingly clear [66, 88]. In the current paper, we specifically focused on the mesolimbic dopaminergic system due to the extensive studies conducted on this system, but it should be borne in mind that other systems like the opioid and the endocannabinoid systems substantially contribute to the brain's response to drug or food rewards as well. Readers are referred to excellent recent reviews on this topic [6, 84, 118, 168]. The present review focused on the homeostatic and hedonic roles of three crucial neuropeptidergic systems: melanocortin system, orexins and BDNF, which act downstream of leptin. Other neuropeptides (e.g. melanin concentrating hormone, Neuropeptide Y) that play a distinct role in overeating have not been discussed here (see [60, 226-228] for excellent reviews on this topic). It is evident that the function of the peptides involved in the feeding regulation is not entirely homeostatic. In fact, the homeostatic and hedonic aspects of feeding are not mutually exclusive and one influences the other.

The melanocortin system is perhaps the principle catabolic modulator of energy balance in animals. αMSH mediates its anorexic effect by affecting several downstream nuclei: paraventricular nucleus (increasing energy expenditure), lateral hypothalamus (via

orexinergic neurons and its connection to the mesolimbic dopaminergic circuit), ventromedial hypothalamus (BDNF and its downstream targets), nucleus of solitary tract/dorsal vagal complex (by interacting with peripheral satiety signal cholecystokinin) and directly influencing the mesolimbic circuit (motivated approach behavior) [57, 141, 142, 144]. Overconsumption can be seen as a secondary phenomenon in response to altered neuropeptidergic systems and rewiring of the brain reward circuitry encountered in leptin-resistant states. Hence, understanding the concrete roles of these neuropeptidergic systems with respect to the reward circuitry in both physiological and leptin resistant states will provide more answers to the leptin resistance-overeating link. It is important to note that although several similarities exist between over-eating and addiction, it does not automatically mean that overeating is a form of addiction. Nevertheless, the booming scientific interest on this field together with a rapidly evolving line of research categorically focusing on overeating as a form of addictive behavior may help us resolve the 'missing link' between these two conditions.

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Figures

Fig. 1: Leptin receptor signaling cascade. Binding of leptin to the extracellular domain of its receptor leads to the activation of janus kinase-2 [20] , which in turn induces the phosphorylation of three downstream tyrosine residues (Try 985, Tyr 1077 and Try 1138) [18]. Jak-2 autophosphorylation further activates several downstream proteins like the insulin receptor substrate (IRS), extracellular signal regulated kinases [21, 22] and the phosphor inositid 3-kinases (PI3-K) [23]. It is through the PI3-K kinase pathway by which Neuropeptide Y (NPY) levels are down regulated in the arcuate nucleus upon leptin action. Furthermore, active PI 3-K leads to the stimulation of the mammalian target of rapamycin (mTOR) pathway via Protein Kinase B (Act) [24]. Tryosine phosphatase protein 1B (PTP1B) acts as a negative modulator of JAK-2 kinase [23]. Tyr 1138 phosphorylation activates the signal transducer activator of transcription-3 (STAT-3) protein which thereby promotes propoiomelanocortin (POMC) expression [25], suppressor of cytokine signaling 3 (SOCS3) synthesis [26-28] and possibly brain-derived neurotrophic factor (BDNF) [29]. SOCS-3 negatively influences the activity of the Try 985 and Jak-2 proteins [30, 31]. At the Try 985 residue, phosphorylation commences the activity of the SHP-2 protein, which competes with SOCS-3 to bind to Try 985 and further promote ERK activity [28]. The function of active signal transducer activator of transcription-5 (STAT-5), at the Try1077 still needs to be clarified [32].

Fig. 2: Leptin Resistance. The possible mechanisms of leptin resistance in obesity. For references see text. Additional references [45-48].

Fig. 3: Neuroanatomy of energy homeostasis: Schematic representation the principle hypothalamic sub-nuclei involved in feeding behavior. MCH: melanocyte concentrating hormone, CRH: corticoitropin releasing hormone TRH: thyrotropin releasing hormone OxY: Oxytocin SF-1: orphan nuclear receptor SF-1 (for references see text).

Fig. 4: The interaction of the dopaminergic, endogenous opioid and GABAergic systems in brain reward circuitry. Dopaminergic neurons in the ventral tegmental area send afferent fibers to the nucleus accumbens , prefrontal cortex, orbitofrontal cortex and the amygdala (not shown). The prefrontal cortex and the orbitofrontal cortex are essential sites for the cognitive control of reward-associated behavior, which send glutamergic projections to i.e. the nucleus accumbens and amygdala. The role of these neocortical structures has been widely studied with respect to addiction. Lateral hypothalamus sends orexinergic signals to the nucleus accumbens, details of which will be discussed in one of the following sections. The nucleus accumbens also receives synaptic inputs from several brainstem nuclei (parabrachial nucleus, nucleus of solitary tract: not shown), carrying gustatory and satiety signals. The opioid system (arcuate nucleus, nucleus accumbens, ventral tegmental area) forms a part of both feed forward and feedback loop, to modulate activity within the reward circuitry [5, 84].

Chapter 3

The role of melanocortins and neuropeptide Y in food reward

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Abstract

The Neuropeptide Y and the melanocortin peptides are two well-described hypothalamic feeding peptides regulating energy balance. Predominantly expressed within the arcuate nucleus, these neurons project to different brain areas and modulate various aspects of feeding. Hedonic feeding, where one overindulges in palatable food consumption beyond one's nutritional necessities, is one such aspect regulated by NPY/melanocortin signaling. Research suggests that NPY/melanocortin regulate hedonic aspects of feeding through its projections to the brain reward circuitry (ventral tegmental area, lateral hypothalamus, nucleus accumbens etc.), however exact target areas have not yet been identified. The current work explores literature to provide a mechanistic explanation for the effects of these peptides on food reward.

Introduction

The discovery of leptin in the early 90's was one of the hallmark events in the field of obesity [1]. This breakthrough led to a cascade of novel findings deciphering the key neuronal and molecular elements within the mammalian feeding circuitry. One of the earliest findings included the identification of two first-order antagonistically acting neuronal populations within the arcuate nucleus as immediate downstream targets for leptin. These were the neuropeptide Y (NPY) and Melanocortin systems [2]. Negatively regulated by leptin, the NPY system comprises of the orexigenic peptide NPY and it's ubiquitously expressed receptors within the mammalian brain [2]. The melanocortin system on the other hand comprises of the anorexigenic peptide αmelanocyte stimulating hormone (αMSH), a derivative of the pro-opiomelanocorticotin (POMC) peptide and it's centrally expressed: melanocortin3, and melanocrtin4 receptors [3-5]. NPY containing neurons of the arcuate nucleus co-synthesize agouti-related peptide (AGRP), a potent orexigenic peptide and an inverse agonist of the melanocortin receptors [6]. Additionally, a subset of NPY/AGRP neurons co-secrete inhibitory neurotransmitter γamino butyric acid (GABA) and project to the POMC neurons, indicating an overall orexigenic overdrive at the level of the arcuate nucleus [7, 8].

Following the identification of NPY and melanocortin systems as downstream targets of leptin, a plethora of studies has focused on the role of these peptides in regulating feeding and energy expenditure [9, 10]. However what had been initially overlooked is their specific role in relation to food reward i.e. how do these two peptidergic systems influence palatable feeding? The arcuate NPY/AGRP and αMSH neurons project to various hypothalamic and limbic sites that form a part of the brain reward system [11] which regulates hedonic feeding. In contrast to homeostatic feeding where food consumption is driven by the organism's need to sustain life and reproduction, hedonic consumption refers to voluntary overconsumption of palatable food items beyond one's biological or nutritional requirements. Interestingly, environmental triggers (starvation, availability of palatable food) that increase motivational drive for palatable food rewards similarly alter hypothalamic NPY/POMC levels [12-14] suggesting that neuropeptidergic systems like the NPY and melanocortins are susceptible to environmental triggers and presumably modulate food reward. The past decade witnessed a surge in studies investigating the role of feeding peptides in relation to food and drug rewards. The current review assimilates the available data on NPY and melanocortin systems to provide a comprehensive overview on how these systems influence reward mechanisms.

Neuropeptide Y and reward

Before zooming into the role of NPY in food reward, it is important to have a neuroanatomical overview of the brain reward circuitry that regulates the hedonic and motivational aspects of natural and artificial rewards. The reward circuitry is a

conglomeration of diverse nuclei spread over fore- and mid-brain structures. It principally includes the lateral hypothalamus, prefrontal cortex, ventral tegmental area, amygdala and the striatum [15]. One of the key elements indispensable to the functioning of this system is the mesolimbic dopaminergic system. Originating in the ventral tegmental area (VTA) the dopaminergic neurons, among other areas project to the nucleus accumbens (NAc), pre-frontal cortex and amygdala. The VTA and NAc connection has been historically implicated in motivation for food and natural rewards [15, 16]. For example, animals depleted in ventral striatal dopamine reduce their motivation to obtain a food reward, without subsequently affecting their appetite [17, 18]. Peripheral injections of cis-Flupentixol, a non-selective dopamine receptor antagonist, likewise attenuate lever pressing for both preferred and non-preferred food pellets while free feeding remains unaffected [19]. And, optogenetic stimulation of the dopaminergic neurons during a fixed ratio task increases the number of active lever presses but not the total consumption of food-rewards [20]. Collectively these data underline the significance of the VTA-NAc dopaminergic connection with respect to food-reward. Besides dopamine other players like opioids and cannabinoids substantially influence the brain reward circuitry [21]. Despite the individual importance of each system, the functioning of the reward circuitry is a product of complex neuronal and neuropeptidergic interactions regulating multiple aspects of reward processing.

The connection between NPY and food intake is well-established. Brain nuclei such as the paraventricular nucleus and the lateral hypothalamus have been identified as loci mediating the orexigenic actions of NPY [22-25]. But whether these orexigenic effects of NPY are due to increased hunger, motivation, rewarding value of food, decreased satiation or a combination, remains unclear. It may well be that the different aspects of NPY-induced hyperphagia are mediated by different neuronal circuitries. For example, studies suggest that the hyperphagic effects of NPY are in part mediated by the brain reward circuitry. States that increase arcuate NPY levels (caloric deprivation, genetic models of obesity, exposure to obesogenic diets), simultaneously induce hyperphagia and heighten motivation [12, 13, 26, 27]. Apart from being expressed in the hypothalamic nuclei, NPY receptors are widely distributed throughout the brain including areas regulating decision making, motivation and reward sensitivity [28]. As the arcuate nucleus is the most prominent source of NPY and has notable projections to reward related brain nuclei, multiple studies have focused on the role of arcuate NPY on food reward. Animals exposed to a free-choice high-fat high-sucrose diet, where animals have a choice of a high-fat and a high-sugar dietary components besides regular chow, exhibit palatability-induced hyperphagia [29]. This hyperphagia in turn is accompanied by increased arcuate NPY levels [14]. Intriguingly, animals exposed to either one of the palatable components besides chow i.e. [30] either sugar or fat are not hyperphagic nor demonstrate augmented arcuate NPY levels, thus coupling palatability-induced hyperphagic response to elevated arcuate NPY levels [14]. In agreement with these

findings, Levin et al reported that obesity resistant animals when exposed to a high caloric palatable drink (Ensure™) become hyperphagic and display enhanced arcuate NPY levels. Upon withdrawal from Ensure, the animals decrease 60% of their caloric consumption, lower their body weight and normalize their arcuate NPY levels to chow control levels [31]. Contrarily, obese-prone animals when food restricted, decrease their body weight, adiposity, circulating leptin levels and consequently increase arcuate NPY levels [31]. Through this study the authors could dissociate NPY-mediated hedonic feeding from homeostatic feeding. In the obesity-resistant group, augmented NPY levels signal hyperphagia and are therefore normalized in the absence of a palatable diet. Oppositely in the obesity-prone group elevated NPY levels warn of a calorie deprived state and is a signal to normalize energy balance. This interaction between NPY and palatability-induced hyperphagia is further supported by the fact that NPY knock-out animals fail to show a hyperphagic response to palatable diet [30].

Besides triggering palatable overconsumption, central NPY also enhances motivation to obtain a food reward. Food deprivation enhances both arcuate NPY levels and lowers the threshold for lateral hypothalamic self-stimulation (LHSS). In order to evaluate the relation between NPY and brain reward in general, two studies examined the effects of central NPY infusion on LHSS. The authors unanimously demonstrate that a dose of NPY that enhances food intake fails to lower LHSS thresholds, as would be expected for a rewarding appetitive agent [32, 33]. This indicates the unlikelihood of NPY mediating the rewarding effects of food restriction or on LHSS. While these studies negated the effects of NPY on rewarding brain stimulation, they lacked specificity in terms of a) not using local injections; therefore the net effect of NPY acting on diverse nuclei might be null and b) not studying the effect on NPY on food reward itself. Subsequently, by studying the effects of NPY on food itself researchers confirmed that central NPY infusion is indeed rewarding. Rats exposed to a lever-pressing paradigm to obtain food, increase the number of responses upon NPY infusion [34, 35]. Similarly, mice centrally treated with NPY overindulge on a palatable milk solution coupled to a light electric shock and overindulge on quinine-adulterated milk solution [36]. This confirms that the central effects of NPY on hedonic feeding can override the associated aversive consequences, a feature predominantly native to drugs of abuse [37, 38]. To further delineate the brain areas mediating these rewarding effects, Brown et al (1998) in their paper locally infused NPY in the perifornical hypothalamus and studied motivation of animals for a palatable reward [39]. The perifornical hypothalamus is an area reciprocally connected to the arcuate nucleus on one hand and VTA and the NAc on the other, thus serving as a neuroanatomical interface between homeostatic and hedonic brain centers. The authors report that NPY infusion in the perifornical hypothalamus enhances motivation for a sucrose reward in a progressive ratio paradigm of reinforcement while simultaneously augmenting sucrose consumption in a free-feeding paradigm. Extending these findings, they demonstrate that a low dose of NPY in the perifornical hypothalamus

besides stimulating free-feeding, conditions a place preference [40]. Oppositely, the same dose of NPY in the NAc conditions a place preference without evoking hyperphagia [40]. Thus, NPY-mediated overconsumption is not a sole consequence of an enhanced state of hunger. Instead, NPY drives both homeostatic and hedonic aspects of food intake and via different neuronal networks (perifornical hypothalamus vs. nucleus accumbens).

Since motivation, food reward and dopamine are closely coupled; studies have addressed the NPY-dopamine interaction to explain the rewarding effects of NPY. A series of evidence affirms this interaction. Firstly, NPY and its receptors are expressed in dopaminergic neurons within the VTA [41] and in the NAc [28, 42]. Secondly, several independent studies show that both intracerebroventricular and intra-NAc administered NPY increases extracellular striatal dopamine levels and more specifically dopamine levels within the NAc [43-46]. As augmented accumbal dopamine levels are coupled with enhanced motivation [47, 48], it is fair to assume that NPY could potentially modulate motivation through its interaction with the dopaminergic system. Finally, behavioral findings endorse these neuroanatomical and neurochemical observations. Josselyn and Beninger demonstrate that conditioned place preference upon NPY infusion in the nucleus accumbens can be blocked by cis-Flupentixol, a non-selective dopamine receptor antagonist, illustrating the dopamine dependency of the rewarding effects of NPY in the NAc [49]. However, Brown and colleagues report that the increased motivation for sucrose pellets following NPY infusion in the peri-fornical hypothalamus is independent of dopamine action [39]. The authors base their conclusion on the fact that cis-Flupentixol itself decreased active lever presses and rewards earned and therefore its effects on NPY-induced motivation are non-specific. Nonetheless these data do not exclude the possible interaction between NPY and dopamine at the level of the perifornical hypothalamus. The motivation attenuating effect of cis-Flupentixol implies that the dopaminergic antagonist has a stronger suppressive effect on motivation/goal-directed behavior that fails to override the stimulating effects of NPY.

NPY-dopamine interactions are not exclusive for food-rewards. Studies indicate that NPY potentiates other dopamine-dependent behaviors. Central infusion of NPY also increases cocaine induced locomotor activity and lever pressing for cocaine, both well-known dopamine dependent behaviors [50]. Rats exposed to dopaminergic stimulants (Apomorphine and Amphetamine) increase their locomotor activity, which can be blocked by an NPY1 receptor antagonist [51]. Likewise, both self-administration of cocaine and cocaine-induced hyper-locomotion are reduced upon pretreatment with an Y5 antagonist. Similar effects are observed in mice lacking Y5 receptors [52]. In fact, the decrease in cocain-induced hyper-locomotion is accompanied by lower accumbal dopamine levels in both Y5 antagonist treated and Y5 knock-out animals. Based on their findings that Y5 receptors co-localize with VTA dopaminergic neurons and that Y5 knock-out animals have lower

accumbal dopamine levels following cocaine administration, the authors suggest that NPY acts on VTA dopaminergic neurons via Y5 mechanisms to increase dopamine output to the nucleus accumbens, thus potentiating reward-related behaviors. Collectively these data support NPY as a general appetitive stimulus which interacts with the mesolimbic dopaminergic system to modulate rewarding aspects of both food and drugs of abuse [52]. In conclusion, the rewarding aspects of NPY are not only limited to food rewards but extends to other drugs of abuse. NPY acts as a general peptidergic signal that mediates brain reward mechanisms through its interaction with the dopaminergic system. Nevertheless the cross-talk between NPY and other peptidergic systems (like the opioid system) although not discussed here, exists and cannot be overlooked. Furthermore, specific studies evaluating the site-specific role of NPY on food or drug reward are needed to provide a mechanism underlying NPY's action on food intake and motivation.

Melanocortins and reward

The central melanocortin system forms another well-characterized downstream target of leptin signaling. Discovered prior to leptin itself, this system comprises of (1) neurons expressing the cleaved peptidergic products of precursor pro-opio melanocorticotrophic hormone (POMC): α, β- and γ- melanocyte stimulating hormone (MSH) (2) neurons expressing AGRP, an inverse agonist for the melanocortin receptors and (3) the central melanocortin3 receptor and melanocortin4 receptor [9]. The melanocortin system is a well-defined anorexigenic system as melanocortin agonism is associated with decreased body weight [53]and antagonism has the opposite effect [54-56].

The exact mechanism by which melanocortins regulate body weight is multi-faceted and involves regulation of various aspects of feeding and locomotion [57]. Although studied to a lesser extent, one mechanism by which melanocortins regulates feeding is through its action on hedonic consumption. Third ventricular application of AGRP in rats apart from inducing hyperphagia increases c-Fos expression within the hypothalamus (PVN, DMH) and reward related nuclei (lateral hypothalamus, nucleus accumbens and central nucleus of amygdala) [58]. Simultaneously, attenuation of melanocortin signaling by central infusion of AGRP increases motivation for food rewards [59, 60] although the role of melanocortin receptors mediating these effects remain unidentified. Contrasting studies have demonstrated that melanocortin4 receptor knock-out mice are less motivated when lever pressing for food pellets in a two-lever seeking-taking fixed ratio task [61] while enhancing their lever presses on a shallow progressive ratio schedule [53, 62]. Taken together, these data indicate that hyperphagic effects of dampened melanocortin signaling can be in part mediated by the brain reward circuitry.

Several lines of studies indicate that much of the reward related effects of melanocortins are mediated through its interaction with the dopaminergic system. Firstly, neuroanatomical

studies illustrate that both POMC and AGRP containing neurons of the ARC project to reward related brain nuclei including ventral tegmental area [63, 64], nucleus accumbens [65], and lateral hypothalamus [66]. Secondly, the neurobiological interaction observed between the two systems serves a functional consequence. Central infusion of AGRP augments c-Fos expression in dopaminergic neurons [67]. In contrast, dampening of AGRP signaling or infusion of αMSH within the VTA enhances dopamine levels within basal forebrain areas, specifically within the NAc. This effect in turn can be blocked by a selective melanocortin4 receptor antagonist, confirming a melanocortin4 receptor mediated mechanism [64, 68]. Treatment with γMSH, a melanocortin ligand with a higher affinity for the melanocortin3 receptor likewise enhances accumbal dopamine levels underpinning the role of melanocortin3 receptor [69]. Lastly, dopamine-dependent behaviors like lever pressing for a reward and grooming are modulated by melanocortin signaling. Melanocortin4 receptor knock-out animals and double melanocortin3 receptor and melanocortin4 receptor knock-out animals when lever pressing for food in increasing fixed ratio paradigms, are sensitive to a ratio-strain [70]. Here animals substantially decrease their lever presses at increasing cost of rewards, a characteristic typical for dopamine depleted mice [71]. Similarly grooming, a dopamine dependent behavior is potentiated by αMSH and is mediated by the melanocortin4 receptor [72-74]. Studies have confirmed that αMSH simultaneously increases grooming and striatal dopamine levels [75], an effect blocked by dopamine antagonists [4].

Although these studies establish a functional modulation of dopaminergic signaling by melanocortins within the VTA, currently no data exists that outlines the mechanism by which melanocortins does so. Predominantly melanocortin3 receptor but also melanocortin4 receptor are expressed within the VTA [5, 76], however the identity of the VTA-neurons remains unestablished. One possible explanation is proposed by De Barioglio and colleagues. In the presence of a GABAA antagonist, αMSH augments grooming behavior. Although speculative, this suggests that even in the absence of GABA signaling, αMSH directly acts on dopaminergic neurons to stimulate dopamine-related behavior [77]. Similar to the VTA, melanocortin receptors are expressed within the NAc. In vitro studies on striatal slices show that melanocortin agonists increases cAMP signaling, a second messenger downstream the melanocortin3 receptor and melanocortin4 receptor. While this cAMP increase can be blocked by a dopamine1 receptor antagonist, lesioning of incoming striatal dopaminergic projections does not attenuate cAMP levels [78]. This indicates 1) the existence of a cross-talk between the melanocortin and dopaminergic systems at the level of the NAc and 2) that melanocortin receptors are not located in the dopaminergic terminals within the striatum but rather post-synaptically. In contrast to the NAc and the VTA, where the neuronal identity of melanocortin containing neurons is largely unknown, in a recent publication authors successfully map the identity and projections of melanocortin4 receptor

containing neurons within the LH [79]. The authors demonstrate that the melanocortin4 receptors-containing neurons within the LH co-express neurotensin and the leptin receptor but not orexin or melanin concentrating hormone. Counter intuitively, these melanocortin4 receptor- expressing neurons project to nucleus of the solitary tract and the parabrachial nucleus rather than to the NAc and VTA, indicating melanocortin4 receptor signaling within the LH might not play a role in melanocortin-mediated motivation. Nevertheless the role of melanocortin3 receptor in this area remains yet to be determined.

Further evidence supporting interaction between the melanocortin and the dopaminergic system is mirrored in drug reward studies. When looking at reward mechanisms, the effect of melanocortin receptor ligands on food and drug reward is divided. Treatment with melanocortin agonists enhance motivation for palatable food rewards [60]. Diet-induced obese animals besides augmenting their motivation for palatable rewards demonstrate decreased hypothalamic POMC and enhanced AGRP levels [14, 29, 60]. Therefore the prevailing concept that attenuation of melanocortin signaling enhances feeding is subsequently reflected in motivation for food as well. But when it comes to drug rewards, the effects are counterintuitive. MTII, a melanocortin agonist fails to show an effect on LHSS by itself, but decreases the threshold-lowering effects of amphetamine on LHSS [80]. Similarly, locomotor sensitizing effects of cocaine are mitigated by melanocortin antagonists or in melanocortin4 knock-out animals suggesting that the reward sensitizing effects of melanocortin are mediated by the melanocortin4 receptor. Despite existing inconsistencies, studies report alterations in POMC levels and melanocortin receptor bindings within the hypothalamus and striatal regions upon cocaine administration [81, 82]. Summing up evidence from drug studies thus imply that potentiation of melanocortin signaling consequently potentiates drug sensitization. An intriguing question however arises: how does the melanocortin system regulate rewards for food and drugs differently?

One possibility is that melanocortin signaling can act on different neuronal networks when comparing drugs of abuse with food rewards. Cross-sensitization experiments suggest that food and drugs share overlapping pathways [83-85]. However, when considering food rewards, central melanocortin peptides may influence both appetite and motivation while for drug rewards the appetite component is absent. Hence, the increased motivation for a drug reward observed after central melanocortin agonist infusion is presumably mediated by the VTA. Contrarily, the enhanced motivation for food rewards observed after melanocortin agonist infusion can be predominantly mediated by another brain area but also might be a reflection of augmented appetite rather than solely motivation. Finally, majority of studies on melanocortin system and reward mechanisms focus on the melanocortin4 receptor pathway and therefore effects mediated by the melanocortin3 receptor remains understudied. Furthermore, differences between doses, ligands and

experimental protocols can influence experimental outcomes. Thus in a given context (food reward or drug reward) intracerebroventricular administration of melanocortin peptides can act via some shared and some separate pathways to have a net effect.

To demarcate the locus of melanocortin action on motivation, a study by Hsu and show that infusion of melanocortin antagonists in the core region of the NAc attenuate both lever presses and place preference for cocaine [86]. Based on their findings that accumbal dopamine1 receptor neurons express melanocortin4 receptor and that both dopamine1 receptor and melanocortin4 receptor increase intracellular cAMP levels, the authors suggest that melanocortin4 receptor and dopamine1 receptor act in concert to potentiate the dopamine1 receptor pathway and modulate drug reward sensitization. This assumption is backed up by the fact that potentiation of the dopamine1 receptor pathway is essential for sensitization for cocaine [87]. Although the model put forward by Hsu and colleagues fits with the available literature, the model by itself is speculative as the authors do not demonstrate whether αMSH potentiates the dopamine1 receptor pathway. Furthermore the doses used by Hsu et al. locally within the NAc (SHU9119 1μg) were similar to the doses used by Cabeza de Vaca and colleagues i.c.v, implying that the effects noted by the former group can in fact be non-specific [80, 86]. In contrast, a recent study on slice electrophysiology shows that αMSH blunts excitatory synaptic transmission by decreasing dopamine1 receptor neuronal AMPAR/NMDA receptor ratio, a marker of long term depression [87]. Furthermore, pretreatment with αMSH occludes NMDAR dependent LTD through melanocortin4 receptor mechanisms. The authors additionally report that increases in cAMP generated upon melanocortin4 receptor activation by αMSH, interacts with EPAC2, a protein downstream of cAMP. This EPAC2 protein has been shown to be involved in AMPA receptor internalization and reduction of excitatory neurotransmission and thus mediates LTD [88]. The authors therefore conclude that αMSH signaling in the NAc leads to mitigation of the dopamine1 receptor pathway by possible internalization of AMPAR. Thus when considering food rewards, this explanation would hold true, if NAc would mediate the effects of αMSH.

The discrepancy between the effects of melanocortin on motivation for food and drug rewards makes the formulation of a general model explaining melanocortin effects on reward mechanisms, difficult. However, based on the available literature, a model of melanocortin effects on reward mechanisms can be chalked out where αMSH acts on the VTA dopaminergic neurons to increase dopamine release within the NAc. Within the NAc, melanocortin act via melanocortin4 receptor to reduce synaptic transmission in dopamine1 receptor neurons and thus inhibiting these neurons, however the consequence of NAc inhibition for food/drug rewards is yet to be determined [89]. Similarly, the role of the lateral hypothalamus in this model is unclear (Fig. 1).

Concluding remarks

In today's society enriched with palatable foods, hedonic overconsumption is a familiar term. Consequently in the past decade our interest in the mechanisms underlying hedonic feeding has surged. Following the discovery that drug or food rewards share overlapping pathways and that metabolic molecules such as leptin influences brain reward mechanisms [83, 84], attention was shifted to decipher the role of peptides lying downstream of the leptin pathway. Work from several labs show that indeed both these neuropeptidergic systems influence hedonic feeding and that they act in a pathway-specific manner. Furthermore, the mesolimbic dopamine system emerges as a crucial interactor of the NPY/melanocortin system. However, regardless of the vast evidence supporting the cross talk between the melanocortin/NPY and the dopaminergic systems, the molecular mechanisms underlying these neuroanatomical, functional and behavioral findings remain elusive. Another finding that unanimously stands out is the importance of the NAc in food-reward. Receiving incoming projections from excitatory glutaminergic cortical neurons, modulatory dopaminergic and hypothalamic neurons while abundantly expressing opioids, this nucleus is the seat for interaction between various systems. Nevertheless only further studies will elucidate how these peptides interact with each other to influence reward-related behaviors.

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Figures

Fig. 1. AGRP and POMC neurons within the arcuate nucleus (ARC) project to the Ventral Tegmental Area (VTA), Lateral Hypothalamus (LH) and Nucleus Accumbens (NAc). Within the VTA, AGRP neurons are shown to synapse on DA neurons [64]. Presumably, POMC neurons do the same [77]. Stimulation of melanocortin signaling in this area increases dopamine levels within the NAc [68]. Within the lateral hypothalamus, POMC neurons predominantly project to Neurotensin neurons which also expresses the leptin receptor [79]. POMC projections from the ARC to the NAc synapse on dopamine1 receptor containing neurons which co-express melanocortin4 receptor. αMSH dampens excitatory synaptic transmission on dopamine1 receptor neurons [90], a pathway involved in drug sensitization [87, 91].

Chapter 4:

Limbic substrates of the effects of Neuropeptide Y on intake of and motivation for palatable food

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Abstract

Neuropeptide Y (NPY), given centrally augments food-intake and the motivation to work for palatable food. Here, we aimed to identify the brain regions through which NPY increases food-intake and motivation. NPY was infused into three brain regions implicated in food-intake and motivation: the lateral hypothalamus (LH), nucleus accumbens shell (NAc) and ventral tegmental area (VTA). Motivation for sucrose was assessed using a progressive-ratio schedule of reinforcement in which the effort to obtain successive rewards increased incrementally. To disentangle the effects of NPY on motivation for palatable food from food consumption, free-feeding experiments were performed in which animals had ad-libitum access to sucrose-pellets. NPY infusion into either VTA or NAc increased the motivation to respond for sucrose, whereas infusion of NPY in either NAc or LH increased sucrose consumption. In addition, the effect of intra-VTA NPY on motivation for food was attenuated after pretreatment with the dopamine receptor antagonist αflupentixol. These data identify specific limbic substrates through which NPY influences consumption of and motivation for palatable food. The motivational effects of NPY are exerted through the VTA, its consummatory effects through the LH, and the NAc is involved in both.

Introduction

Predominantly expressed in the arcuate nucleus of the hypothalamus, Neuropeptide Y (NPY) plays a prominent role in the regulation of food-intake [1]. Through projections to various hypothalamic and extra-hypothalamic regions, arcuate nucleus NPY-expressing neurons modulate food-intake [2]. In addition to its role in hypothalamic homeostatic regulation of food-intake, a few studies have also shown a role for NPY in food-motivated behavior [3, 4]. Several hypothalamic regions are known to be involved in NPY-mediated food-intake, but there is only scarce information on the brain areas that mediate the effects of NPY on food-motivation [3, 5]. We here identified the neural substrates of NPY-driven motivation for food by infusing NPY into three brain regions implicated in food-intake, reward or motivation, i.e. the lateral hypothalamus (LH), nucleus accumbens shell (NAc) and ventral tegmental area (VTA), all of which contain NPY immunoreactivity and receptors [6].

The effect of NPY on motivation for sucrose pellets was determined using a progressive-ratio schedule of reinforcement [7]. To dissociate food motivation from consumption, the effect of intra-LH, -NAc and -VTA NPY infusion was assessed in a sucrose free-feeding experiment. Since NPY receptors are expressed on dopaminergic neurons within the VTA [8] and that central infusion of NPY increases extracellular striatal dopamine levels [9], we further investigated whether the effects of intra-VTA NPY infusion were dopamine-dependent. Here animals were pretreated with saline or a dopamine receptor antagonist prior to NPY infusions in the VTA.

Methods and Methods

For details of surgical procedures, microinfusion of drugs and experimental designs we refer to the supporting data.

Animals

Male Wistar rats (Charles River, Germany) were maintained on ad-libitum chow (3.31 Kcal/g, SDS, UK) and housed individually in temperature (21±2 °C) and light controlled rooms (lights-off 0700-1900 hr). All experiments were approved by the Animal Ethics Committee, Utrecht University.

Drugs

Rat NPY (H-6375, Bachem, Germany) was dissolved in sterile saline and infused bilaterally in a counterbalanced fashion at doses of 0, 78,156 and 235 picomoles/300 ηl /side. Each animal received all doses of the drug. The doses were based on a previous study in which the effect of NPY infusion into the perifornical hypothalamus on motivation for food

was studied [3].

Statistics
All data were analyzed using SPSS (IBM, USA). Progressive-ratio data were analyzed using nonparametric Friedman's repeated measure analysis followed by Wilcoxon signed rank test corrected for multiple testing. Free-feeding data were analyzed using a repeated measures ANOVA followed by post-hoc Bonferroni tests. A p-value below 0.05 was considered statistically significant.

Histology
Following sacrifice, cannula placements were histologically verified using cresyl-violet staining. Few animals (LH:2, NAc:1) were excluded from the study due to incorrect cannula placement (Fig. S1).

Results

Effect of NPY on motivation for sucrose
Infusion of all doses of NPY into the VTA significantly increased the number of ALP ($\chi^2(3)$ =18.25 p<0.001, Fig 1A) and reinforcers earned ($\chi^2(3)$ =13.44, p<0.05, Fig 1B). Comparable to the VTA, infusion of NPY into the NAc significantly increased active lever presses ($\chi^2(3)$ =10.46, p<0.05, Fig 1A) and reinforcers earned ($\chi^2(3)$ =10.46, p<0.05, Fig 1B) at doses 78 and 156 picomoles. NPY infusion into the LH did not affect ALP ($\chi^2(3)$ =4.44, p>0.05, Fig 1A) or rewards earned ($\chi^2(3)$ =6.00 p>0.05, Fig 1B). Inactive lever presses was not affected by NPY infusion into any area (data not shown).

Effect of NPY on free-feeding of sucrose
The amount of sucrose consumed was significantly higher following of NPY infusion into the LH at the doses of 156 and 235 picomoles (F (3, 21) =12.29, p<0.05, Fig 1C). Similar to the LH, NPY infusion into the NAc enhanced the free intake of sucrose at the doses of 156 and 235 picomoles (F (3, 18) =6.27, p<0.05, Fig 1C). In contrast, infusion of NPY into the VTA did not alter free-feeding (F (3, 18) =0.36, p>0.05, Fig 1C). The 24hr chow-intake following NPY infusion into all three brain areas remained unchanged (Fig S3).

The motivational effect of intra-VTA NPY is dopamine-dependent
Infusion of NPY increased the number of ALP and rewards earned (Fig 2). While αflupentixol pretreatment did not have an effect by itself, it attenuated the NPY driven increase in ALP ($\chi^2(3)$ =12.60, p<0.05, Fig 2A), and rewards earned ($\chi^2(3)$ =14.68, p<0.05, Fig 2B).

Discussion

We here demonstrate a site-specific dissociation between the effects of NPY on sucrose consumption and motivation for sucrose after administration into different limbic regions. Infusion of NPY into either VTA or NAc increased motivation to respond for sucrose, while it failed to do so after infusion into LH. In contrast, NPY infusion into LH and NAc, but not VTA, enhanced sucrose consumption in the free-feeding paradigm.

In line with the literature [5], we demonstrate that when fed ad-libitum, NPY signaling in LH enhances palatable food-intake, however, incentive motivation for sucrose remains unaffected. This underlines the role of LH in NPY-mediated hyperphagia, but the downstream pathways remain unknown [5]. Although NPY in LH enhanced sucrose free-feeding, the 24-hr chow intake remained unaltered. As chow intake was measured 24hr after NPY treatment, any orexigenic effects of NPY at an earlier time-point cannot be excluded.

NPY receptors are expressed on orexin- and MCH-containing neurons in the LH [10, 11]. These are orexigenic neuropeptides that act downstream of the leptin-sensitive NPY/POMC neurons. Orexin-containing projections from the LH to the VTA are known to modulate motivation for palatable rewards [12] without affecting free-feeding [13]. Since NPY infusion into LH failed to enhance motivation for sucrose, it is unlikely that intra-LH NPY activated this population of orexin-containing neurons to the VTA. Interestingly, both orexin and MCH neurons project to the NAc and infusion of these peptides within the NAc provokes food consumption [14, 15], implicating a role for LH-NAc connections in mediating NPY effects on sucrose free-feeding.

Our current findings are supported by the fact that orexin and MCH neurons lie downstream of the arcuate NPY neurons and possibly mediate the orexigenic effects of NPY. However, studies have also shown that applied ex vivo, NPY inhibits orexin and MCH neurons [10, 11]. This evidence and the fact that multiple NPY receptor subtypes are expressed within the LH (both post- and presynaptically), calls for neuronal projection-specific studies as a next step to unravel the role of NPY in the LH.

Similar to the NAc, NPY infusion into the VTA increased motivation for palatable food. However, free-feeding remained unaffected, indicating an exclusively motivational role of NPY within the VTA. Given the role of mesolimbic dopamine in the motivation for food [16, 17], it is conceivable that the effect of intra-VTA NPY on responding for sucrose was mediated by dopaminergic neurotransmission. Central NPY administration increases striatal dopamine levels, especially within the NAc, an effect mediated by Y5 receptors [9]. Since (a) NPY receptors are expressed on the dopaminergic neurons in the VTA [8] and (b) that increased NAc dopamine levels are associated with enhanced incentive motivation [18], we hypothesized that NPY administration into VTA potentiates dopamine release and enhanced

motivation. Indeed, the augmented motivation for sucrose observed upon NPY infusion into VTA was attenuated after pretreatment with a dopamine receptor antagonist, supporting the role of dopamine in NPY-induced motivation for sucrose.

Interestingly, NPY infusion into the VTA increased the motivation for sucrose but not its intake. Thus, after intra-VTA NPY infusion, animals were willing to work for a sugar reward but did not consume more when it was provided ad-libitum. These results are reminiscent of previous work implicating dopamine in motivation for food, rather than in consumption [16, 17, 19].

NPY infusion into the NAc augmented free-feeding, and it also increased operant responding for sucrose. The effect of NPY within the NAc is therefore both on consumption of and motivation for sugar. Our data are in line with findings of Josselyn & Beninger (1993) showing NPY effects within the NAc on conditioned place preference. Moreover, they provide evidence that these effects are dopamine dependent (22).

The NAc can be considered a critical hub connecting limbic, cortical and hypothalamic circuits regulating food-intake. As the NAc mediates both increased motivation and free-feeding, one possibility is that NPY signaling in the NAc induces these separate effects via different neuronal circuits (e.g. ventral pallidum vs. LH). Alternatively, the increase in motivation for sucrose may be secondary to an increase in the positive subjective value ('pleasure') of sucrose, which may result in both an increase in intake and in the motivation to respond.

The data presented support the notion of region-specific diversity of NPY action on food-directed behavior. In addition to earlier studies demonstrating the motivational and hyperphagic effects of central NPY infusion, we identified specific neural substrates underlying NPY-enhanced operant responding for sucrose. Our studies further identify NPY-dopamine interaction at the level of the VTA as a key player for NPY driven motivation for food rewards.

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Figures

Fig. 1: (A) active lever presses and (B) sucrose pellets earned upon NPY infusion into the VTA (n=11), NAc (n=14) and LH (n=10) expressed as percentage of saline infusion. (C) Free-feeding of sucrose pellets in a 30-minute period after saline vs. NPY infusion (expressed in grams). * p<0.05 compared to saline injections. Values represent as Mean± SEM.

Fig 2: (A) Active lever presses and (B) rewards earned after treatment with αflupentixol (Flu, 0.125 mg/kg, i.p.) prior to infusion of NPY (156 picomoles/side) into the VTA (n=10). * indicate statistically significant (p<0.05) differences between the various treatment conditions. Values expressed as Mean± SEM.

Supplemental Data

Fig. S1: Cannula placements within the NAc, LH and the VTA. Numbers indicate relative location from the bregma.
Relative to the bregma, the following coordinates were used: LH (AP: -2.80 ML: +1.70 DV: -8.60), NAc (AP: +1.20 ML: +2.80 DV: -7.50, angle10°) and the VTA (AP: -5.40, ML: +2.20, DV: -8.90, angle10°).

Fig. S2: Scheme of the experimental designs.

Fig S3: 24-hr chow intake following NPY infusion. (LH: $F_{(3, 21)} = 3.01$, $p>0.05$, NAc: $F_{(3,18)} =25.31$, $p<0.05$, post hoc tests non-significant, VTA: $F_{(3, 18)}=6.99$, $p<0.05$, post hoc tests non-significant)

Materials and Procedures

Operant Testing

First, rats were trained under a fixed-ratio 1 schedule of reinforcement in two-lever operant conditioning chambers (30.5 x 24.1 x 21.0 cm, Med-Associates, USA), in which each active lever press lead to retraction of the levers, illumination of a cue light above the active lever and delivery of a 45-mg sucrose pellet (Noyes precision pellets Formula F, Research Diet, USA). Twenty seconds after the pellet was delivered, the lever was reinserted into the chamber. Presses on the inactive lever were counted but had no programmed consequences. Sessions lasted 30 min or until the rats earned 60 pellets, whichever occurred first. The position of the active and inactive levers was counterbalanced between animals. Next, the animals were trained under a progressive- ratio schedule of reinforcement. Here the number of lever presses required to obtain successive rewards increased according to the following equation: response ratio =(5e (0.2 × reward number))-5 (6) through the following series: 1, 2, 4, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 603, 737.

The session ended when the animal failed to earn a reward within 60 min. Microinfusions were performed once responding had stabilized, i.e., when the number of active lever presses (ALP) did not differ more than 15% between three consecutive sessions, and there was no up- or downward trend. Figure S2 gives an overview of the experimental protocol.

Chapter 5

Central melanocortins regulate the motivation for sucrose reward

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Abstract

The role of the melanocortin (MC) system in feeding behavior is well established. Food intake is potently suppressed by central infusion of the MC 3/4 receptor agonist αmelanocyte stimulating hormone (αMSH), whereas the MC 3/4 receptor inverse-agonist Agouti Related Peptide (AGRP) has the opposite effect. MC receptors are widely expressed in both hypothalamic and extra-hypothalamic brain regions, including nuclei involved in food reward and motivation, such as the nucleus accumbens (NAc) and the ventral tegmental area. This suggests that MCs modulate motivational aspects of food intake. To test this hypothesis, rats were injected intracerebroventricularly with αMSH or AGRP and their motivation for sucrose was tested under a progressive ratio schedule of reinforcement. Food motivated behavior was dose-dependently decreased by αMSH. Conversely, AGRP increased responding for sucrose, an effect that was blocked by pretreatment with the dopamine receptor antagonist αflupentixol. In contrast to progressive ratio responding, free intake of sucrose remained unaltered upon αMSH or AGRP infusion. In addition, we investigated whether the effects of αMSH and AGRP on food motivation were mediated by the NAc shell. In situ hybridization of MC3 and MC4 receptor expression confirmed that the MC4 receptor was expressed throughout the NAc, and injection of αMSH and AGRP into the NAc shell caused a decrease and an increase in motivation for sucrose, respectively. These data show that the motivation for palatable food is modulated by MC4 receptors in the NAc shell, and demonstrate cross-talk between the MC and dopamine system in the modulation of food motivation.

Introduction

The central melanocortin (MC) system, which functions downstream of the leptin pathway, forms an integral part of the hypothalamic feeding circuitry [1]. Within the arcuate nucleus of the hypothalamus, pro-opiomelanocortin (POMC) neurons express α-melanocyte stimulating hormone (αMSH), an agonist for the MC3 and MC4 receptors. A neighboring neuronal population expresses Agouti Related Peptide (AGRP), an inverse agonist for the same MC receptors [1-4]. The opposing effects of αMSH and AGRP on feeding are well established.

In states of hunger, hypothalamic αMSH levels are decreased while AGRP levels are increased [5;6], whereas the reverse pattern is observed in states of obesity or positive energy balance following overfeeding [7;8]. In line with these observations, central infusion of αMSH or its cyclic analogue MTII robustly suppresses food intake [9;10], whereas treatment with AGRP or the MC3/4 receptor antagonist SHU9119 has the opposite effect [9;11]. These studies clearly demonstrate the role of MCs in food intake. However, it remains incompletely understood to what extent these effects are the result of changes in hunger, satiety or motivation to obtain food.

Animals exposed to an obesogenic diet display increased motivation for food accompanied by lower POMC levels [12;13]. Furthermore, MC4 receptor knock-out mice become obese and exhibit augmented food-motivated behavior in non-food-deprived states [14]. Together, these findings suggest that lower MC receptor activity is associated with an enhanced motivational drive for food. Thus, there is some evidence that MC ligands modulate the motivation for food [15;16], but the neural mechanisms underlying these effects remain unclear.

One brain area of particular interest is the nucleus accumbens (NAc), through which dopaminergic neurotransmission regulates food-motivated behavior [17]. Activation of the NAc shell has been reported in animals lever pressing for sucrose pellets under a progressive ratio schedule of reinforcement [18]. Moreover, central infusion of AGRP leads to activation of the NAc shell, indicating that the behavioral effects of AGRP involve this brain area [19]. Intriguingly, overexpression of AGRP in the NAc shell has no effects on food-intake and body weight [20], suggesting that the NAc mediates other effects of MCs on eating, such as motivation for food and food palatability. Combined, these pieces of evidence identify the NAc shell as a candidate region to mediate the effects of MCs on motivation for food.

Importantly, food reward or reward-related stimuli have been shown to increase dopamine levels within the NAc, whereas responding for a palatable food reward was inhibited by

administration of a dopamine receptor antagonist into the NAc [21;22]. We therefore hypothesized that the effects of MC ligands on food-motivated behavior are dopamine mediated. Support for this hypothesis is provided by studies showing that the NAc receives projections from hypothalamic MC neurons and abundantly expresses MC4 receptors [2;23-26]. In addition, microdialysis experiments have shown that, compared to the NAc core, the NAc shell is more responsive to dopamine increases when animals are engaged in an operant task [27].

In the present paper, we therefore investigated the role of the MC system in food motivation by determining the effect of central infusion of MC 3/4 receptor ligands on the motivation to obtain a palatable food reward (i.e., sucrose) under a progressive ratio schedule of reinforcement [28;29]. The involvement of dopamine neurotransmission in the effects of MC 3/4 ligands on food-motivation was determined using the dopamine receptor antagonist αflupentixol. Finally, in order to determine whether the NAc shell mediates the effect of MC signaling on food reward, we first assessed whether MC receptors were expressed within the NAc shell and subsequently studied the behavioral effects of MC ligands injected directly into the NAc shell.

Materials and Methods

Animals
Male Wistar rats (Charles River, Sulzfeld, Germany) weighing 200-225 g on arrival were individually housed (Macrolon cages; 40x26x20 cm) with ad libitum access to rat chow (3.31Kcal/gram, Standard Diet Service, UK) and tap water. Animals were kept in a temperature (21±2 °C) and humidity (60-70 %) controlled room under a 12 h reversed light/dark cycle (lights on at 19.00 h). All experimental procedures were approved by the Animal Ethics Committee of Utrecht University and were in agreement with Dutch laws (Wet op Dierproeven 1996) and European regulations (Guideline 86/609/EEC).

Surgery
Surgery was performed when animals weighed between 275-300 g. Rats were anaesthetized with 0.1 ml/100 g i.m. fentanyl/fluanisone (Hypnorm, Janssen Pharmaceutica, Belgium). For implantation of intracerebroventricular (i.c.v.) cannulas, the head was shaved and the skull exposed by a midline incision of the skin. After preparation of a small craniotomy (approximately 1 mm in diameter) , a 5 mm stainless steel guide cannula (Plastics One, USA) was inserted into the lateral ventricle (1 mm lateral and 1 mm posterior from bregma). Cannulas were fixed to the skull with two stainless steel screws and dental cement. For implantation of cannulas aimed at the NAc shell, animals were positioned in a stereotaxic apparatus (David Kopf, USA) and stainless steel guide cannulas (26 GA, 8 mm; Plastics One,

USA) were implanted bilaterally, 1 mm above the NAc shell. Coordinates relative to bregma were as follows (in mm): AP: +1.20 ML: + 2.80 DV: - 7.50, angle 10° (Paxinos and Watson, 1998). Perioperatively, rats received carprofen (5 mg/kg s.c.) and saline (3 ml s.c.). Behavioral experiments commenced following a 10-14 days recovery period.

At the end of the experiment, cannula placement was verified using histological methods. Animals equipped with i.c.v. cannulas were euthanasized with carbon dioxide and received an i.c.v. injection with 2 μl of ink. Brains were then removed and the lateral ventricles opened to check for ink staining. Rats equipped with NAc shell cannulas were decapitated and their brains removed and frozen on dry ice. Next, cryostat sections (16 μm) were stained with cresyl violet to determine cannula locations. Data from animals with cannula placements outside the target area were excluded from the analysis.

Drugs and microinfusions

αMSH (Bachem GmbH, Germany) and AGRP (83-132) Amide (Phoenix Pharmaceuticals, USA) were dissolved in sterile saline. For i.c.v. infusions, rats were briefly restrained and 2μl of drug solution was slowly (10-15 seconds) injected into the ventricular cavity. Infusions into the NAc shell were performed through an injector (8 mm, 33 GA, Plastics One, USA) inserted into the guide cannula. Bilateral infusions (300 ηl over 30 sec) were given using a syringe pump with the injectors left in place for another 30 sec to allow for diffusion. Behavioral testing commenced 5 min after drug infusions. αflupentixol dihydrochloride (Sigma Aldrich, USA) was dissolved in saline and injected i.p. 30 min prior to i.c.v. infusions. All animals received all drug doses/combinations, according to a latin square design. Furthermore, a minimum 2-day drug-free period was maintained between infusions during which the animals were trained but not tested.

Experimental design

Central effects of αMSH and AGRP on motivation for sucrose

Apparatus: Operant conditioning chambers (30.5 cm x 24.1cm x 21.0 cm; Med-Associates, USA) situated in light- and sound-attenuating cubicles equipped with a ventilation fan were used. Each chamber had a metal grid floor, two retractable levers with white stimulus lights above it and a food dispenser, which delivered 45 mg sucrose pellets (Noyes Precision Pellets Formula F, Research Diets, USA) to the food receptacle. Chambers were illuminated by a white house light. Data collection and processing was controlled by MED-PC software.
Training: Following recovery from surgery, the rats were first trained under a fixed ratio (FR) 1 schedule of reinforcement, with 2 sessions/day for five consecutive days. Under this schedule, a single press on the active lever resulted in the delivery of one 45 mg sucrose pellet, illumination of the light above the lever and retraction of the lever. Twenty seconds

after the pellet was received, the lever was reinserted into the chamber. Sessions lasted 30 min or until rats earned 60 pellets, whichever occurred first. Presses on the inactive lever were recorded, but had no programmed consequences. Positions of the active and inactive levers were counterbalanced between animals. After 10 sessions under the FR1 schedule, the progressive ratio (PR) schedule of reinforcement was introduced. Under a PR-schedule, the cost of a reward is progressively increased over successive trials to determine the effort the rat will emit for it. The response requirement increased according to the following equation [28;29]: response ratio= (5 X e(0,2 x infusion number))– 5 through the following series: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 603, 737. The session ended when the animal failed to earn a reward for 60 min. Responding was considered stable when the number of food pellets earned per session did not differ more than 15 % for three consecutive sessions and up- or downward trends were absent. After 10 training sessions, rats were tested during a period of 3-5 weeks with 5-6 training sessions/week including training on non-testing days. All behavioral testing was performed between 8.00 and 17.00 h.

In a group of 12 rats, the effects of αMSH on PR-performance were tested. On test days, rats received either saline (2 µl) or αMSH (0.66 ηmol or 1.2 ηmol) according to a latin square design. Data from 5 rats were excluded from analysis due to instable responding under the PR schedule (3 rats) and misplaced cannulas (2 rats).

To study the effects of AGRP on responding for sucrose under the PR schedule of reinforcement, a separate group of 12 rats were either infused with saline or AGRP (0.66 ηmol or 1.0 ηmol) according to a latin square design. Data from 5 rats were excluded from analysis due to instable responding under the PR schedule (2 rats), unexplained weight loss following testing (2 rats) and a misplaced cannula (1 rat).

Central effects of αMSH and AGRP on free-feeding of sucrose

Free-feeding experiments were conducted to determine whether the effects of αMSH and AGRP were specific for the animal's motivation for palatable food or secondary to an effect on feeding behavior in general. In this setup, animals had free access to sucrose pellets for 60 min every day, i.e. the animals did not need to perform an operant task to obtain the sucrose pellets. Ad-libitum fed rats were introduced into an empty cage with a suspended steel receptacle containing 45 mg sucrose pellets. Sessions took place in the beginning of the dark period. After 1 h, sucrose pellet intake was measured. To accustom rats to the procedure and stabilize sucrose intake, 4-5 training sessions were performed before testing. A separate group of 12 rats was used to determine the effects of αMSH on free-feeding of sucrose. Rats were infused with saline (2 µl) and two doses of αMSH (0.66 ηmol or 1.2 ηmol) according to a latin square design. Data of 5 rats were excluded from analysis due to

unexplained weight loss after injection and testing (4 rats) and a misplaced cannula (1 rat). In a separate experiment, effects of AGRP on free-feeding of sucrose were studied. Twelve rats were infused with saline (2 µl) and AGRP (0.66 ηmol or 1 ηmol) according to a latin square design. Data of 3 animals were excluded from analysis due to unexplained weight loss following i.c.v. injection.

Interaction between dopamine and MC systems in the motivation for sucrose

To investigate the role of dopamine in the effects of AGRP on food-motivated behavior, a pilot experiment was first performed to determine the dose of the dopamine receptor antagonist αflupentixol to be used. A separate group of rats (n=6) was injected with αflupentixol according to a latin square design (all rats received 0, 0.125, 0.25, 0.5 mg/kg i.p.) 30 minutes prior to a PR session (data not shown). The dose used for the interaction experiment (0.125 mg/kg) was chosen because it had no effect on PR performance, whereas higher doses reduced responding under a PR-schedule of reinforcement [30] .
A separate group of eighteen rats was injected i.p. with saline or αflupentixol (0.125 mg/kg, 0.5 ml) 30 min prior to i.c.v. saline or 1 ηmol of AGRP infusion according to a latin square design. Five minutes after i.c.v. infusions, responding for sucrose under a PR schedule was tested as outlined above. Data of 6 rats were excluded from analysis due to unexplained weight loss after injection (1), misplaced cannulas (1), and unstable responding during training and testing (4).

The role of the NAc in MC-dependent motivation for sucrose

Fluorescent in-situ hybridization to characterize MC4 receptor expression in the NAc
In order to understand whether the effect of αMSH and AGRP on food motivation is mediated through the NAc, we first studied the expression patterns of MC3 or MC4 receptors in the NAc, and whether they were expressed by dopamine D1 and/or D2 receptor positive cells. For this purpose, a triple in-situ hybridization experiment with 3 different fluorophores was performed.

For this experiment, a naive group of rats was used that was not subjected to behavioral testing. Following decapitation, brains were frozen on dry ice. Coronal sections (16 µm) were fixed with 4% paraformaldehyde (10 min), subsequently washed with PBS (3x, 10min) and further acetylated (10 min). Following acetylation, slices were preincubated with a prehybridization mix (50 % deionized formamide, 5x SSC, 5x Denhardt's solution, 250 µg/ml tRNA baker's yeast, 500 µg/ml sonicated salmon sperm DNA final concentrations in MilliQ). Next, sections were incubated overnight at 40 ºC in 120 µl hybridization mix (Panomics) containing the probe sets (concentration D1/D2 1:33, MC3 receptor/MC4 receptor 1:50) and washed. Probe sets used to detect the desired rat mRNAs we designed by Panomics

(Santa Clara, USA), using published sequences (see S1 table and [31]). The probes were designed to hybridize at adjacent regions of the target mRNA, allowing the hybridization of a preamplification oligo (Panomics) that spans the hybridized probe pair, thus ensuring signal specificity. This specific signal was further amplified by hybridization of amplification oligo's (Panomics) and visualized by label oligo's (Panomics), resulting in an amplification of up to 500 times for low abundant mRNAs.

Sections were then incubated for 1.5 h at 40 ᵒC in 120 μl PreAmplifier Mix (Panomics) containing preamplification oligos (PreAmp TYPE4 1:20, PreAmp TYPE 6 1:50, PreAmp TYPE 8 1:33) and washed. Then, sections were incubated for 1.5 h at 40 oC in 120 μl Amplifier Mix (Panomics) containing amplification oligos (Amp TYPE4 1:20, Amp TYPE 6 1:50, Amp TYPE 8 1:33) and washed. Next, sections were incubated for 1.5 h at 40 ᵒC in in 120 μl in Label Probe Mix (Panomics) containing label oligos (LP TYPE4 1:20, LP TYPE 8 1:33, LP TYPE 6 1:50). After washing, sections were incubated in 750 μl PBS supplemented with 4',6-diamidino-2-phenylindole (DAPI) (6.7 μg/ml, Sigma Aldrich, St. Louis, USA) for 5 min. Finally, slices were washed and embedded in Mowiol. Sections were visualized and images were obtained with a Zeiss AxioScope A1 microscope (Carl Zeiss, , Jena, Germany) equipped with Chroma filter sets (Chroma, Bellows Falls, USA) and Zeiss AxioVision Rel. 4.8 acquisition software. DAPI images were acquired using the 31000v2 filter block of Chroma. The Chroma FITC filter block 410001 was used to acquire the 488 nm conjugated TYPE4 label. The 550 nm conjugated TYPE8 label was acquired using a Chroma TRITC 41002b filter block containing a narrowband excitation filter. A custom Chroma Cy5 infrared filter was used for the acquisition of the 650 nm conjugated TYPE6 label. This label was excited at 650 nm using an HQ650/45x filter (Chroma) and light was directed by a Q680LP dichroic mirror (Chroma) through a HQ690LP emission filter (Chroma). Images were processed and analyzed using ImageJ 1.43r software.

The (co)expression of the mRNA transcripts of the different receptors was quantified within the core and shell sub-regions of the NAc. At least 8 images per sub-region, spanning the entire rostro-caudal axis of the NAc (300 μm x 220 μm) were used for quantification of mRNA with the DRD1 and DRD2 transcripts. Cells were identified on the basis of nuclear DAPI staining and were counted as expressing a certain mRNA if one or more fluorescent dots were present in, or in close vicinity of (defined as a circle with twice the diameter of the DAPI staining) the area of DAPI staining. The percentage of cells expressing a certain mRNA was determined by dividing the amount of cells expressing a certain mRNA by the total amount of DAPI stained nuclei. The percentage of cells co-expressing dopamine D1 or the dopamine D2 receptor mRNA with MC3 receptor or MC4 receptor mRNA was determined by dividing the amount of cells positive for the dopamine D1 or the dopamine D2 receptor and MC3 receptor/MC4 receptor mRNA by the total amount of dopamine D1 and dopamine D2

receptor positive cells.

POMC fibers innervate the NAc Shell

In order to study whether POMC neurons innervate the NAc, we performed an immunohistochemistry study. Two rats were perfused with ice-cold 0.9 % NaCl followed by 4 %PFA in PBS. Following perfusion, brains were removed, post-fixated overnight in 4 %PFA, and then embedded in 30 % sucrose solution in PBS. Free-floating 40μm slices were stained for POMC peptide. Briefly, slices were washed with 0.5M Tris-Buffered solution (TBS; 3x10min), and then blocked with super mix (0.5M TBS, 0.25 % gelatin and 0.1 %TritonX) for 30 minutes. Following blocking, slices were incubated with primary antibody against POMC (Rabbit, H-029-30, Phoenix Pharmapseuticals, USA) (4°C, overnight) on a shaker. Subsequently slices were washed (TBS, 3x10min) and incubated with fluorescent secondary antibody (chicken anti rabbit, 488, A-21441 Alexa Fluor, Life Technologies, USA) for 1 hour at room temperature. Slices were further washed (3x10min) with TBS, put on glass slides and embedded with Flurosave (Merk Milipore, USA). Images were made using Zeiss AxioScope A1 microscope (Carl Zeiss, Jena, Germany) and processed with ImageJ sofatware.

Effects of NAc infusions of αMSH and AGRP on motivation and free-feeding of sucrose

To determine the role of the NAc shell in the effects of αMSH and AGRP on the motivation for sucrose, animals received bilateral infusions of saline and αMSH (0.2 ηmol) or AGRP (0.1 ηmol) in a counterbalanced fashion (300 ηl in 30 seconds). Subsequently, the animals were tested as outlined in earlier. Data from 1 animal was excluded from the final analysis due to incorrect cannula placement. Free-feeding experiments were conducted in a separate group of rats as described in earlier. Here, 11 animals received bilateral NAc shell infusions with saline, αMSH (0.2 ηmol) and AGRP (0.1ηmol) in a counterbalanced manner and tested for sucrose consumption for one hour.

Statistical analyses

For the final analyses, data were excluded from animals with 1) instable responding under the PR schedule of reinforcement (i.e., > 15 % variation between three consecutive sessions and/or a consistent upwards or downwards trend in the number of rewards earned); 2) unexplained body weight loss (>20 g); 3) incorrect cannula placements. Unless otherwise indicated, data from all experiments were analyzed using repeated measures ANOVA followed by a Tukey post-hoc test where appropriate. Data from experiment with αflupentixol were analyzed using two-way repeated measures ANOVA with i.p. injections (saline vs αflupentixol) and i.c.v. infusions (saline vs AGRP) as within subject's factors. Data from experiments with infusions of MC ligands in the NAc shell were analyzed using a paired

samples t-test. Differences were considered significant at p<0.05. All statistical analyses and graphical representations were performed using Graphpad software (v 6.03, USA). All data are expressed as Mean ± SEM.

Results

Central effects of αMSH and AGRP on the motivation for sucrose

I.c.v. infusion of αMSH (1.2 ηmol) significantly reduced both the number of active lever presses ($F_{(2,12)}=4.5$, p<0.05, Fig. 1A) and rewards earned ($F_{(2,12)}=6.0$, p<0.05, Fig. 1B). The amount of inactive lever presses following infusion of αMSH remained unchanged ($F_{(2,12)}=5.0$, p>0.05, Fig. 1A). Conversely, the highest dose of AGRP (1.0 ηmol) significantly increased the number of active lever presses ($F_{(2,12)}=10.6$, p<0.05, Fig. 1D) and rewards earned ($F_{(2,12)}=11.5$, p<0.05, Fig. 1E). The number of inactive lever presses following infusion of AGRP was enhanced ($F_{(2,12)}=5.0$, p<0.05, Fig. 1D).

Central effects of MCs on free-feeding of sucrose

In the free-feeding paradigm, infusion of αMSH or AGRP had no effects on sucrose intake ($F_{(2,24)}=2.3$, p>0.05, Fig. 1C), ($F_{(2,28)}=3.1$, p>0.05, Fig. 1F).

Interaction between dopamine and MC systems in the motivation for sucrose

In order to assess the interaction between the MC and dopaminergic systems, rats were pre-treated with either αflupentixol or saline, prior to infusion of saline or 1ηmol of AGRP. Infusion of 1ηmol of AGRP increased the number of active lever presses and rewards earned under the PR schedule of reinforcement. The effect of AGRP on both these measures was attenuated by pre-treatment with αflupentixol, whereas αflupentixol failed to affect PR performance on its own (Fig. 1G and Fig. 1H), Two-way repeated measures ANOVA revealed a significant effect of AGRP on both active lever presses ($F_{(1,11)}=8.4$, p<0.05) and rewards earned ($F_{(1,11)}=18.2$, p<0.05). There was a significant interaction between αflupentixol treatment and AGRP infusion for the number of rewards earned ($F_{(1,11)}=9.4$, p<0.05), and a trend towards an interaction for the number of active lever presses ($F_{(1,11)}=3.4$, p=0.08).

Expression of MC4 receptors but not MC3 receptors in the NAc

To understand whether MC ligands exert their effects on food motivation via the NAc, fluorescent in-situ hybridization was conducted for the MC3 receptor and MC4 receptors. In line with literature, Fig. 2 A-D shows prominent expression of MC4 but not MC3 receptors in the NAc [32]. Co-localization of MC4 receptors was observed with both dopamine D1 and D2 receptors. The distribution of MC4 receptor expressing cells was uneven and showed a decreasing gradient from ventral to dorsal parts of the striatum. The percentage of MC4 receptor positive cells in the NAc shell and core was 5.7 ± 1.6 % and 8.3 ± 1.8 % respectively.

Within the NAc shell, 12.9 ± 3.3 % of MC4 receptors were co-expressed in dopamine D1 expressing neurons and 10.9 ± 2.4 % within dopamine D2 receptor positive neurons. These data support a postsynaptic interaction between MC4 receptor and dopamine D1/D2 receptors, although a presynaptic interaction cannot be entirely excluded. Furthermore, Fig. 2E-G identifies POMC neuronal projections specifically in the shell region of the NAc, indicating the NAc shell as a crucial site for MC action.

The role of the NAc shell in MC-dependent motivation for sucrose

Bilateral infusion of 0.2 ηmol of αMSH into the NAc shell decreased both the number of active lever presses ($t(6)=2.9$, $p<0.05$) and reinforcers earned ($t(6)=2.7$, $p<0.05$) without affecting the number of inactive lever presses ($p>0.05$, Fig. 3A and Fig. 3B). Conversely, infusion of 0.1ηmol of AGRP increased both active lever presses ($t(7)=4.1$, $p<0.05$) and reinforcers obtained ($t(8)=3.5$, $p<0.05$, Fig. 3C and Fig. 3D) without affecting the number of inactive lever presses ($p>0.05$). Free-feeding of sucrose was not significantly affected by 0.2 ηmol of αMSH or 0.1ηmol of AGRP ($F(2,20)=0.9$, $p>0.05$, Fig. 3E). Placement of the NAc canulas is depicted in Fig. 2H.

Discussion

Here we show that rats treated i.c.v. with αMSH show decreased motivation for sugar, while infusion of AGRP had the opposite effect. Furthermore, increase in responding for sucrose by AGRP was blocked by αflupentixol, indicating that this stable effect of AGRP was dopamine dependent. We further identify the NAc shell as an important site of action for the motivational effects of MCs. Thus, the NAc shell is innervated by POMC-positive fibers and expresses MC4, but not MC3 receptors. In addition, infusion of αMSH and AGRP into the NAc shell mimics their i.c.v. effects on responding for sucrose, indicating that MC signaling in the NAc shell modulates the motivation for palatable food.

The willingness to work for a food reward is thought to be one of the mechanisms through which MCs influence feeding [15;16;20]. Here, we therefore investigated the role of the MC system on the motivation for sucrose in ad libitum fed rats. As mentioned previously, food restriction results in alterations in neuropeptidergic signaling involved in feeding [33] and as a result, alterations in food-motivated behavior in general [34]. We therefore chose to infuse MC ligands under ad libitum conditions. In this manner, we aimed to avoid any confounding effects of food restriction on food motivation and to specifically determine the effect of MC receptor activation on the motivation for palatable food.

Our findings confirm that alterations in food-motivated behavior play a role in the effects of the MC system on the regulation of food intake. Rats that received i.c.v. infusions with MC ligands demonstrated opposing effects on motivation, with αMSH suppressing operant

responding for sucrose and AGRP increasing it. These results are in line with previous findings from other laboratories, where, in addition to enhancing chow intake, central AGRP infusion has also been shown to increase motivation for both sugar [16] and fat [35].
In order to determine whether central MCs influenced sugar intake in general as well as food motivation, free feeding experiments were also conducted. In these experiments, animals could freely consume sucrose pellets without having to perform an operant task. Our results failed to demonstrate any effects of αMSH or AGRP on free feeding of sucrose, indicating that MCs can modulate motivation independent of food intake. The lack of effect of AGRP might seem surprising as AGRP is known to stimulate (palatable) feeding. However, most evidence is based on studies using fat feeding [35]. It could therefore well be that sugar intake is differentially modulated by MC signaling. Indeed, in rats given the choice between saturated fat, liquid sugar and chow, AGRP enhances chow and saturated fat intake but not sugar intake (unpublished data, see S1 Figure). The finding that sucrose intake is influenced by AGRP under a PR schedule of reinforcement but not under free feeding conditions, further supports the notion that AGRP influences food-motivation in general. Nevertheless based on the current experimental paradigm, possible αMSH effects on sucrose intake at a different time point cannot be excluded.

In order to identify the neural substrates underlying MC-driven food motivation, we focused on the NAc shell, as its role in mediating motivation for rewards is well known [36]. Our results show that although MC signaling within the NAc shell does not affect chow intake and body weight [20], it does regulate motivation for palatable food. It is known that the NAc receives projections from dopaminergic neurons in the VTA and from hypothalamic POMC neurons [2;23-26;32;37]. Furthermore, the NAc shell but not the core region is activated in rats responding for food under a PR schedule of reinforcement [18]. Similarly, central infusion of AGRP increases activity of the shell region of the NAc [19]. In line with these findings, our immunohistochemistry study shows extensive POMC neuronal fibers in the shell region only, indicating the importance MC signaling within the shell region. Using fluorescent in situ hybridization techniques, we first demonstrated pronounced expression of MC4 but not MC3 receptors in this region. In addition, MC4 receptors were expressed on both dopamine D1 and D2 receptor expressing neurons. Interestingly, activation of D1-positive neurons within the NAc is associated with an enhanced behavioral response to drug reinforcers [38]. Conversely, optogenetic inhibition of these neurons or infusion of a D1 receptor antagonist into the NAc decreases responding for sucrose and cocaine [39;40].

The role of dopamine in the motivation for food reward is well established [21;36;41]. For instance, ingestion of sucrose increases dopamine levels within the NAc [42], whereas blocking dopamine signaling decreases operant responding for sucrose, especially when response requirements are high [17]. Since AGRP infusion enhanced operant responding for

sucrose, we hypothesized that the effect of AGRP on the motivation for sucrose was mediated by dopamine and thus would be blocked by pretreatment with a dopamine receptor antagonist. Indeed, the AGRP-induced increase in operant responding for sucrose under a PR schedule was prevented by a low dose of αflupentixol that failed to affect general locomotion or motivation for sucrose on its own. These data suggest that AGRP-enhanced motivation for palatable food rewards is dopamine dependent, which is in line with the finding that infusion of MC agonists modulates dopamine receptor levels in various brain areas [43].

Although not studied extensively, MC signaling in the NAc has been associated with attenuation of excitatory postsynaptic currents and synaptic strength. Lim et al. reported a decrease in AMPA/NMDA ratio specifically in dopamine D1 but not D2 receptor expressing NAc neurons following αMSH treatment, indicating decreased activity of these neurons after αMSH treatment [44]. Based on these findings [39;44] and our current observations, it is reasonable to speculate that αMSH acts on the dopamine D1 receptor-positive population of neurons within the NAc to attenuate motivation for sucrose, and that antagonizing MC4 receptors with AGRP results in opposite behavioral effects. However, our data also demonstrate MC4 and dopamine D2 receptor co-localization, and αflupentixol acts on both D1 and D2 receptors. Thus, it is possible that melanocortin signaling in D2 positive neurons partly mediate melanocortin dependent motivation. Taken together, these data suggest that the NAc mediates the effect of MCs on food-motivated behavior. Consistent with our hypothesis, intra-NAc infusion of αMSH and AGRP mirrored their effects on motivation for sucrose after i.c.v infusion, indicating that MC action within the NAc is sufficient to modulate food-motivated behavior.

Interestingly, in the free-feeding paradigm of the present study, infusion of MC ligands into the NAc failed to alter the consumption of sucrose. This is in line with the finding that overexpression of AGRP [20] and infusion of a MC4 antagonist [39] within the NAc does not alter food-intake or meal patterns. In summary, our findings further identify the NAc shell as a prime target for MC action and demonstrate an interaction between dopamine and MCs in the motivation for palatable food. Our data provide a clear picture of the motivational effects of MCs on food reward, thereby adding to the existing knowledge of their role in food satiation [20;45], anticipation [46] and consumption [9].

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Chapter 5

Figures

Fig. 1. Effect of αMSH and AGRP on motivation and sucrose free feeding: Lever presses, rewards earned and number of sucrose pellets consumed under free feeding conditions upon central infusion of αMSH (A-C) or AGRP (D-F). Active lever presses (G) and rewards earned (H) during a PR schedule of reinforcement upon pretreatment with systemic saline or αflupentixol (0.125 mg/kg) prior to central AGRP (1ηmol/2μl) or saline (2μl) infusion. Data are mean ± SEM. * denotes statistically significant difference (p<0.05) between saline and test conditions. α denotes statistically significant difference between the two test conditions.

Fig. 2. Melanocortin receptor signaling in the nucleus accumbens: Fluorescent in-situ hybridization with MC3 and MC4 receptors on rat brain slices containing the NAc shell. Nuclei were stained with DAPI and pseudocolored in blue and MC receptors are shown in red and D1/D2 receptors in green. Arrow indicates cells with prominent mRNA co-localization (A-D). POMC expression in the ARC (Bregma -3.30mm) (G) and in the NAc shell but not core regions (Bregma =+1.2mm) (E and F). Cannula placements within the NAc shell. Numbers indicate relative location from bregma (H).

Fig. 3 Effects of intra-NAc αMSH and AGRP infusion on motivation and free-intake:
Lever presses and rewards earned during a PR schedule of reinforcement uponintra NAc
injections of αMSH (A and B) or AGRP (C and D). Number of 45 mg sucrose pellets consumed
under free-feeding conditions following central infusion of saline, αMSH and AGRP (E). Data
are mean ± SEM. * denotes statistically significant difference (p<0.05) between saline and
test conditions

Supplemental Data

Table S1: Regions and accession numbers used for synthesis of the FISH probes

Fig.S2: Effect of AGRP infusion in the lateral ventricle on food intake when rats are subjected to a free-choice high-fat high-sugar diet. Fat intake was significantly increased, whereas chow and sugar intake were not affected by AGRP. Rats were injected in a cross over design, and order of injection did not affect outcome. Data are expressed as mean ± SEM and *: p<0.05 with paired t-test.

Method: After recovery from ICV surgery (as described in manuscript), rats were switched to a free choice high-fat high sugar diet (fcHFHS) for which rats were exposed to a cup of saturated fat (Ossewit, Vandermorteelte, Belgium), a bottle of 30% sucrose water, a bottle of

tap water and familiar chow (see for description of this diet: la Fleur et al, Int J Obes 2010&2011). After a week on the diet, rats were injected with 2ul saline icv to get accustomed to the method.
Seven days later, at the beginning of the light peroid, 4 rats were injected with saline and 5 rats with AGRP (83-132) Amide (5.6 ug/2ul saline) (Phoenix Pharmaceuticals Inc., Burlingame, USA) and 5h and 24h later lard, sugar and chow intake were measured. Four days later, a similar experiment was performed, this time injecting the saline animals with AGRP and vice versa. In this way every animal was used as its own control. At the end of the experiments, rats were decapitated and brains were checked for canula placement. Data from 2 of the 9 animals were not included in the analysis due to unexplainable weight loss after the icv infusion and a misplaced canula.

Results: AGRP (1ηmol) injected in the lateral ventricle increased 5h caloric intake in rats on the fcHFHS diet significantly (20.0 kcal ± 2.7 in saline injected rats compared to 35.4 kcal ± 4.3 in AGRP injected rats; $p<0.04$). Below the effects of AGRP on chow, fat and sugar are depicted at the 5h time point. AGRP significantly increased fat intake compared to a saline injection, whereas chow and sucrose intake were not affected by AGRP (Fig 1A). After 24h, fat intake was still significantly increased in AGRP injected rats and intake of chow and sucrose was again not different from saline injected rats (data not shown).

Conclusion: Inhibiting MC receptors in the brain increases fat intake, whereas sugar intake is not affected.

Chapter 6

Melanocortin 3 receptor signaling in midbrain dopamine neurons increases the motivation for food reward

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Abstract

The central melanocortin (MC) system mediates its effects on food intake via MC3 and MC4 receptors (MC3R/MC4R). While the role of MC4R in meal size determination, satiation, food preference and motivation is well established, the involvement of MC3R in the modulation of food intake has been less explored. Here, we investigated the role of MC3R on the motivation for food reward, which is a crucial component of feeding behavior. To this aim, rats responded for sucrose under a progressive ratio schedule of reinforcement. We started by showing that αMSH suppressed motivation for a sucrose reward in the absence of a MC4R selective antagonist, while increased motivation when co-administered with it. Intracerebroventricular administration of the selective MC3R agonist γMSH increased the motivation to respond for sucrose but not free consumption of sucrose or chow. Since dopamine (DA) neurons within the ventral tegmental area (VTA) play a crucial role in the motivation for food rewards, we focused on MC3R signaling in VTA DA neurons. We found that MC3R are expressed in a sub-population of VTA DA neurons and that infusion of γMSH directly into the VTA increased the motivation for sucrose but not free consumption. Furthermore, ex-vivo electrophysiological recordings showed increased VTA DA neuronal activity upon γMSH treatment. Consistent with a DA mechanism of action of γMSH, the increased motivation for sucrose after intra-VTA infusion of γMSH was blocked by pretreatment with the DA receptor antagonist α-flupentixol. Together, these data reveal a function of MC3R and their interaction with DA in the modulation of the incentive motivation for food rewards.

Introduction

The central melanocortin (MC) system plays an integral role in regulation of food intake [1]. MCs are products of the pro-opiomelanocortin (POMC) gene, which is expressed in neurons within the arcuate nucleus of the hypothalamus (ARC) and the nucleus of the solitary tract (NTS) [1]. Through projections from the ARC and NTS to various brain nuclei, MCs regulate various aspects of food intake such as satiation [2-4], food motivation [5-8] and food preference [9, 10]. MCs mediate their effects within the brain via the MC3 and MC4 receptors (MC3R/MC4R) that are differentially expressed in brain structures including the hypothalamus and the limbic system [11, 12].

Interestingly, next to regulating homeostatic aspects of feeding, the MC system has also been implicated in the hedonic and incentive motivational properties of food [7, 13]. In the past decade, pharmacological and genetic manipulations have been used to investigate the role of MC4Rs in regulation of hedonic food intake [13-15]. Furthermore, we recently showed that central infusion of αMSH, a potent MC4R agonist, decreases the motivation for sucrose and that this effect is mediated by MC4R in the nucleus accumbens (NAC) [16].
In contrast to the well-established role of MC4R in the motivation for food, the involvement of MC3Rs in the modulation of food intake and especially food motivation remains largely unexplored. Peripheral administration of the selective MC3R agonist γMSH causes an increase in food intake in freely moving animals [17]. Within the ARC, MC3Rs are expressed in POMC neurons where they function as auto-receptors. Ex vivo application of γMSH decreases firing of POMC neurons, which is mainly through an increase of inhibition on these neurons from Neuropeptide Y/ GABA terminals [18]. However, the direct effect of activation of MC3R on POMC neurons seems to be an excitatory effect [19].

MC3Rs are also expressed in dopamine (DA) neurons within the ventral tegmental area (VTA) [12, 20], a neuronal population that plays a key role in the motivation for food and drug rewards [21, 22]. Several studies have suggested a cross-talk between MC3Rs and the DA system. Thus, MC3R knock-out mice show decreased sucrose preference [20] and lower spontaneous and running-wheel induced locomotor activity [23-25], a phenotype reminiscent of VTA DA depleted animals [26]. Similarly, overexpression of the α and γMSH precursor POMC within the VTA increases tyrosine hydroxylase expression [27], suggesting modulation of dopamine signaling by MCs. In line with this, global deletion of MC3R decreases dopamine release within the NAC [20]. In contrast, pharmacological activation of intra-VTA MC3Rs by infusion of γMSH or αMSH increases NAC dopamine levels [28, 29]. Although these data suggest that MC3R signaling within the VTA potentiates dopaminergic signaling, direct evidence to support a role for MC3R signaling in the motivation for food is lacking. Therefore, in the present study we investigated the role of MC3Rs within the VTA on the motivation to respond for a sucrose reward under a progressive ratio (PR) schedule of

reinforcement. We hypothesized that stimulation of MC3R within the VTA increases motivation for palatable food rewards.

Methods

Animals

Male Wistar rats (Charles River, Sulzfeld, Germany) weighing 200-225 g on arrival in our laboratory were used for behavioral studies and TH::Cre heterozygous transgenic rats on a Long-Evans background (1-2 months old) were used for slice electrophysiology and retrograde tracing experiments. Animals were individually housed (Macrolon cages; 40x26x20 cm) in a temperature (21±2 °C) and humidity (60-70 %) controlled room under a 12 h reversed light/dark cycle (lights on: 19.00 h). Rats had ad libitum access to rat chow (3.31 Kcal/gram, Standard Diet Service, UK) and tap water. All behavioral experiments were performed in the dark phase of the day-night cycle (8:00 h to 17:00 h). Experimental procedures were approved by the Animal Ethics Committee of Utrecht University and were in agreement with Dutch laws (Wet op Dierproeven 1996) and European regulations (Guideline 86/609/EEC).

Surgery

Surgery was performed when the animals weighed between 275-300 g. Rats were anaesthetized with 0.1 ml/100 g intramuscular injection of fentanyl/fluanisone (Hypnorm®, Janssen Pharmaceutica, Beerse, Belgium). For implantation of intracerebroventricular (i.c.v.) cannulas, the skull was exposed and a 10 mm stainless steel guide cannula (Plastics One, Roanoke, USA) was inserted into the lateral ventricle (1 mm lateral and 1 mm posterior from bregma) via a craniotomy. Cannulas were fixed to the skull with stainless steel screws and dental cement. For VTA cannulations, animals were positioned in a stereotaxic apparatus (David Kopf, Tujunga, USA) and stainless steel guide cannulas (26 GA, 8 mm; Plastics One) were implanted bilaterally, 1 mm above the VTA (AP: -5.40, ML: + 2.20, DV: - 7.90, angle 10°, Paxinos and Watson, 1998). Perioperatively, rats received carprofen (5 mg/kg, s.c.) and saline (3 ml, s.c.). Behavioral experiments commenced following a 10-14 day recovery period.

For electrophysiology experiments, TH::Cre rats (40-70 g) were deeply anaesthetized and placed in a stereotaxic frame and craniotomies were performed using VTA coordinates (AP: -4.8, ML: 1.0, DV: -7.1 from bregma, angle 5°). AAV-DIO-mCherry (UNC Vector Core, USA) was bilaterally injected in the VTA as described previously [30]. Briefly, the virus was injected using a 2 μl Hamilton syringe at a rate of 0.2 μl/min for a total volume of 1 μl. Injection needles were left in place 5 min to prevent backflow. After the injection, the skin was sutured and the rats allowed to recover for at least two weeks before electrophysiological recordings were performed.

Drugs and microinfusions

αMSH, HS014, D-trp8-γMSH (γMSH) (all from Tocris, Bristol, UK), AGRP (83-132) Amide (Phoenix Pharmaceuticals, Burlingame,USA) and α-flupentixol dihydrochloride (Sigma Aldrich, St. Louis, USA) were dissolved in sterile saline solution. 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX,and bicuculline (both from Tocris) were prepared as a stock solution. For i.c.v. infusions, rats were briefly held and 2 μl of drug solution was slowly (over 10 sec) injected into the ventricular cavity. Intra-VTA infusions were performed through an injector (9 mm, 33GA, Plastics One) inserted into the guide cannula. Bilateral infusions (300 ηl over 30 sec) were made using a syringe pump with the injectors left in place for another 30 sec to allow for diffusion. α-Flupentixol dihydrochloride was injected intraperitoneally (i.p.) 30 min prior to intra-VTA infusions. All animals received all drug doses/combinations, according to a Latin-square design. Behavioral testing commenced 5 min after drug infusions and a minimum 1 day drug-free period was maintained between infusions during which the animals were trained but not tested.

Electrophysiology

Horizontal slices of the midbrain (300 μm) were prepared from TH::Cre rats (5-7 weeks) using a vibratome (HM650V; Microm) in ice-cold modified artificial cerebrospinal fluid (ACSF) containing (in mM): 92 mM N-methyl-D-glucamine (NMDG), 2.5 mM KCl, 1.25 mM NaH_2PO_4, 30 mM $NaHCO_3$, 20 mM HEPES, 25 mM glucose, 2 mM thiourea, 5 mM Na-ascorbate, 3 mM Na-pyruvate, 0.5 mM $CaCl_2.4H_2O$, and 10 mM $MgSO_4.7H_2O$, bubbled with 95% O_2 and 5% CO_2 (pH 7.3–7.4). Slices were initially recovered in carbogenated modified ACSF for 15 min at 34°C and then transferred into a holding chamber containing standard ACSF (in mM): 126 NaCl, 3 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 10 glucose, 1.25 NaH_2PO_4 and 26 $NaHCO_3$ bubbled with 95% O_2 and 5% CO_2 (pH 7.3) at room temperature for at least 1 h. The slices were transferred one at a time to the recording chamber perfused with standard ACSF continuously bubbled with 95% O2 and 5% CO2 at 30–32°C. Whole-cell patch-clamp recordings were made from VTA dopamine neurons visualized with an Olympus BX61W1 microscope using infrared video microscopy and differential interference contrast (DIC) optics. VTA dopaminergic neurons were identified by mCherry fluorescence. Patch electrodes were pulled from borosilicate glass capillaries and had a resistance of 3-5 MΩ when filled with intracellular solutions. Internal solution contained (in mM): 140 K-gluconate, 1 KCl, 10 HEPES, 0.5 EGTA, 4 MgATP, 0.4 Na2GTP, 4 phosphocreatine (pH 7.3 with KOH). Signals were amplified filtered at 3 KHz and digitized at 10 KHz using an EPC-10 patch-clamp amplifier and PatchMaster v2x73 software. Series resistance was constantly monitored, and the cells were rejected from analysis if the resistance changed by >20%. No series resistance compensation was used. Resting membrane potential was measured in bridge mode (I=0) immediately after obtaining whole-cell access.

Experimental design

Central effects of MC3R stimulation on the motivation to respond for sucrose

Motivation for sucrose: To investigate the effects of MC3R stimulation on the motivation for sucrose, rats were tested under a progressive ratio (PR) schedule of reinforcement [31], according to previously published procedures [32]. Briefly, following post-surgical recovery, rats were trained under a fixed ratio 1 schedule of reinforcement in two-lever operant conditioning chambers (30.5 x 24.1 x 21.0 cm., Med-Associates, St Albans City, USA), whereby each active lever press (ALP) led to retraction of the levers and the delivery of a 45 mg sucrose pellet (Noyes precision pellets Formula F, Research Diet, New Brunswick, USA). Twenty seconds after pellet delivery, levers were reinserted into the chamber. Inactive lever presses (ILP) were counted but had no programmed consequences. Allocation of the left and right lever as active and inactive lever was counterbalanced between animals. After acquisition of responding under the fixed ratio 1 schedule, a PR schedule of reinforcement was introduced, in which the number of lever presses required to obtain successive rewards increased according to the following equation: response ratio 5e(0.2 × reward number))-5 through the following series: 1, 2, 4, 9, 12, 15, 20, 25 [32, 33]. Sessions ended whenever the animal failed to earn a reward within 60 min. After 10 training sessions, rats were tested during a period of 3-5 weeks with 5-6 training sessions/week. All infusions were performed once responding under the PR schedule had stabilized. Responding was considered stable when the number of ALP did not differ more than 15% between three consecutive sessions and there was no consistent up- or downward trend. To investigate the role of MC3R stimulation on food motivation, rats were treated with αMSH (2 ηmol, 2 μl), a MC3R/MC4R agonist with or without HS014 (1.2 ηmol, 2 μl), a MC4R antagonist [34], 5 min prior to operant testing. A separate group of rats also received either saline (2 μl) or γMSH (0.5 or 1 ηmol) according to a Latin square design. After completion of operant testing, animals were placed back in their home cages with a pre-weighed amount of chow. Chow consumption was determined 20 h later.

Central effects of γMSH on sucrose free-feeding

Free-feeding experiments were conducted to determine whether the effects of γMSH were specific to the animal's motivation for palatable food or secondary to an effect on feeding behavior in general. Here, animals had free access to sucrose pellets for 60 min every day, i.e. the animals did not need to perform an operant task to obtain the sucrose pellets. Ad-libitum fed rats were introduced into an empty cage with a suspended steel receptacle containing 45 mg sucrose pellets. After 60 min, sucrose intake was measured. To accustom rats to the procedure, 4-5 training sessions were performed before testing. Following stabilization of sucrose intake, rats were injected with saline (2 μl) and two doses of γMSH (0.5, 1 ηmol) according to a Latin square design.

MC3R expression in the VTA

To establish whether dopaminergic neurons within the VTA express MC3R, an in-situ hybridization study was conducted. It is known that dopamine D2 receptors within the VTA function as auto-receptors [35]. Thus, in order to establish the identity of dopaminergic neurons, a probe against dopamine D2 receptors was used using published sequences [36]. In this experiment, a separate group of rats was used that were not subjected to behavioral testing. Following decapitation, brains were frozen on dry ice. Coronal sections (16 μm) were fixed with 4% paraformaldehyde (10 min), subsequently washed with PBS (3x, 10 min) and further acetylated (10 min). Following acetylation, slices were preincubated with a prehybridization mix (50% deionized formamide, 5x SSC, 5x Denhardt's solution, 250 μg/ml tRNA baker's yeast, 500 μg/ml sonicated salmon sperm DNA final concentrations in MilliQ). Next, sections were incubated overnight at 40 oC in 120 μl hybridization mix (Panomics, Santa Clara, USA) containing the probe sets (concentration D1/D2-1:33, MC3R/MC4R-1:50) and washed. Probe sets used to detect the desired rat mRNAs we designed by Panomics, using published sequences (see supplementary data). Sections were then incubated for 1.5 h at 40°C in 120 μl PreAmplifier Mix (Panomics) containing preamplification oligos (PreAmp TYPE4 1:20, PreAmp TYPE 6 1:50, PreAmp TYPE 8 1:33) and washed. Then, sections were incubated for 1.5 h at 40 oC in 120 μl Amplifier Mix (Panomics) containing amplification oligos (Amp TYPE4 1:20, Amp TYPE 6 1:50, Amp TYPE 8 1:33) and washed. Next, sections were incubated for 1.5 h at 40 oC in in 120 μl in Label Probe Mix (Panomics) containing label oligos (LP TYPE4 1:20, LP TYPE 8 1:33, LP TYPE 6 1:50). After washing, sections were incubated in 750 μl PBS supplemented with 4',6-diamidino-2-phenylindole (DAPI) (6.7 μg/ml, Sigma Aldrich, St. Louis, USA) for 5 min. Finally, slices were washed and embedded in Mowiol. Sections were visualized and images were obtained with a Zeiss AxioScope A1 microscope (Carl Zeiss, Jena, Germany). Images were processed using ImageJ (version1.43r) software.

To determine the physiological relevance of MC signaling within the VTA dopaminergic neurons, we performed a double labeling immunohistochemistry study. For this, rats were perfused with ice-cold 4 % paraformaldehyde (PFA) in PBS. Brains were removed and overnight post-fixated in 4 % PFA and then embedded in a 30% sucrose solution in PBS. Brains were subsequently sliced using a cryostat to obtain free-floating 40 μm thin slices. Briefly, slices were washed with 0.5M Tris-Buffered solution (TBS) (3x10 min), and then blocked with super-mix (0.5M TBS, 0.25 % gelatin and 0.1 % Triton X) for 30 min. Following blocking, slices were incubated with primary antibody against POMC (1:1000, Rabbit, H-029-30, Phoenix Pharmaceuticals) and tyrosine hydroxylase (1:1000, Mouse, MAB318, Millipore, USA) (4° C, overnight) on a shaker. Subsequently, the slices were washed (TBS, 3x10 min) and incubated with fluorescent secondary antibody mix (chicken anti rabbit, 488, A-21441 and goat anti mouse 568, A-11004, Alexa Fluor, Life Technologies, USA) for 1 h at

room temperature. Slices were further washed (3x10 min) with TBS, put on glass slides and embedded with Flurosave (Merk Milipore, USA). Images were made using Zeiss AxioScope A1 microscope (Carl Zeiss) and processed with ImageJ software.

To determine whether VTA dopaminergic neurons are innervated by POMC neurons, we used a rabies virus based trans-synaptic retrograde tracing approach as described earlier [37]. Briefly, TH::Cre rats were injected with cre-dependent helper viruses AAV-DIO-TVA-mCherry and AAV-DIO-RG unilaterally into the VTA (AP: - 5.40, ML: + 2.20, DV: - 8.90, angle 10°, Paxinos and Watson, 1998) that would restrict the rabies virus expression to Cre-positive TH cells. Two weeks later rats were injected in the VTA with rabies virus SAD dGmCherry (ENVA) virus that could now infect TH cre positive cells and be retrogradely transported across a single synapse. Thus, neurons providing synaptic input to TH positive neurons can be visualized using this technique. Seven days following the second injection, rats were perfused with 4 % PFA as described earlier. Immunohistochemistry was performed and staining was visualized for POMC and mCherry (1:500, monoclonal mouse, Abcam, Cambridge, UK) with an Olympus Fluoview FV1000 confocal microscope (Olympus, Tokyo, Japan).

The role of VTA MC3Rs in motivation for sucrose

To investigate the effects of specific activation of MC3R stimulation on motivation for sucrose, rats were bilaterally injected with saline or γMSH (0.1 or 0.2 ηmol) directly into the VTA and tested under the PR schedule of reinforcement. The effects of VTA MC3R stimulation on responding for sucrose was also investigated using a non-specific MC3/MC4 agonist (αMSH). PR testing was also conducted following intra-VTA infusion of the MC3R/MC4R inverse agonist AGRP. In a separate group of animals, effect of MC3R stimulation by γMSH on sucrose free-feeding was also determined. Furthermore, the interactions between MC3R and dopamine signaling were investigated by pre-treating rats with saline or αflupentixol (0.125 mg/kg, i.p.) 30 min prior to intra-VTA saline or γMSH (0.2 ηmol) infusions. The dose of αflupentixol was based on a previous study [38]. Five minutes after intra-VTA infusions, responding for sucrose under the PR schedule was tested as outlined above.

Cannula placements

At the end of the experiment, animals equipped with i.c.v. cannulas were euthanatized with carbon dioxide and received an i.c.v. injection with 2 µl of ink. Brains were then removed and the lateral ventricles opened to check for ink staining. Rats equipped with VTA cannulas were decapitated, the brains removed and frozen on dry ice. Next, cryostat sections (16 µm) were stained with cresyl violet to determine cannula locations (See Supplementary Fig. S1-2).

Statistical analyses

All data were analyzed using repeated measures ANOVA followed by a Dunett's post-hoc test, unless otherwise indicated. To study the interactions between αflupentixol and γMSH, an additional two-way repeated measures ANOVA was conducted using i.p. injections (saline vs αflupentixol) and i.c.v. infusions (saline vs γMSH) as within-subjects factors. Differences were considered significant at p<0.05. All statistical analyses and graphical representations were performed using Graphpad software (v 6.03, USA). Data are expressed as Mean ± SEM.

Results

Central stimulation of MC3Rs increases motivation and free-intake of sucrose

As reported earlier [16], i.c.v infusion of αMSH decreased the number of ALP ($F_{(3,16)}=4.03$, p=0.036, **Figure 1a**) and rewards earned ($F_{(3,16)}=7.62$, p=0.002, **Figure 1b**). Interestingly, co-injection of MC4R specific antagonist HS014 together with αMSH, increased both ALP and rewards earned. This suggests the involvement of another MC receptor, most likely the MC3R, since this is the most abundant receptor in the brain. The number of ILP following combined HS014 and αMSH injections was also increased ($F_{(3,16)}=4.06$, p=0.029, **Figure 1c**).

In line with this, i.c.v. infusion of the selective MC3R agonist γMSH increased both ALPs ($F_{(2,7)}=10.36$, p=0.002) and rewards earned ($F_{(2,7)}=6.19$, p=0.012) in a dose dependent manner (**Figure 1d** and **1e**). The number of ILPs remained unchanged ($F_{(3,7)}=1.44$, p=0.270, Figure 1f). In the free feeding paradigm, γMSH did not affect sucrose ($F_{(2,7)}=2.56$, p=0.112, **Figure 1g**) or overnight chow $F_{(2,7)}=0.75$, p=0.489, **Figure 1h**) consumption.

MC3Rs are expressed in mesolimbic dopaminergic neurons

Figure 2a shows abundant expression of MC3Rs in VTA DA neurons. Approximately 43% of all D2 receptor-expressing cells expressed MC3R (**Figure 2b**). Additionally, all MC3Rs co-localized with D2 receptor containing DA cells. We did not find MC4Rs in D2 receptor-expressing cells (data not shown). Immunohistochemical studies further revealed the presence of POMC fibers in the vicinity of dopaminergic neurons within the VTA (**Supplementary Figure S3**). Furthermore, synaptic connectivity between the VTA dopaminergic neurons and the ARC POMC neurons was established by using trans-synaptic retrograde tracing experiments. Here, rabies virus injection [37] in VTA-TH positive neurons retrogradely transported mCherry in a sub-set POMC neurons within the ARC (**Supplementary Figure S4**).

γMSH modulates dopamine neuronal firing in the VTA

Whole-cell current clamp recordings were made from VTA dopamine neurons from TH::Cre rats. Spontaneous action potentials were recorded at resting membrane potential in the

presence of inhibitors of synaptic transmission (CNQX, 10 μM and bicuculline, 20 μM). The average frequency of action potentials and resting membrane potentials from all recorded dopamine neurons were 1.57± 0.16 Hz and -46.1 ± 2.7 mV, respectively (n =14). D-trp8-γMSH (10 nM) was applied to the recorded dopamine neurons via bath application. D-trp8-γMSH depolarized dopamine neurons by 4.3± 0.55 mV and increased the frequency of action potentials to 147% ± 4.5% of baseline in 9 out of 14 neurons which was reversed upon washout of γMSH (F(2,16)=14.62, p=0.0006, **Figure 2c** and **2d**).

Intra-VTA MC3R stimulation increases motivation but not free feeding of sucrose

Similar to its central effects on motivation, intra-VTA infusion of γMSH increased ALPs for sucrose (F(2,8)=10.57, p=0.001, **Figure 3a**) and rewards earned (F(2,8)=11.60, p=0.001, Figure 3b) without increasing ILPs (F(2,8)=5.33, p=0.465, Figure 3c). In contrast to its effects on motivation, intra-VTA injections of γMSH failed to increase free-intake of sucrose (F(2,6)=1.18, p=0.340, Figure 3d) or chow (F(2,5)=2,72, p=0.114, Figure 3e). Interestingly, the motivational effects of intra-VTA γMSH infusion could be replicated using αMSH, a MC3R ligand with lower specificity (ALP: F(2,8)=12.10, p=0.001, **Figure 3f**; rewards earned: (F(2,8)=8.25, p=0.003, Figure 3g). ILPs after αMSH treatment (F(2,8)=1.44, p=0.267) remained unaltered (**Figure 3h**).Furthermore, infusion of AGRP, an inverse agonist for the MC3R had antagonistic effects on motivation (ALP: t(10)=2.83, p=0.018, **Supplementary Figure 5a**); rewards earned: t(10)=2.51, p=0.031, **Supplementary Figure 5b**, ILP: t(10)=0.91, p=0.380, **Supplementary Figure 5c**).

MC3R mediated motivation for sucrose is dopamine dependent

Pretreatment of animals with αflupentixol prior to infusions of γMSH prevented the increase in responding for sucrose by γMSH as reflected by the amounts of ALP (F(3,20)=8.09, p=0.000, **Figure 3i**) and rewards earned (F(3,20)=11.11, p=0.001, **Figure 3j**) while the ILP remained unchanged (F(3,20)=0.99, p=0.397, Figure 3k). A two-way ANOVA further showed an interaction between γMSH and αflupentixol for both ALP (F (1, 20) = 5.92, p=0.024) and rewards earned (F (1, 20) = 14.12, p=0.001), but not ILP (F (1, 20) = 2.86, p=0.107).

Discussion

We here show that, the motivation lowering effects of central αMSH infusion (MC4R/MC3R agonist) was reversed by co-administration of a MC4R antagonist, suggesting a role for MC3Rs in increasing motivation for sucrose. Furthermore, rats centrally treated with γMSH increase motivation for sucrose but not free consumption of sucrose or chow, indicating that central MC3R stimulation is sufficient to enhance motivation for sucrose. We know that the VTA dopaminergic neurons projecting to the NAC play a crucial role in incentive motivation for rewards [39-42]. Therefore, we determined whether MC3R signaling within the VTA

influences motivation for sucrose. In order to determine the physiologic relevance of MC3R signaling within the VTA, we first confirmed the presence of POMC immunoreactivity within the VTA. Secondly, we confirmed the expression of MC3Rs in this area as earlier reported [12]. Interestingly, MC3R co-localized with D2 receptor containing neurons, suggesting that MC3R are expressed in a subset of dopaminergic neurons. Similar findings were recently reported by Lippert et al., showing MC3Rs to be extensively co-localized (72%) with dopaminergic neurons [20]. Furthermore, using trans-synaptic retrograde tracing technology we found that POMC neurons from the ARC project to and make synaptic contact with dopamine neurons in the VTA, indicating that these neurons can directly regulate dopaminergic activity within the VTA.

Expanding on these neuroanatomical findings, we investigated the physiological effects of MC3R stimulation in dopaminergic neurons. Similar to the effects observed in POMC neurons [19], γMSH depolarized and increased the firing frequency of dopaminergic neurons. Since phasic firing of dopamine neurons underlies reward seeking behavior [43-45] we hypothesized that stimulation of VTA- MC3Rs would increase the motivation for sucrose. Indeed, intra-VTA infusion of γMSH increased responding for sucrose under a PR schedule of reinforcement and intra-VTA infusion of the non-selective MC3R agonist αMSH also increased motivation for sucrose. Finally, the increase in incentive motivation after intra-VTA γMSH treatment was blocked by pretreatment with the dopamine receptor antagonist αflupentixol, implying that the motivational effects of intra-VTA γMSH infusion are dopamine dependent. The finding that free-feeding remained unchanged upon γMSH infusion into the VTA, is consistent with the notion that VTA dopaminergic neurons mediate food motivation rather than consumption [44]. We also report MC3R co-localization with D2 receptor-containing neurons. Since D2 receptor-containing neurons are known to project to the NAC [35], and stimulation of VTA-NAC projections increase motivation for sucrose ([39, 42, 46], our data strongly suggest that the motivational effects of intra-VTA MC3Rs are mediated via dopamine signaling in the NAC. In line with this notion, Lippert et al. found decresed NAC dopamine levels in MC3R knock-out mice [20].

In the current study, the effects of central MC3R stimulation was limited to food motivation and did not affect free consumption of sugar or chow, suggesting an exclusive role of MC3Rs in food motivation. In contrast, Marks and colleagues reported that peripheral injections of γMSH increased food intake in wild type but not MC3R knock-out mice, indicating that γMSH works via MC3Rs to increase food intake [17]. This discrepancy between results might be due to peripheral infusions versus central infusion used in the present study and effects on food intake could therefore be due to e.g. peripheral or brainstem mediated effects of MC3Rs. In addition, species differences between rats and mice cannot entirely be excluded. Considering the physiological role of a MC3R-dopamine system interaction, one might find

the effects of γMSH on food motivation confusing especially since MCs in general are considered as anorexigenic peptides [1]. Our results viewed in light of a recent publication, where ARC POMC neurons increase their firing frequency following food presentation [47], would further suggest that the motivation to eat following food presentation could be mediated by the VTA MC3Rs. Furthermore, as opposed to the motivation enhancing effects of MC3 signaling in the VTA, MC4R signaling in the NAc decreases motivation for palatable rewards, implying that melanocortins can fine tune motivation for palatable rewards and this is exclusively regulated by MC receptor expression in different brain nuclei.

All together, the current study puts forward at least one mechanism through which central MC3Rs influence food intake, i.e. by affecting motivation for food. Furthermore, these data adds and extends our existing understanding about MC3R-dopamine crosstalk where absence of MC3R signaling is correlated with attenuated dopamine-dependent behaviors like decreased locomotion and sucrose preference [14, 20]. The current study also shows that next to other feeding peptides like Gherlin and Neuropeptide Y [48, 49], MC signaling within the VTA regulates motivational aspects of food intake.

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Figures

Fig. 1: Central stimulation of MC3Rs increases motivation and free-intake of sucrose.
Effects of combined αMSH and MC4R blocker (HS014) i.c.v. infusion on ALPs (a), rewards
earned (b) and ILPs (c) (n=17). Effects of i.c.v. infusion of saline or γMSH on ALPs (d),
rewards earned (e), ILPs (f) sucrose free feeding (g) or 24 h chow intake (h) (n=8). One Way
ANOVA, *<p<0.05 **p<0.01, ***p<0.001 compared to saline condition.

Fig. 2: MC3Rs are expressed in mesolimbic dopaminergic neurons. Co-localization of MC3R within VTA dopaminergic neurons identified by the presence of D2 receptors (D2R). Inset gives a digitally zoomed in image showing co-localization (a). Percentage of D2R positive, MC3R positive and MC3R in D2R positive cells (b) Sample traces of spontaneous action potentials recorded from dopamine neurons in the absence and presence of γMSH (c). Effect of activation of MC3Rs on the frequency of spontaneous action potentials in VTA dopamine neurons before, during, and after bath application of γMSH (n = 9) (d). One Way ANOVA, ***p<0.001 compared to baseline condition.

Fig. 3: Intra-VTA MC3R stimulation increases motivation but not free feeding of sucrose. Effects of intra-VTA injections of γMSH on ALPs (a), rewards earned (b) and ILPs (c), sucrose free-intake (d) and 24 h chow intake (e) (n=7-9). Effects of intra-VTA injections of αMSH on ALPs (f), rewards earned (g) and ILPs (h) (n=9). Effects of αflupentixol (FLU) pretreatment on the number of ALPs (i), rewards earned (j) or ILPs (k) following intra-VTA γMSH infusion (n=21). One Way ANOVA,*<p<0.05 **p<0.01, ***p<0.001 compared to saline condition.

Supplemental Data

Fig. S1: Experimental scheme and cannula placements. General experimental scheme for behavioral testing (progressive ratio & free feeding) following i.c.v. infusion or intra-VTA infusion of γMSH (a). Cannula placements for intra-VTA infusions followed by progressive ratio for γMSH (b), αMSH (c), AGRP (d) and combined γMSH and FLU injections (e). Cannula placements for intra-VTA infusions followed by free-feeding for γMSH (f).

Fig. S2: Cannula placements. Showing cannula placement (c) over the VTA dopaminergic neurons (identified by tyrosine hydroxylase staining).

Fig. S3: Melanocortin signaling in the VTA. Presence of POMC fibers (green) surrounding VTA dopaminergic neurons (red, labelled by tyrosine hydroxylase staining). Inset in Figure b gives a digitally zoomed image showing close proximity of POMC immunoreactive fibers with dopamine neurons, marked by yellow arrows.

Fig. S4: POMC neurons within the arcuate make synaptic contact with VTA. POMC cells in the arcuate nucleus co-localize with mCherry positive neurons indicating that these POMC nerons have synaptic contact with dopaminergic cells from the VTA. In the digitally zoomed images shows colocalizations are indicated with yellow arrows.

Fig. S5: MC3R antagonism in the VTA decreases motivation. Infusion of AGRP into the VTA decreased active lever presses (a), rewards earned (b) without affecting inactive lever presses (c) (n=9). One-way ANOVA, *<p<0.05, compared to saline condition.

Section II

Chapter 7

The neural mechanisms underlying thermogenic and cardiovascular effects of leptin

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Chapter 7

Abstract

The adipocyte derived hormone leptin is a peripheral signal that informs the brain about the metabolic status of an organism. Although traditionally viewed as an appetite suppressing hormone, studies in the past decade have highlighted the role of leptin in energy expenditure. Leptin has been shown to increase resting energy expenditure in particular through its effects on the cardiovascular system and thermogenesis. The current review specifically addresses the role of leptin signaling in various hypothalamic nuclei and its effects on the sympathetic nervous system to influence blood pressure, heart rate and brown adipose tissue thermogenesis. Specifically, the role of leptin signaling on four different hypothalamic nuclei, the dorsomedial hypothalamus, the ventromedial hypothalamus, the paraventricular hypothalamus and the arcuate nucleus is reviewed. It is known that all these brain regions influence the sympathetic nervous system activity and thereby regulate thermogenesis and the cardiovascular system. Thus, the current work focuses on how leptin signaling in specific neuronal populations within these hypothalamic nuclei influence certain aspects of resting energy expenditure.

Introduction

With the discovery of leptin in 1994, began a new era in the field of obesity research [1, 2]. Until this landmark discovery, the concept of a peripheral messenger signaling the brain about the energy status of an organism remained theoretical [3-5]. The years following leptin's discovery witnessed a surge in studies addressing the effects of leptin on food intake; a major focus of these studies being the hypothalamic arcuate nucleus (ARC). As a hypothalamic nucleus bordering the third ventricle, the ARC serves as a suitable gateway for peripheral molecules to access the brain tissue. Within the ARC, two antagonistically acting neuronal populations, the neuropeptide Y (NPY) and Pro-opiomelanocortinergic (POMC) neurons, were identified as immediate downstream targets of leptin. Even though leptin receptors are expressed on both neuronal populations [6], leptin stimulation of NPY neurons decrease their firing and attenuates food intake while its action on POMC neurons are opposite [7-9]. The years that followed unraveled the role of leptin in hypothalamic areas outside the ARC; however the majority of these studies were focused on the appetite-suppressing effects of leptin or its effects on glucose metabolism [10-12]. In comparison with the well-defined effects of leptin on food intake, our knowledge on the mechanisms underlying leptin's role in energy expenditure is fairly limited. Nevertheless, a sufficient volume of work addressing leptin's role in energy expenditure has surfaced in the recent years. The current mini-review summarizes and provides a brief overview of the most recent developments in this field to give a clear picture on leptin's role in energy expenditure, especially focusing on non-shivering thermogenesis. We know that total energy expenditure comprises of various components i.e. non-resting energy expenditure or physical activity, resting energy expenditure and the thermic effect of food [13]. Importantly, resting energy expenditure can be further classified into shivering and non-shivering thermogenesis. Shivering thermogenesis, which is primarily induced by cold, occurs due to contraction of antagonistic groups of skeletal muscles. In contrast, non-shivering thermogenesis refers to the heat generated by the brown adipose tissues (BAT), the pumping action of the heart and the organism's basal metabolic rate. [14, 15]. In the current review, we look into leptin's role in influencing two specific aspects of resting energy expenditure i.e. its effect on BAT thermogenesis and cardiovascular regulation. We here specifically focus on the contribution of leptin signaling in different neuronal populations within four different hypothalamic nuclei: dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), the paraventricular hypothalamus (PVH) and the ARC, on mediating resting energy expenditure via the sympathetic nervous system (SNS).

Leptin as a starvation signal

The initial evidence on leptin's role in thermogenesis comes from studies on leptin deficient ob/ob mice. When these mice are pair-fed to their ob/ob littermates treated with leptin, the decrease in their body weight and adiposity is to a lesser extent compared to their leptin-

treated littermates. This suggests that other factors, besides food intake, mediate the weight-reducing effects of leptin [16]. Interestingly, the absence of leptin signaling in ob/ob or db/db mice next to triggering hyperphagia, also leads to hypothermia [17], implying that leptin deficiency affects both food intake and energy expenditure. The fact that leptin replacement in ob/ob mice but not db/db mice (ie. mice lacking leptin receptors) corrects both food intake and hypothermia, further confirms this theory [18, 19]. However, leptin replacement in ob/ob mice increases body temperature, but not beyond that in lean controls, implying that under physiological conditions leptin is essential for normothermia rather than causing hyperthermia [20]. Similar findings by Flier and colleagues showed that the drop in leptin levels during starvation is accompanied by metabolic adaptations that limit energy expenditure [19]. Moreover the fact that leptin replacement in starved mice corrected these metabolic changes led to the theory that leptin, besides its role as an appetite suppressor, acts as a starvation signal. Thus, the drop in leptin levels during starvation, signals the brain about pending threats to energy reserves and triggers metabolic and behavioral adaptations directed at minimizing energy loss and maximizing the chances of survival [21]. These adaptations range from decreased thyroid hormone levels and SNS tone to reduced fertility, i.e. suppression of all systems that promote energy expenditure. Through a series of elegant studies Leibel and colleagues further extrapolate these findings to humans, where they demonstrate that the attenuated leptin levels in weight-reduced obese subjects decreased thermogenesis to an extent that favored restoration of previous body weight and could be restored with leptin replacement [22, 23].   Kraklin et al., similarly reported a decrease in nocturnal body temperature in dieting overweight women [24]. As mammals spend a substantial percentage of their energy intake (50-60%) to maintain homeothermy [25], any alterations in thermogenesis would significantly affect the total amount of expended energy and thereby influence body weight. Such adaptations are for example encountered in hibernating animals that decrease their energy expenditure, by decreasing thermogenesis in order to conserve energy [26, 27]. Besides decreasing thermogenesis, decrease in body mass and thus leptin levels also affects the cardiovascular system by decreasing heart rate and blood pressure, both in humans and rodents [28, 29].

Leptin as an overfeeding signal

In contrast to leptin deficiency that decreases resting energy expenditure, an increase in circulating leptin correlates with increased resting energy expenditure [30]. Studies show that an increase in body weight accompanies increased resting energy expenditure both in humans and rodents [23, 31, 32]. Moreover, this increase in resting energy expenditure occurs via the SNS and is in the form of increased brown adipose tissue thermogenesis, heart rate or blood pressure [33, 34]. Multiple studies have in fact established direct link between leptin levels and SNS activity. For example, leptin infusion in rodents stimulates SNS activity, BAT thermogenesis, heart rate and mean arterial pressure [35-37]. These

effects of leptin are also reflected in epidemiological data that gives a clear relationship between leptin levels and cardiovascular parameters such as heart rate and blood pressure [38, 39]. Furthermore, the fact that human subjects with congenital leptin deficiency, although obese, have normal to decreased SNS activity, suggest that high leptin levels underlie the hypertensive effects of obesity [40, 41]. However, it is interesting to note here that the high leptin levels accompanying obesity, although has no effects on food intake, increase the SNS activity, indicating that leptin resistance during obesity is limited to its effect on food intake and does not involve the thermogenic and cardiovascular effects of leptin [42]. In another study, Aizawa-Abe and colleagues show that transgenic mice overexpressing leptin despite their 'skinniness' have elevated heart rate, blood pressure and urine norepinephrine output. When these 'skinny' mice were food restricted, a drop in body weight was noted, but no changes were observed in their systolic blood pressure [43].

Altogether these studies establish an intricate relationship between leptin and the SNS, where increases in leptin drives SNS activity and its decrease has an opposite effect, suggesting that the SNS system serves as a mediator for leptin's effect on energy expenditure. Consensus exists that heightened SNS tone could be a compensatory mechanism by which the brain tries to counteract for increased caloric load by increasing energy expenditure [21, 44]. However, the degree of this compensation is dependent on several factors including genetic makeup and diet [45, 46]. In line with this theory, failure to increase energy expenditure in response to experimental overfeeding has been associated with increased obesity [32], indicating that enhanced energy expenditure in some individuals forms a defense barrier against obesity.

Leptin signaling within the hypothalamus regulates energy expenditure

The hypothalamus with the highest expression of leptin receptors mediates both the appetite-suppressing and energy expenditure aspects of leptin. Initial evidence on hypothalamic regulation of thermogenesis and the cardiovascular system comes primarily from lesion studies. For example lesions of the dorsomedial hypothalamus (DMH) and the ventromedial hypothalamus (VMH) were associated with decreased core body temperature. [47-49]. In contrast, electrical or chemical activation of the DMH, VMH and the paraventricular hypothalamus (PVH) had opposite effects on core body temperature [50-53]. Moreover, these cardiovascular and thermogenic effects of their stimulation were shown to be sympathetically mediated [47, 48, 50, 51, 54, 55]. These initial studies connected distinct brain nuclei to regulation of body temperature and the cardiovascular system and future studies categorically looked into the role of leptin in this regard.

Leptin in the DMH: Till date the role of leptin signaling in the DMH in relation to energy expenditure is perhaps most accurately described. Intra-DMH injections of leptin in rodents increase body temperature, heart rate and blood pressure [56, 57]. Similar effects are also observed in obese mice that are resistant to the anorexigenic effects of leptin [33, 42]. In agreement with these findings, chemogenetic activation of leptin receptor positive neurons within the DMH stimulates BAT thermogenesis and body temperature without influencing food intake [57]. This subsequently culminates in body-weight loss [57]. Interestingly, recent data from Dodd and colleagues show that a distinct population of leptin receptor positive neurons within the DMH, the prolactin releasing peptide (PrRP) neurons mediates leptin's thermogenic effects [58]. This was shown by disrupting leptin receptors from PrRP expressing neurons, which attenuated the thermogenic effects of peripheral leptin and resulted in body weight gain [58]. In another recent study, leptin's role in cardiovascular regulation was outlined where antagonism of leptin receptors within the DMH correlated with decreased heart rate and blood pressure [33]. Taken together, these studies establish that modulation of leptin signaling within the DMH is sufficient to influence energy expenditure, ultimately influence body weight.

A bulk of studies has established that the DMH controls resting energy expenditure through the SNS [14, 55, 59]; however only recent findings confirm that the cardiovascular and thermogenic effects of leptin within the DMH are also sympathetically mediated. Enriori and colleagues reported that the increase in body temperature following intra-DMH leptin infusion is blocked by pretreatment with a B3 agonist [42]. Similarly, hyperthermia following chemogenetic activation of leptin receptor containing neurons within the DMH was minimized by a B3 agonist [57]. Despite the concrete evidence on leptin's role in thermogenesis and cardiovascular regulation via the DMH, ambiguity remains on the pathways by which leptin modulates the SNS and thereby influence energy expenditure. We know that leptin activates DMH neurons [33], but as leptin receptors are expressed both in GABA and glutamatergic neuronal populations, the precise neuronal identities mediating leptin's effects on thermogenesis, heart rate and blood pressure remains unclear. Leptin receptor containing DMH neurons are known to project to the paraventricular nucleus (PVN), the peri-aqueductal grey (PAG) and the raphae pallidus (RPa), areas involved in the SNS regulation of body temperature and the cardiovascular system [60, 61], but the exact role of these projections remain yet to be deciphered. It is also not known whether leptin via different neuronal populations mediate separate aspects of energy expenditure i.e. thermogenesis, blood pressure and heart rate. Although the PrRP neuronal population has been linked to thermogenesis, its neurotransmitter phenotype, projections or effects on the cardiovascular system is yet to be studied.

Leptin in the VMH: Although limited, a number of important works defines the contribution of leptin signaling in VMH region with respect to energy expenditure. Perhaps the most direct evidence of leptin's action within the VMH is provided by Marsh and colleagues [56]. Here, leptin infusion within the VMH increased blood pressure and renal sympathetic activity, findings that were subsequently replicated by others [62]. Both intravenous and VMH administration of leptin increased plasma catecholamine levels, an effect that was abolished in VMH lesioned animals, thus recognizing the VMH as crucial site for leptin action on the SNS. In agreement with this finding, leptin infusion within the VMH increased glucose uptake in BAT, that was blocked upon sympathetic denervation of BAT, indicating that increased SNS activity underlies the increased BAT activity [63].

Having established the relevance of leptin signal within the VMH on energy expenditure, later works mostly focused on the role of the SF-1 neuronal population within the VMH in relation to thermogenesis. These neurons express leptin receptors and form the bulkiest neuronal population within the VMH [64, 65]. Mice with leptin receptors deleted from the SF-1neuronal population display obesity without hyperphagia, suggesting that lower energy expenditure in these mice could explain their obese phenotype. Interestingly, when exposed to a high fat diet, these mice display defective adaptive thermogenesis and subsequently become obese [65, 66]. This led to the hypothesis that leptin signaling within these neurons in the VMH acts as a feed-back mechanism to counter body weight gain during high fat feeding by promoting energy expenditure [65]. Since the SF-1 neurons within the VMH are largely glutamatergic [64, 67] and that glutamatergic outputs from the VMH regulate SNS outflow [68, 69], deletion of these neurons would directly decrease sympathetic outflow and thus decrease thermogenesis. The fact that another set of glutamatergic VMH neurons projecting to the ARC innervate the anorexogenic POMC neurons, implies that activation of this excitatory pathway would potentiate the anorexogenic effects of the POMC neurons [69]. Consistent with this theory, food restriction dampened the activity of this VMH-ARC projection implying that glutamatergic output from the VMH, at least to the ARC is decreased in a situation of negative energy balance, when conservation of energy is crucial [69]. However, whether VMH glutamatergic projections to the hind-brain show similar dampening in food-deprivation conditions, remains to be delineated. Also, whether VMH glutamatergic neurons projections to the ARC POMC neurons regulate thermogenesis via the MC system, is still unknown.

In another set of studies, neuron specific ablation of the LIM domain only 4 (LMO4) protein extensively expressed in the VMH decreased SNS tone, thermogenesis and resulted in obesity in mice. The fact that leptin injection in LMO4 knock-out mice increased BAT

thermogenesis to a lesser extent compared to wild-type littermates, indicates that the presence of LMO4 signaling in the VMH is permissive for the energy expenditure aspects of leptin [70]. In contrast to the permissive actions of LMO4 on leptin signaling, the transcription factor foxhead protein FOXO1, a negative regulator of leptin's feeding effects within the AGRP neurons [71], was recently shown to negatively regulate the thermogenic effects of leptin within the ventromedial hypothalamic SF-1 neurons. The targeted deletion of FOXO1 from SF-1 neurons increased SNS activity, thermogenesis and energy expenditure generating a lean phenotype [72]. Moreover, leptin sensitivity and energy expenditure upon HFD exposure was boosted in FOXO1 knock-out mice. Thus exclusion of FOXO1 signaling from hypothalamic neurons counteracts diet-induced obesity by promoting thermogenesis [72]. Collectively, these studies outline leptin's role in the VMH on thermogenesis and suggest that leptin signaling within the VMH is protective against diet-induced obesity during weight gain. Despite the substantial volume of research devoted on the role of the VMH in regulation of thermogenesis, no specific studies have addressed the question whether signaling within the VMH is essential or permissive for thermogenesis and cardiovascular regulation. Accordingly, future studies with peripheral leptin injections in animals lacking leptin receptors in specific neuronal populations could answer this question.

Studies have also identified the role of brain derived neurotrophic factor (BDNF) containing neurons within the VMH in mediating thermogenesis [73]. Starvation that decreases thermogenesis simultaneously reduces VMH BDNF levels. Rats infused with BDNF in the VMH increase their body temperature and energy expenditure that results in lower body fat contents [73, 74]. However, no co-expression between leptin receptors and BDNF neurons has been reported so far, leaving the possibility open that the thermogenic effects of leptin in the VMH occurs through mechanisms other than BDNF [75]. Furthermore as BDNF lies downstream of the central melanocortin system [76], its thermogenic effects could be mediated via POMC projections from the ARC.

Leptin in PVH: Lesions of the PVH are associated with obesity as a consequence of decreased energy expenditure [77]. The general consensus is that the PVH increases thermogenesis either via peripheral catabolic hormones like thyroxin and cortisol or by directly influencing the sympathetic outflow. Tracing studies confirm the existence of poly-synaptic pathways between the PVH and the BAT [78]. Furthermore, these studies also show that PVN neurons that project to the spinal cord are activated during fever [79]. The fact that the chemical activation of the PVN that increases BAT thermogenesis can be blocked by a sympathetic ganglion blocker but not hypophysectomy, suggests that the thermogenic effects of PVH stimulation is dependent on sympathetic outflow [51]. Despite the relation between the PVH and SNS activity, evidences on the thermoregulatory effects of leptin in this brain area are mostly indirect, speculative and sometimes contrasting [80]. Direct

infusion of leptin within the PVH had non-significant increases in blood pressure, heart rate and SNS activity [56]. In line with this a study by Shih and colleagues, injecting leptin receptor antisense oligonucleutides within the PVN failed to show any cardiovascular or thermogenic effects, whereas the effects on food intake were prominent [81]. Nevertheless, it is known that neuronal populations such as the oxytocin and the thyroxin releasing hormone (TRH) neurons within the PVH express leptin receptors and influence resting energy expenditure. The TRH containing neurons influence thermogenesis via endocrine and neuronal mechanisms. It is known that leptin receptors are expressed in TRH neurons and that leptin enhances TRH biosynthesis [82, 83]. Starvation that decreases leptin levels also suppresses thyroid hormone levels [84]. In line with this, dieting individuals who reduce their energy expenditure, show decreased thyroid hormone levels, an effect reversed upon leptin replacement [22, 23, 85, 86]. This phenomenon is also observed in food restricted rodents subsequently treated with leptin [19]. This implies that decreased thyroid hormone levels due to a drop in leptin levels have immediate consequences for thermogenesis. The mechanisms by which thyroid hormones increase thermogenesis are numerous, including its effect on total basal metabolic rate, heart rate and BAT thermogenesis [87, 88]. Independent of these endocrine effects, studies demonstrate that TRH neurons through its neuronal projections regulate thermogenesis. These neurons send projections to the median preoptic area and the peri-aquecuctal grey area, brain regions that mediate thermogenesis and cardiac functions [89]. Central application of TRH increases both BAT and core body temperature. Furthermore, this increase in thermogenesis was independent of increases in plasma thyroxin levels and is reversed upon sympathetic denervation, indicating a direct involvement of the sympathetic outflow [90]. This effect of central TRH on thermogenesis is also reflected in its cardiovascular effects i.e. increases in heart rate and blood pressure [91]. Despite these evidences, the neural-mediated effects of TRH neurons in thermogenesis remain speculative and controversial [89, 92]. As TRH is also expressed in extra-hypothalamic sites [93], it is difficult to conclude whether the thermogenic and cardiovascular effects of TRH are exclusively mediated by the PVH.

A second population of PVH neurons, the oxytocin containing neurons, expressing leptin receptors have been implicated in mediating thermogenesis [94]. Leptin is shown to modulate these neurons as PVH levels of oxytocin are decreased upon food restriction and reversed upon leptin treatment [94]. In a recent publication, Blevins and colleagues demonstrated that rhesus monkeys chronically treated with oxytocin decreased their food intake and energy expenditure [95]. Since mice lacking the oxytocin gene are obese and have reduced SNS activity [96], the authors suggest that the increased energy expenditure seen in these monkeys is a result of decreased SNS activity [95]. The authors further implicate that the increase in energy expenditure could be mediated by oxytocin projections to the NTS [94, 95]. These findings on oxytocin and thermogenesis were replicated by another group,

where mice lacking the oxytocin receptors showed a steeper drop in body temperature upon cold exposure compared to their littermates [97]. Interestingly, the hypothermic phenotype could be reversed upon expression of oxytocin receptors within the hypothalamus, indicating that oxytocin signaling within the hypothalamus might form a poly-synaptic pathway regulating SNS activity and thermogenesis [98, 99]. In conclusion, although there are several indications that leptin signaling in these two PVN neuronal populations affect SNS activity and thermogenesis, no systemic studies have been conducted that determined whether manipulation of leptin signaling specifically in these neuronal populations affects thermogenesis.

Leptin in ARC: Finally, evidence suggests that leptin can indirectly influence thermogenesis through the first order NPY or POMC neurons within the ARC. Ablation of leptin receptor positive neurons specifically within the ARC decreases SNS activity and diet-induced thermogenesis [100], whereas infusion of leptin within the ARC increases SNS activity and blood pressure [101]. Given that the ARC is home to two antagonistically acting neuronal populations, the contributions of these two neuronal populations on cardiovascular and thermogenic regulation are also antagonistic.

Known for its potent orexigenic effects, acute central NPY infusion is associated with a hypometabolic state with increased food intake and decreased energy expenditure and thermogenesis [102, 103]. Mice overexpressing NPY in the ARC decrease energy expenditure, thermogenesis and BAT UCP-1 levels, effects known to be mediated by the SNS activity, as they are blocked upon BAT denervation [104]. Similarly, central infusion of NPY decreases SNS activity in BAT in rats [105, 106]. These effects are mediated by the Y1 receptors, as central infusion of anti-sense Y1 receptor peptides boosts thermogenesis [106]. In literature, NPY-ergic neuronal projections to the PVN and the VMH have been implicated in mediating the thermogenic effects of NPY. Infusion of NPY within the PVN suppresses SNS mediated BAT activity and decreases BAT UCP-1 levels and thermogenesis [107-110]. Shi and colleagues show that these hypothermic effects are probably mediated by NPY-ergic projections to   tyrosine hydroxylase (TH) containing neurons in the PVH [104]. The fact that anti-sense oligonucleotide injections of NPY-1 receptors within the VMH increase thermogenesis, implies that NPY-ergic projections to the VMH in part mediates the thermogenic effects of NPY [105]. Nevertheless, these findings are not absolute as results claiming the contrary also exists [111, 112]. Furthermore, it is important to note that ambient temperature has been identified as a crucial factor in NPY mediated thermogenesis, a factor ignored by many of the above-mentioned studies [113].

The role of the melanocortin system in cardiovascular and temperature regulation is opposite to that of the NPY system. Knocking out leptin receptors specifically from POMC neurons reversed the SNS effects of leptin infusion on heart rate and blood pressure [114].

As POMC neurons within the NTS are leptin unresponsive [115], it is possible that the cardiovascular effects of the melanocortins are mediated by the POMC neurons of the ARC. Similar to infusion of leptin, infusion of MC4R agonist Melatonan II (MTII) increases SNS activity, indicating that leptin signaling in POMC neurons in part regulates the SNS [116]. Furthermore, the augmentation in SNS activity following leptin infusion is attenuated in MC4 receptor knockout mice, implying that the SNS effects of leptin are in part MC4 receptor dependent [101]. In line with this, SNS activation following leptin injection could be blocked by the MC4 receptor antagonist, SHU9119 [116]. Mice without MC4 receptors are normotensive despite obesity and increased leptin levels, implying that MC4 receptors are needed to mediate the hypertensive effects of obesity [117]. Furthermore, MC4 receptor mutation carriers have a higher body mass but lower muscle SNS activity, heart rate, blood pressure and urinary catecholamine excretion [118, 119]. Similarly, healthy volunteers treated with MC4 receptor agonists show an increase in blood pressure and heart rate [119]. These studies collectively validate the fact that melanocortin signaling, lying downstream of leptin, mediates leptin's effects on energy expenditure via the SNS.

Recent data however challenges the solely catabolic actions of melanocortin signaling by showing that central infusion of melanocortin agonists lead to a biphasic response on temperature and blood pressure i.e. an initial hypothermic/hypotensive effect is subsequently followed by a hyperthermic/hypertensive effect [120]. Furthermore, the hypothermia following MTII injection was due to decreased BAT thermogenesis, and was present in MC3 and MC4 receptor knock-out mice, indicating that these receptors did not mediate the hypothermic effects of MTII. In contrast, the hyperthermic effects of MTII were lost in the MC4 receptor knock-out mice, confirming the findings of other authors that MC4R signaling essentially mediates the thermogenic and cardiovascular effects of melanocortins [101, 116, 121, 122]. For example, MC4 receptor knockout mice show no UCP-1 up-regulation following leptin injections [122]. Similarly, the general increase in energy expenditure as a response to a high fat diet was also abolished in MC4 receptor knockout mice [123].

Although retrograde tracing techniques show a wide intra- and extra hypothalamic distribution of MC4 receptor containing neurons that project to the BAT, including the DMH, medial preoptic nucleus (MPO), posterior hypothalamus, NTS, PVH, sub Zona Incerta (sub ZI) and the RPa [124-126], the exact contribution of MC4R signaling on SNS activity has not been studied thoroughly. Injection of MTII within the sub ZI increased BAT, an effect that could be blocked with a MC4 receptor antagonist [127]. Similarly, peripheral injections of MTII that increased BAT thermogenesis, could be blocked by injecting a MC4 receptor antagonist into the DMH [42]. Another hypothalamic nucleus, the MPO through its inhibitory projections to the DMH is known to modulate the excitatory DMH neurons projecting to the

RPa [128, 129]. Interestingly, the MPO expresses high amounts of MC4 receptors and infusion of MTII within this area increased thermogenesis that could be blocked by lesioning the DMH [130]. MC4 signaling in the PVH has been similarly implicated in thermogenesis, as injection of MTII into the PVH increases BAT thermogenesis [125], however not much is known on the underlying neuronal mechanisms. Relatively recent data challenge the earlier viewpoint that ARC POMC neurons mediate its thermogenic and cardiovascular activity exclusively via second order neurons in various hypothalamic and extra-hypothalamic nuclei [131]. Rescuing of MC4 receptor signaling in ARC POMC neurons in MC4 receptor deficient mice increased body temperature and BAT thermogenesis [131], implying that MC4 receptors on POMC neurons are equally crucial for thermogenesis. Lastly, as MC4 receptor knockout animals still respond to the thermogenic actions of leptin [42], indicates that the sympatho-excitatory actions of leptin is not exclusively mediated by melanocortin signaling.

Conclusion

Based on the existing evidences, we can conclude that multiple parallel neuronal pathways mediate the effects of leptin on thermogenesis. Even though leptin's action on various hypothalamic nuclei has been clearly defined, a major question that surfaces is the identity of the neuronal projections from these hypothalamic nuclei to hind brain structures that ultimately regulates heart rate, blood pressure and thermogenesis. The majority of literature reviewed here does not address the question whether the effects of leptin on resting energy expenditure i.e. on thermogenesis or cardiovascular regulation are distinctly mediated by separate neuronal populations. Furthermore, body temperature regulation is a delicate balance between heat production and heat loss, parameters affected by distinct neuronal circuitries [14, 128, 129]. However, most studies considered increased body temperature synonymous with increased heat production, thus ignoring the effects of heat loss. With the advent of modern technologies such as opto- and chemogenetics, it is now possible to address these questions in order to shed more light on the neurocircuitry of leptin's role in energy expenditure. Equally important is the identification of neuronal populations bearing leptin receptors that contribute to resting energy expenditure, as this might provide further insights and possible drug targets in order to curtail the potentially damaging effects of leptin in diseases such as obesity mediated hypertension.

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Chapter 8

Deficient leptin action in the dorsomedial hypothalamus underlies dieting–induced hypothermia

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Abstract

Weight loss in obesity is hampered by counter regulatory mechanisms such as reduced thermogenesis that can be reversed by injecting leptin peripherally. We discovered that restoring leptin signaling in the dorsomedial hypothalamic nucleus (DMH) is sufficient to normalize the reduced thermogenesis caused by withdrawal from an obesogenic diet. In support of this, inhibition of leptin signaling in the DMH reduces thermogenesis and promotes adiposity independent of food intake. Leptin's effect on thermogenesis involves DMH neuronal projections to the lateral/dorsolateral periaqueductal grey areas; these neurons express prolactin-releasing peptide, and when depolarized by leptin or chemogenetically activated by CNO, increase core body temperature and brown adipose tissue thermogenesis. These studies collectively demonstrate that a state of relative leptin deficiency during dieting reduces leptin signaling in the DMH resulting in reduced thermogenesis and provides a mechanistic explanation for the preservation of adiposity despite lowered caloric intake during dieting.

Introduction

In the book "Dieting makes you fat", published in 1983, the paradox was put forward that the regain of body weight following dieting is often in the form of fat mass [1]. Obesity is associated with increased intake of saturated fat and sugar-sweetened beverages, and thus the time preceding a dieting-to-lose-weight period is that of a period with high energy intake. The brain adapts to these diets high in fat and sugar by mechanisms that include a lessened efficacy of leptin to reduce food intake [2, 3]. Thus, decreased caloric intake in an attempt to lose weight is subsequently followed by physiological counter regulatory processes [4]. Reduced thermogenesis is one such mechanism during dieting [5]. However, the mechanisms underlying the thermogenic adaptations to dieting remain poorly understood.

Obese subjects that are maintained at a 10% lower body weight by dieting, decrease their resting energy expenditure by dampening sympathetic and thyroid axis activity which counteracts the weight loss [6-8]. Interestingly, decreased thermogenesis in these individuals coincided with low circulating leptin levels and was reversed by peripheral leptin treatment [8, 9]. Similar to the human studies, mice temporarily exposed to an obesogenic diet, retained their obese phenotype despite being switched to a normal diet. Regardless of the higher adiposity, leptin levels in these mice were comparable to control animals [10]. These studies indicate that a state of relative leptin deficiency following withdrawal from an obesogenic diet [7] drives metabolic adaptations in an effort to defend a higher adiposity.

The role of the adipocyte-derived hormone leptin in thermoregulation, an integral component of resting energy expenditure, is well documented. A deficiency in leptin action, either in leptin deficient ob/ob mice or leptin receptor deficient db/db mice, is associated with hypothermia [11, 12]. Interestingly, hypothermia in ob/ob mice but not db/db mice is reversed upon leptin treatment [11, 13], indicating that a lack of leptin signaling in these mice underlies the hypothermia. Within various neuronal populations of the hypothalamus leptin signaling regulates feeding behavior, locomotion and thermoregulation and collectively these influence the body weight of an organism [14]. Rodent studies demonstrate that leptin signaling within the dorsomedial hypothalamic nucleus (DMH) is critical for thermogenesis [15, 16]. Leptin infusion into the DMH of ob/ob mice corrects their hypothermia [17] while ablation of DMH leptin receptors results in hypothermia and body weight gain [18]. In addition, these thermogenic effects of leptin are mediated by prolactin-releasing peptide (PrRP) containing neurons within the DMH, as deletion of leptin receptors selectively from PrRP neurons decreases thermogenesis and causes obesity [19]. Collectively, these data highlight the significance of leptin signaling within the DMH in relation to thermogenesis. However, it is not known whether altered leptin signaling within

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this area underlies the reduced thermogenesis during dieting. Here we describe a novel physiological role of leptin signaling within the DMH in relation to thermogenesis following withdrawal from an obesogenic diet. Rats exposed to a free choice high-fat high sugar (fcHFHS) diet and subsequently switched to a chow-only diet, lower their caloric consumption and become hypothermic. We demonstrate that this hypothermia is a result of decreased leptin signaling within the DMH. Additionally we show that leptin-responsive DMH neurons projecting to the periaqueducatal grey form an important pathway mediating the thermogenic effects of leptin.

Results

Withdrawal from a fcHFHS diet decreases thermogenesis

As reported previously [20], rats exposed to a fcHFHS diet became hyperphagic, and obese (**Figures 1A-1C, S1A**). Following withdrawal from the saturated fat and liquid sugar components, caloric consumption of rats fed the fcHFHS diet was substantially reduced and, at week 7, was normalized to that of caloric consumption of control chow animals (**Figure 1C**). Despite the lower caloric intake, rats withdrawn from the fcHFHS displayed higher adiposity (**Figure S1A**) and leptin resistance compared to control rats as determined by the failure of peripheral leptin injections to decrease food intake (**Figure 1D**). Furthermore, whereas body weight gain in chow fed animals remained constant, fcHFHS fed rats stopped gaining weight following fat-sugar withdrawal reaching similar levels of body weight as the control group at week 6 (**Figure 1B**). Withdrawal from the obesogenic diet resulted in hypothermia as indicated by a decrease in core body temperature (**Figure 1E**). This 0.2°C fall in core body temperature was independent of locomotor activity (**Figure 1F**) and persisted throughout the entire withdrawal phase in spite of the normalized chow intake. The increase in brown adipose tissue (BAT) thermogenesis during fcHFHS exposure normalized upon withdrawal, suggesting that decreased BAT thermogenesis contributed to the decreased core body temperature (**Figure 1G**). This was further confirmed by attenuated BAT UCP-1 and B3 receptor mRNA levels (**Figure 1H**). Similarly, heart rate, which also contributes to core body temperature [21] was increased following four weeks of diet and normalized upon withdrawal **(Figure 1I)**. Withdrawal from the fcHFHS diet had no effects on arcuate neuropeptide expression, mRNA levels of thyrotropin-releasing hormone (TRH) or peripheral glucose levels (**Figures S1C-E**).

Reduced leptin signaling within the DMH during dieting underlies dieting-induced hypothermia

Elevated leptin levels in rats fed a fcHFHS diet, measured at the end of the obesogenic phase (W4), were normalized to levels exhibited in chow control rats at the end of the withdrawal phase (W8) (**Figure 2A**). This was also confirmed by leptin mRNA levels in abdominal fat

tissue which were not significantly different in rats withdrawn from the fcHFHS diet compared to rats on chow at W8. (**Figure S1B**). In order to establish whether the fall in leptin levels in the dieting phase explained the hypothermia observed in these animals, rats were peripherally injected with leptin (i.p. 2 mg/kg/ml) during the withdrawal phase (W7). Hypothermia in rats peripherally injected with leptin was reversed following leptin treatment (**Figure 2B**). Since leptin signaling within the DMH has been implicated in thermogenesis [17], we studied whether the DMH was activated in withdrawal animals receiving leptin treatment. Leptin replacement in fcHFHS animals in W7 significantly increased FOS and pSTAT3 protein expression within the DMH compared to controls (**Figure 2C**), suggesting an enhanced responsiveness of DMH neurons to an i.p. leptin challenge. Also, the degree of change in body temperature correlated positively with FOS expression in the fcHFHS group (**Figure S1F**). This positive correlation between increased core body temperature and FOS activation was seen only in the fcHFHS withdrawal group and not in the chow group Thus, the thermogenic response to peripheral leptin correlated with activation of the DMH only in rats withdrawn from the obesogenic diet.

To evaluate if the increased activity of the DMH was physiologically relevant, we infused leptin directly into the DMH via intra-DMH cannulae (bilateral/300ηg/300ηl/30sec) during the withdrawal phase (W7). Similar to peripheral injections (**Figure 2B**), intra-DMH injections of leptin rescued the hypothermia in fcHFHS withdrawal animals, whereas in control rats, leptin injections in the DMH did not have any effect (**Figure 2D**).

We further confirmed the role of leptin signaling within the DMH with respect to thermogenesis independently from our fcHFHS withdrawal model. Rats were food restricted animals to lower their body temperature and subsequently treated with intra-DMH leptin (**Figure S2A**). Similar to the fcHFHS withdrawal group (**Figure 1D**), food restricted rats showed a drop in body temperature (Fi**gure S2B**) that could be reversed upon intra-DMH leptin replacement (bilateral/300ηg/300ηl/30sec) **(Figure S2C**). Subsequently, we established the requirement of DMH leptin signaling for thermogenesis, by treating food restricted rats peripherally with leptin (i.p. 2 mg/kg/ml) or saline vehicle 15 min prior to intra-DMH administration of a leptin antagonist (bilateral, 0.3ηg/ηl, 300nl). Intra-DMH leptin receptor antagonism blocked the peripheral thermogenic effects of leptin **(Figure S2D**).

Leptin signaling in the DMH regulates body adiposity independent of caloric consumption

Next, we investigated the long-term effects of intra-DMH leptin receptor antagonism on thermogenesis. Over-expression of a virally expressed leptin antagonist [22] in the DMH increased body weight (**Figure 3A**) and adiposity (**Figure S3A**) by increasing food consumption (**Figure 3B**) and decreasing core body temperature (**Figure 3C**). Food restriction (2 days, 10 g chow) decreased core body temperature in control rats and in rats

treated with a virally-expressed leptin antagonist. Peripheral leptin injections (i.p. 2 mg/kg/ml) increased core body temperature in control rats, but failed to increase body temperature in rats with leptin receptor antagonist delivery in the DMH (**Figures 3D-F**), confirming previous results (**Figure S2D**) that leptin signaling within the DMH is essential for thermogenesis.

As intra-DMH leptin antagonism had effects on body temperature and food intake, a pair-feeding experiment was conducted to tease apart the effects of temperature on body weight and adiposity. Here, despite the lower caloric intake (**Figure S3C**), pair fed rats with leptin receptor antagonist in the DMH did not lose body weight (**Figure 3G**) but still had similar levels of adiposity compared to ad libitum fed leptin receptor antagonist-treated animals (**Figure 3I**).

Leptin receptor positive neurons projecting to the periaqueductal grey mediate thermogenesis

Given the importance of leptin receptors in the DMH on thermogenesis [18, 19], we aimed to identify the neuronal pathways through which leptin-sensitive neurons within the DMH drive core body temperature. It is known that disinhibition of the DMH increases core and BAT temperature [23]. This increase in body temperature involves DMH projections to the periaqueductal grey (PAG) and the raphe pallidus (RPa), amongst other areas [24]. Within the PAG, the lateral/dorsolateral PAG (l/dl PAG) part has been implicated in the regulation of core body temperature, as inhibition of this area is associated with a decrease in core body temperature [25]. Interestingly, cold exposure results in activation of this area [26]. The increase in thermogenesis upon disinhibition of the DMH is dramatically reduced following inhibition of the l/dl PAG [25], pointing to a role for l/dl PAG in mediating, in part, the thermogenic effects of DMH. However, it is not known whether the DMH neurons projecting to the l/dl PAG are leptin-sensitive and whether activating these specific neuronal projections affects core body temperature and body weight.

Based on these evidences and the fact that withdrawal from a fcHFHS diet decreases core body temperature, we next determined whether activating DMH neurons that specifically project to the l/dl PAG increases core body temperature. In order to do so, AAV-hSyn-DIO-hM3D (Gq)-mCherry was bilaterally injected into the DMH and a retrogradely transported virus driving expression of Cre recombinase (Cav-Cre 2) was injected into the l/dl PAG.

Double labeling of pSTAT3 and mCherry proteins confirmed the leptin-responsiveness of DMH neurons that project specifically to the l/dl PAG (**Figure 4A**). Consequently, chemogenetic activation of this pathway with injections of CNO (i.p. 0.3 mg/kg/ml) substantially increased core body temperature in rats (**Figures 4B, S4D**) independent of

locomotor activity (**Figures S4B-C**). The increase in BAT temperature was modest but significant (**Figure S4E**). Although CNO treatment had no effect on food intake (**Figure 4D**), it curbed the 24-hr body weight gain (**Figure 4C**). Given the role of intra-DMH PrRP neurons on core body temperature [19] using double immunohistochemistry we confirmed that multiple DMH neurons projecting to the l/dl PAG are PrRP-positive (**Figure S4A**).

Leptin modulates the activity of DMH neurons projecting to the PAG

To determine effects of leptin on DMH neurons projecting to the PAG (DMH-PAG), we performed electrophysiological recordings in brain slices on mCherry expressing DMH neurons prepared from rats injected with Cav2-Cre into the PAG and AAV-DIO-mCherry into the DMH. In whole-cell current clamp recordings these neurons showed a resting membrane potential of -52.6±1.4 with frequent spontaneous action potentials with frequency of 1.91± 0.1 Hz (n =16). In the presence of inhibitors of synaptic transmission (CNQX, 10 µM and bicuculline, 20 µM), application of leptin (100 nM) caused depolarization of the membrane potential (4.4± 0.5 mV) and increased the frequency of spontaneous action potentials in 60.5% (10 out of 16) of recorded neurons. The increase in the frequency of spontaneous action potentials after application of leptin was 138% ± 4% of baseline which was reversed upon washout of leptin (F**igure 4F**). Leptin-induced depolarization was also associated with a decrease in membrane input resistance (baseline: 326.5 ± 21 MΩ; leptin 287.1 ± 18 MΩ; n= 12, P = 0.0005; F**igure 4G**). Taken together, these data suggest that leptin modulates the activity of DMH-l/dlPAG neurons through direct postsynaptic effects.

Discussion

Metabolic adaptations following withdrawal from an obesogenic diet defends adiposity

Here we show that rats withdrawn from a fcHFHS diet, mimicking a human obesogenic snacking diet, exhibit decreased core body temperature that persists beyond the normalization of caloric intake and body weight. Since leptin treatment in these rats in the withdrawal phase restores body temperature, we propose that the drop in leptin following withdrawal from the obesogenic diet underlies reduced thermogenesis.

It has been suggested that the fasting-induced drop in leptin levels informs the brain about decreased fat reserves, thereby triggering compensatory responses to increase appetite and decrease energy expenditure [27, 28]. The fact that animals in withdrawal persistently remain hypothermic, suggests that long-lasting physiological adaptations endure in order to defend body weight. Furthermore, as homoeothermic animals use a substantial percentage of their basal metabolic rate (50-60%) to maintain core body temperature [29], lowering body temperature provides an efficient way to conserve energy. Interestingly, calorie-

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restricted humans, similar to the rats withdrawn from fcHFHS diet, have a 0.2°C decrease in core body temperature [30]. Comparable physiological responses are also noted in hibernating animals and in weight-reduced obese individuals [8, 31]. In the current study, peripheral leptin injections increased core body temperature in fcHFHS diet withdrawal animals but not in control animals. Similar observations were made in studies where infusion of leptin in lean ob/+ mice [11] or lean individuals [8] had no effects on body temperature. These data suggest that circulating leptin levels drive thermogenesis (but do not necessarily result in hyperthermia) and that a fall in leptin levels signals the brain to conserve energy by decreasing core body temperature.

Next to being hypothermic, fcHFHS withdrawal animals failed to decrease food intake following peripheral leptin injections. The persistent leptin insensitivity in the background of lowered caloric intake might appear paradoxical. However, seen from an evolutionary perspective where leptin resistance facilitates energy storage, immediate reversal of leptin sensitivity during negative energy balance would be detrimental to survival. In line with this theory, leptin injections in food-deprived mice minimally affected food intake [32], implying that leptin resistance during negative energy balance helps to defend body weight. Furthermore, the dissociation in the effects of leptin during withdrawal on core body temperature versus food intake is in line with findings of others where leptin resistance following exposure to an obesogenic diet is restricted to the ARC, while the DMH, mediating thermogenesis, remains sensitive [2, 17].

Decreased leptin action in the DMH underlies hypothermia following withdrawal from an obesogenic diet

Lesioning of the DMH or chemical inhibition of the DMH is accompanied by hypothermia [23, 33], suggesting that activation of DMH neurons stimulates heat production. Here, we identify the DMH as a crucial site for leptin action during withdrawal from an obesogenic diet. Leptin treatment during withdrawal from the fcHFHS diet increased thermogenesis and increased FOS and pSTAT3 expression in the DMH. Furthermore, leptin injected into the DMH in the dietary withdrawal phase was able to normalize body temperature, suggesting that stimulation of leptin signaling within the DMH is sufficient to normalize body temperature. The fact that the thermogenic effects of peripheral leptin injections in hypothermic animals were blocked by intra-DMH leptin antagonist injections, confirms the requirement of intra-DMH leptin signaling in mediating thermogenesis.

Next to the acute effects, long term antagonism of intra-DMH leptin receptors with an AAV leptin antagonist resulted in hypothermia and hyperphagia leading to higher adiposity. Also, pair-feeding animals treated with an intra-DMH leptin receptor antagonist increased adiposity indicating that hypothermia itself is sufficient to defend adiposity in animals. This

supports the notion that a disruption in intra-DMH leptin signaling, as noted in withdrawal animals, is sufficient to defend adiposity. These results are further supported by recent publications in which the role of intra-DMH leptin receptors were investigated with respect to thermogenesis [18, 19], heart rate and blood pressure [16]. To note, the increased sensitivity of the DMH to leptin indicated by the higher FOS and pSTAT3 activation during the withdrawal phase hints at a physiological response to falling leptin levels. These data are in line with previous findings where caloric restriction following an obesogenic diet-increased leptin-stimulated hypothalamic pSTAT3 and leptin receptor levels [34, 35]. Additionally, the fact that high-fat fed mice that are resistant to leptin's action on food intake [2], show increments in body temperature, heart rate and blood pressure [3, 16, 17] indicates a different sensitivity to leptin action with respect to food intake and thermogenesis depending on the site of action.

Leptin responsive DMH-l/dl PAG projections mediate thermogenesis
The DMH is known to project to hind-brain pre-sympathetic neurons in the RPa and PAG to regulate BAT thermogenesis, core body temperature and heart rate [15, 21, 23-25]. While the role of DMH-RPa connections for thermogenesis were addressed in recent publications [15, 18], the contribution of the DMH-PAG connection in thermogenesis has remained elusive. Interestingly, projection studies report more abundant DMH leptin receptor-containing neurons projecting to the PAG than to the RPa, underscoring the potential importance of the former area in leptin-induced thermogenesis [36]. Thus, based on these evidences and coupled with the fact that intra-DMH leptin replacement in hypothermic animals restores euthermia, we investigated the contribution of DMH-l/dl PAG connections in regulating core body temperature.

In the current study, activation of the DMH- l/dl PAG pathway increased core body temperature and BAT temperature (although the latter to a lesser degree). Additionally, activation of DMH-l/dl PAG connections decreased body weight gain independent of caloric intake, establishing that increasing core body temperature is sufficient to reduce body weight. We further report that DMH neurons projecting to the PAG are leptin-responsive, indicating that next to the leptin-responsive DMH-RPa pathway [15] that primarily regulates BAT thermogenesis [37], the DMH-l/dlPAG projections provide a parallel pathway influencing core and BAT temperature. Furthermore, based on the recent finding that leptin signaling in DMH PrRP neurons regulates core body temperature [19], we identify DMH neurons projecting to the l/dl PAG to be PrRP positive.

Within the DMH, leptin receptors are expressed in both glutamatergic [38] and GABAergic neurons [39], suggesting that the observed thermogenic effects of leptin maybe either due to activation of glutamate or GABA neurons. Since inhibition of l/dl PAG decreases core body

temperature [25]; it implies that activating glutamatergic DMH-l/dl PAG projection stimulates glutamatergic dl/PAG neurons to increase core body temperature. This hypothesis is supported by the fact that knocking out glutamate from leptin receptor positive neurons causes hypothermia [38]. Alternatively, activating intra-DMH GABA neurons that synapse on PAG GABA neurons projecting to the RPa would also increase BAT thermogenesis [40].

Dampening of sympathetic nervous system underlies hypothermia in withdrawal animals

BAT activity and heart rate are influenced by the sympathetic nervous system (SNS) and modulated by dietary manipulations [41, 42]. The dietary withdrawal phase in the current experiment was accompanied by decreased BAT thermogenesis (temperature and UCP-1 levels) and decreased heart rate in the fcHFHS animals, pointing to a suppression of the SNS. Our results resonate with the findings of other labs where decreased body weight is accompanied by lower SNS activity [8]. These studies, in turn, identify leptin as a cause for the attenuated SNS activity [7, 8]. Increases in leptin are known to increase SNS activity in rodents and humans [16, 43, 44]. Conversely, caloric deprivation that decreases leptin levels, attenuates the SNS tone [45, 46], serving as a counter regulatory mechanism to conserve energy [46]. In the current study, exposure to a fcHFHS diet was associated with hyperleptinemia and increased thermic effects of leptin as measured by increased BAT thermogenesis and heart rate which are in line with recently reported findings in obese mice where leptin dependent increases in heart rate and blood pressure were shown [16]. Withdrawal from the fcHFHS diet reduced leptin levels, decreased heart rate and thermogenesis in our rats, which is also in line with this previous work in mice where it was shown that withdrawal from an obesogenic diet decreased leptin levels, decreased heart rate and blood pressure without effecting body weight [16]. Also, in human studies decreased leptin levels and heart rate following weight reduction have been reported [47].

We here confirm the DMH as an important neural hub, connecting the status of energy balance to thermogenesis. Although we focused on one leptin-sensitive efferent DMH pathway, we cannot exclude that other leptin-sensitive projections from the DMH contribute to adaptive thermogenesis during weight loss. Leptin receptor-expressing DMH neurons, for instance, also project to the paraventricular nucleus (PVN) [36], in which TRH has been shown to be important for thermogenesis. Decreased TRH and thyroxin levels encountered during starvation are reversed following leptin administration [48]. Replacing leptin in dieting individuals, besides correcting energy expenditure, also restores the thyroid hormone levels, indicating that decreased thyroid levels contribute to the decreased thermogenesis during dieting [5]. Leptin is known to directly stimulate TRH neurons and increase TRH gene expression [49]. Intra-DMH leptin receptor-positive neurons projecting

to the PVN have been suggested to regulate the neuroendocrine effects of leptin, namely via TRH and CRH peptides [32, 48, 50-52]. Since we did not find alterations in PVN TRH expression and because optogenetic stimulation of the DMH-PVN pathway failed to increase core body temperature[53], we focused on another important efferent leptin-sensitive DMH pathway – that projects to the PAG.

In conclusion, we propose that the drop of leptin during withdrawal from an obesogenic diet triggers counter regulatory mechanisms at the level of the DMH which decrease core body temperature and sympathetic out flow in order to preserve adipose tissue mass.These data further support the hypothesis that leptin treatment during withdrawal from an obesogenic diet [28, 54] provides potential therapeutic options to promote and sustain weight loss during dieting.

Methods

Animals
All experimental procedures were approved by the Animal Ethics Committee of Utrecht University and were in agreement with Dutch laws (Wet op Dierproeven 1996) and European regulations (Guideline 86/609/EEC). Male Wistar rats (Charles River, Sulzfeld, Germany) at seven weeks age on arrival were individually housed (Macrolon cages; 40x26x20 cm) in temperature (21±2 °C) and humidity (60-70 %) controlled room under a 12 h light/dark cycle.

Animal model
Rats were subjected to a free choice high fat high sugar (fcHFHS) diet, where next to chow (3.31 Kcal/g, Standard Diet Service, UK) and tap water, rats had a choice of lard (9.1 Kcal/g Ossewit/Blanc de Boeuf, Belgium) and 30 % sucrose solution (1.0M sucrose mixed from commercial grade sugar and tap water) for four weeks (diet induced obesity phase) and then chow and tap water only for the subsequent four weeks (withdrawal phase). Control rats remained on chow and tap water for the entire duration of the experiment.

Telemetric measurements
Rats were implanted with an intra-abdominal telemetric transmitter (TA10TA-F40, Data Sciences International, New Brighton, USA) to record core-body temperature and locomotor activity under fentanyl/fluanisone (0.1 ml/100 g i.m., Hypnorm®, Janssen Pharmaceutica, Beerse, Belgium) and Midazolam (0.05 ml/100 g i.p., Dormicum, Hoffman-La Roche, Maastricht, The Netherlands) anesthesia. Brown adipose tissue temperature was recorded using a temperature sensitive transponder (IPTT-300, BioMedic Data Systems, Seaford, USA) that was implanted subcutaneously in the BAT. Activity and temperature of animals were

remotely measured using DSI software (Data Science International) and averaged per hour for statistical analysis. Heart rate was measured using TA11CTA-F40 transmitters and implanted as indicated elsewhere [48]. For telemetric measurements following drug injections, recordings were made every 2 min for at least 1 hr before and 5 hr following injections.

Intra-DMH cannulas and nano-infusions

For intra-DMH cannulations, anesthetized (Hypnorm/ Dormicum) rats were positioned in a stereotaxic apparatus and stainless steel guide cannulae (26 GA, 8 mm; Plastics One, Roanoke, USA) were implanted bilaterally, 1mm above the DMH (AP: - 2.40, ML: + 2.20, DV: - 8.30, angle 10°, Paxinos and Watson, 1998). Cannulae were fixed to the skull with stainless steel screws and dental cement. Intra-DMH injections were performed through an injector (9mm, 33GA, Plastics One) inserted into the guide cannula. Bilateral infusions (300 ηl over 30 sec) were performed using a syringe pump with the injectors left in place for another 30 sec to prevent backflow. At the end of the experiments cannula placements were verified with cresyl violet staining.

Intra-DMH AAV leptin antagonist injections

A rat leptin antagonist was obtained by mutating WT leptin gene with several point mutations as published previously [22]. Briefly, HEK293T cells were transfected with leptin antagonist [22] or control GFP [49] plasmids together with helper pDp1. Sixty hours post transfection; cells were harvested, pelleted, lysed and subsequently centrifuged through an iodixanol gradient to harvest viral particles [50]. Titer was determined using GFP (F 5' CACAGACTTGTGGGAGAAGC, R 5' AGCCACTCGTCTTGGCAGT) or leptin antagonist (F 5' AGACCCCAGCGAGGAAAATG, R 5' TACCGACTGCGTGTGTGAAA) primers by methods outlined previously [50]. Rats were stereotaxically injected with either control or leptin antagonist viruses (1.0×10∧9 genomic copies/μl) in the DMH (AP: - 2.50 ML: + 1.40 DV: - 9.30,‹ 5 °) in a volume of 2 μl over 10 min, 0.2 μl/min). Food intake, body weight and telemetric measurements were conducted for five consecutive weeks. At week six, animals were food-restricted for two consecutive days (10 g ofchow) and treated with leptin (2 mg/kg/ml) or saline.

To study the effects of core body temperature on adiposity, a pair-feeding experiment was conducted. Here, next to a control group, two groups of rats were injected with a leptin antagonist into the DMH. Once the hyperphagic phenotype was apparent in the leptin antagonist group, one group of rats was pair-fed to control amounts. For this, the amount consumed by the control animals was given to the pair-fed group the following day divided over two meals ZT 9 (1/3) and ZT 15 (2/3)). To verify injection sites, DIG labelled in-situ hybridization with a GFP antisense probe (720 bp, NCBI gene DQ768212) was performed on

fresh-frozen coronal brain slices (16 μm) using protocols as described previously [51].

DREADD- CAV2Cre virus injections

AAV-hSyn-DIO-hM3D(Gq)-mCherry was obtained from UNC Vector Core (Chapel Hill, USA) and CAV-2Cre from IGMM (Montpellier, France). 1μl (5.0×10∧8 genomic copies/μl) of CAV-2 was injected in the PAG (AP- 5.30 ML:+ 1.40 DV: - 6.70,‹ 10 °) and 1 μl (1.0×10∧9 genomic copies/μl) of AAV-hSyn-DIO-hM3D (Gq)-mCherry was injected into the DMH (AP: - 2.50 ML: + 1.40 DV: - 9.30,‹ 5 °).

Drugs

Recombinant murine leptin (NHPP, Torrance, U.S.A) was dissolved in 0.9% saline and used in all experiments either i.p. (2 mg/kg/ml), intra-DMH (bilaterally 300 ηg/300 ηl/ 30 seconds) or i.v (250 μg/250μl). Clozapine N-Oxide (CNO) was a kind gift of Dr. Bryan Roth (University of North Caroline, USA) and was dissolved to a concentration of 0.3 mg/kg/ml in 0.9% saline. Leptin antagonist peptide (L39/D40/F41 mutant, Protein Laboratories Rehovot Ltd, Rehovot, Israel) was dissolved in 0.9 % sterile saline to an end concentration of 0.3 ηg/ηl and infused into the DMH (300 ηl bilaterally over 30 sec). Experiments where body temperature was measured following acute manipulations with leptin or CNO were performed in the light-phase between ZT4-ZT5.

Leptin sensitivity test

Prior to beginning of the fcHFHS diet, rats were implanted with intra-arterial silicon catheters via the right jugular vein, according to the method of Steffens [52]. To measure leptin sensitivity, following an overnight fast (10 g chow at ZT 11) rats were injected with saline or leptin (250 μg /250μl) via the jugular vein at ZT2 in a latin-square design. Food intake was measured five hours following leptin injections.

Leptin and glucose analysis

Blood (250 μl) was collected in heparinized Eppendorf tubes prior to injection of leptin or saline in jugular vein-cannulated rats. Blood was centrifuged (3000 rpm, 15', 4°C) and plasma was stored at -20°C for further analysis. Blood leptin levels were determined in duplicate using a radioimmunoassay kit (LincoResearch, St. Charles, USA). Blood glucose was determined in duplicates using Biosen C-line plus glucose analyzer (EKF Diagnostics, Magdeburg, Germany).

Immunohistochemistry

Rats were injected with leptin 2 mg/kg/ml i.p. and, 2 hr later, were anesthetized with sodium pentobarbital (Nembutal, 100mg/kg/ml) and perfused with ice cold 0.9 % NaCl followed by 0.4 % PFA. Brains were subsequently removed, overnight post-fixed with 4 %

PFA, and subsequently immersed in 30% sucrose solution in PBS. Immunohistochemistry was performed on 40 µm coronal free-floating slices using protocols described previously: FOS [53], pSTAT3 [54].

For FOS staining, brain slices were first incubated with FOS antibody (1:2000, polyclonal rabbit FOS, sc52, Santa Cruz Biothechnology, Santa Cruz, USA), followed by biotinlylated donkey anti-rabbit (1:1000, Jackson Immunoresearch Laboratories, West Grove, USA) and finally with Avidin-Biotin Complex (1:200). The peroxidase staining was visualized with 3,3-diaminobenzidine solution containing 7.5% nickel ammonium sulfate. pSTAT3 staining was performed using rabbit anti- pSTAT3 (1:1000, rabbit monoclonal, Cell Signalling, Danvers, USA). Following overnight incubation and washing, sections were first incubated with biotinylated anti-rabbit antibody (1:1000), followed by avidin-biotin-complex labeling. For analysis, sections were photographed using a bright-field microscope with a digital camera (Axiocam, Zeiss, Hamburg, Germany). Sections were matched using the stereotaxic brain atlas from Paxinos and Watson (1998). For each animal, one section (Bregma -2.80 to 3.12) was blindly analyzed using fornix as a reference point. The hypothalamus was divided into four quadrants, of which the left upper quadrant formed the DMH.

For double pSTAT3 and mCherry staining, slices were first stained for pSTAT3 (as outlined above), followed by an mCherry staining. For mCherry staining, slices were washed with TBS (3x 10 min) followed by overnight incubation at 4°C with a mCherry antibody (1:500, monoclonal mouse, Abcam, UK) in supermix (0.25 % gelatin, 0.5 % TritonX, TBS). Subsequently, slices were washed (TBS, 3x10 min) and incubated in supermix with a fluorescent goat anti-mouse Alexa Fluor 594 (1:500, Life Technologies, Westburg, Netherlands) secondary antibody. mCherry staining was photographed with a FITC filter while pSTAT3 immunoreactivity was photographed under bright field. Overlays were made using imageJ (National Institutes of Health) where pSTAT3 images were inverted and pseudocoloured green and mCherry images were pseudocolord red. For combined mCrerry and PrRP staining, free floating brain slices were co-incubated with mCherry and PrRP antibody (rabbit, PrRP-31, H-008-50, Phoenix Pharmaceuticals, USA) in supermix overnight at 4°C. Following washing, slices were incubated with chicken fluorescent goat anti-mouse Alexa Fluor 594 and chicken anti-rabbit 488 secondary antibodies (1:500, Life Technologies).

RT qPCR
Following decapitation, BAT and peri-renal white adipose tissue were collected from rats and stored at -20°C. RNA was extracted using Trizol reagent (Life Technologies) according to company-provided protocol. cDNA was made using the Revert Aid™ First Strand cDNA synthesis kit (Fermentas, Westburg, Netherlands). mRNA was quantified by a $\Delta\Delta CT$

quantification method using SYBR Green PCR Kit (LightCycler® FastStart DNA MasterPLUS SYBR Green I, Roche, USA) and Cyclophilin A as a household gene. The following primers were used for RTPCR: Cyclophilin A (F 5'AGCCTGGGGAGAAAGGAATT, R 5' AGCCACTCGTCTTGGCAGT), leptin (F 5' ACATTTCACACACGCAGTC, R 5' TGGTGAGGATCTGTTGATAGAC), UCP-1 (F 5' CAACCTCATGACAGACGAC, R 5' CTCGTCTTGACCACATCAAC), B3 adrenergic receptor (F 5' CGTCTTCTGTGCAGCTACG, R 5'GCCATCAAACCTGTTGAGC).

Peripheral leptin and glucose analysis

Blood (250 µl) was collected in heparinized eppendorf tubes prior to injection of leptin or saline in rats bearing a chronically-implanted jugular vein catheter. Blood was centrifuged (3000 rpm, 15 min, 4°C) and plasma was stored at -20°C for further analysis. Blood leptin levels were determined in duplicate using a radioimmunoassay kit (LincoResearch, St. Charles, USA). Blood glucose was determined in duplicates using Biosen C-line plus glucose analyzer (EKF Diagnostics, Magdeburg, Germany).

Quantitative in-situ hybridization

Radioactive in situ hybridization was performed for agouti-related peptide (AGRP), neuropeptide Y (NPY) and pro-opiomelanocortinergic peptide (POMC) on hypothalamic coronal sections (16 µm) as described previously [55]. The TRH probe was a kind gift of Dr. Perry Barret [56]. 33P labeled antisense RNA probes were made for AgRP, NPY and POMC. The films were developed, scanned (Epson Perfection 4990 Photo flatbed scanner) and expression of the above genes in the ARC were determined. Calibration curves were obtained by measuring the gray values of the 14C microscales and plotting them against the appropriate nCi/g tissue wet weight values supplied by the microscale manufacturer, corrected for decay and tissue equivalent factor microscales. Specific signal was calculated by the subtraction of the background value. All sections with a signal were measured and averaged.

Electrophysiology

Animals were deeply anesthetized by intra-peritoneal injection of sodium pentobarbital and then trans-cardially perfused with carbogenated modified artificial cerebrospinal fluid (aCSF) containing 92 mM N-methyl-D-glucamine (NMDG), 2.5 mM KCl, 1.25 mM NaH_2PO_4, 30 mM $NaHCO_3$, 20 mM HEPES, 25 mM glucose, 2 mM thiourea, 5 mM Na-ascorbate, 3 mM Napyruvate, 0.5 mM $CaCl_2.4H_2O$, and 10 mM $MgSO4.7H_2O$, bubbled with 95% O_2 and 5% CO_2 (pH 7.3–7.4). Rats were then decapitated and the brains were removed and coronal hypothalamic slices (300 µm) were prepared using a vibratome (HM650V; Microm) in ice-cold modified ACSF. Slices were initially recovered in the carbogenated modified ACSF for 15 min at 34° and then transferred into a holding chamber containing standard ACSF

containing (in mM): 126 NaCl, 3 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 10 glucose, 1.25 NaH_2PO_4 and 26 $NaHCO_3$ bubbled with 95% O_2 and 5% CO_2 (pH 7.3) at room temperature for at least 1 hr. The slices were transferred one at a time to the recording chamber perfused with standard ACSF continuously bubbled with 95 % O_2 and 5 % CO_2 at 30–32˚C. Whole-cell patch-clamp recording were performed from DMH neurons projecting to the PAG. These neurons were identified by mCherry fluorescence and visualized with an Olympus BX61W1 microscope using infrared video microscopy and differential interference contrast (DIC) optics. Patch electrodes were pulled from borosilicate glass capillaries and had a resistance of 3-5 MΩ when filled with intracellular solutions. Internal solution contained (in mM): 140 K-gluconate, 1 KCl, 10 HEPES, 0.5 EGTA, 4 MgATP, 0.4 Na_2GTP, 4 phosphocreatine (pH 7.3 with KOH). Signals were amplified filtered at 3 KHz and digitized at 10 KHz using an EPC-10 patch-clamp amplifier and PatchMaster v2x73 software. Data were analyzed with Clampfit 10 (Axon Instrument, Sunnyvale. USA) and Mini Analysis (Synaptosoft, Fort Lee, USA) software. Series resistance was constantly monitored, and the cells were rejected from analysis if the resistance changed by >20%. No series resistance compensation was used. Resting membrane potential was measured in bridge mode (I=0) immediately after obtaining whole-cell access.

Statistics

All data are expressed as Mean±SEM. Data were analyzed using Prism Graphpad (Graphpad Software Inc, La Jolla, USA). Depending on the experiments, significance (p<0.05) was determined using two-sided paired or unpaired t-test, two-way ANOVA (Sidak's post-hoc test) or one-way ANOVA (Newman-Keuls post-hoc test).

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Figures

Fig. 1. Withdrawal from a HFHS diet results in decreased thermogenesis.
(A) Experimental Scheme depicting obesogenic/HFHS diet phase (W1-4; gray area) and withdrawal phase (W5-8).
(B) Relative body weight during the whole experimental period. Obesogenic phase is indicated in grey, (n=9-10, two way ANOVA, * p<0.05, ***p<0.001 compared to controls).
(C) Caloric intake during the whole experimental period. Obesogenic phase is indicated in grey, (n=9-10, two way ANOVA, **p<0.01 compared to controls).
(D) Leptin sensitivity as measured by cumulative 5 hours food intake following peripheral leptin injections (i.v. 250 μg, 1μg/μl) at W4 and W8, (n=7-8, paired t-test, *p<0.05, **p<0.01 compared to saline injection). Dotted line indicates normalized food intake following saline injections for both groups.
(E) Core body temperature averaged per week during the whole experimental period. Obesogenic phase is indicated in grey, (n=10-11, two way ANOVA, * p<0.05, **p<0.01, ***p<0.001 compared to controls).
(F) Locomotor activity averaged per week during the whole experimental period. Obesogenic phase is indicated in grey, (n=10-11, two way ANOVA, p>0.05)
(G) BAT temperature measured by intra-BAT transponders averaged across the obesogenic (W1-4) and withdrawal (W5-8) phase (n=12, two way ANOVA, * p<0.05 between W1-4 and W5-8)
(H) UPC1 and B3R gene expression in BAT at the end of the experiment (n=9-11, t-test, compared to controls). Dotted line indicates normalized mRNA expression to control values.
(I) Heart Rate measured at week 1, 4 and 8 of the experimental period, (n=4-5, two way ANOVA, *p<0.05 compared to controls).

Fig. 2. Relative leptin deficiency during withdrawal from HFHS underlies weight loss-induced hypothermia.

(A) Peripheral leptin levels measured at W4 and W8 of the experiment, (n=7-8, Two way ANOVA, **p<0.01 compared to controls)

(B) Core body temperature following peripheral leptin injections (i.p. 2mg/Kg/ml) at W7 averaged across 2 hr post injection, (n= 6-8 per group, One way ANOVA, *p<0.05 compared to control-saline)

(C) Percentage of DMH cFOS (n=7-8, t-test, *p<0.05 compared to controls) and pSTAT3 (n=3-5, t-test. *p<0.05 compared to controls) positive neurons following peripheral leptin injections (i.p. 2 mg/Kg/ml) at W7. Dotted line indicates normalized values to leptin injections in control animals.

(D) Core body temperature following intra-DMH saline or leptin injection (300ηg, per side, 1 ηg/ηl/30sec) at W7 (n=15-18, two way ANOVA, **p<0.01 compared to controls).

Fig. 3. Prolonged leptinreceptor antagonism by overexpression of AAV leptin antagonist in the DMH increases body adiposity independent of caloric consumption.
(A) Relative body weight following intra-DMH injection of AAV GFP control or leptin receptor antagonist (LepA), (n=6-8, Two way ANOVA, **$p<0.01$, ***$p<0.001$ compared to controls).
(B) Caloric intake following intra-DMH injection of AAV GFP control or leptin receptor antagonist (LepA), (n=6-8, two way ANOVA, ***$p<0.001$ compared to controls).
(C) Core body temperature averaged per week following intra-DMH injection of AAV GFP control or leptin receptor antagonist (LepA), (n=6-8, two way ANOVA, **$p<0.01$ compared to controls).
(D) Area under the curve (AUC) following saline and leptin injections in control and leptin antagonist animals following two days of mild food restriction (10g chow, 2 days) at W5 (two way ANOVA, *$p<0.05$ compared to saline #$p<0.05$ compared to control-saline condition). n
(E-F) Core body temperature following saline or leptin (i.p. 2 mg/kg/ml) averaged over 180 minutes in control and (F) leptin antagonist animals.
(G) Relative body weight following pair-feeding of intra-DMH AAV leptin receptor antagonist treated animals (LepA pair fed) to control animals (Control), (n=8, two way ANOVA, **$p<0.01$, ***$p<0.001$ leptin antagonist ad-libitum vs controls; θ $p<0.05$, θθθ $p<0.001$ leptin antagonist pair-fed vs controls).
(H) Core body temperature averaged per week (n=8, two way ANOVA, *$p<0.05$, **$p<0.01$, ***$p<0.001$ leptin antagonist ad-libitum vs controls θ $p<0.05$, θθ $p<0.01$, θθθ $p<0.001$ leptin antagonist pair-fed vs controls leptin antagonist pair fed vs controls).

(I) Visceral fat pads removed at the end of the experiment, (n=8, one way ANOVA, ***p<0.001 leptin antagonist ad-libitum vs controls θθθ p<0.001 leptin antagonist pair-fed vs controls)

Fig. 4. Leptin receptor positive neurons in the DMH projecting to the periaqueductal grey mediate thermogenesis.

(A) Specific labeling of DMH neurons projecting to the l/dl PAG by injecting AAV-hSyn-DIO-hM3D(Gq) into the DMH and Cav 2-Cre in the l/dl PAG. Neurons in the DMH show immunofluorescence for mCherry (red) and pSTAT3 (green). Lower panel shows digitally zoomed images with co-localizations (yellow arrows) identifying leptin responsive neurons projecting to the l/dl PAG.

(B) Core body temperature over 240 minutes following activation of DMH-l/dl PAG projections with CNO (i.p. 0.3 mg/Kg/ml), (n= 10, **p<0.01, ***p<0.001, compared to saline conditions).

(C) 24 hr body weight gain following injection of saline or CNO (i.p. 0.3 mg/Kg/ml) (n=5-8, t-test, p=0.05).

(D) 24 hr food intake gain following injection of saline or CNO (i.p. 0.3 mg/Kg/ml) (n=5-8, t-test, p>0.05).

(E) Whole cell patch clamp electrophysiology showing changes in firing frequency following bath application of leptin measured from DMH cells projecting to the l/dl PAG.

(F) Action potential frequencies following bath application of leptin measured from DMH cells projecting to the l/dl PAG, (n=10, one-way ANOVA, ***p<0.001 compared to baseline values).

(G) Input resistance of DMH cells projecting to the l/dl PAG following bath application of leptin, (n=12, t-test, *** p<0.001 compared to baseline values).

(H) mCherry immunofluorescence used to identify DMH neurons projecting to the l/dl PAG.

Supplementary Data

Supplementary Fig. 1, related to Figure 1. Characterization of the withdrawal phase in control and HFHS withdrawal animals.

(A) Visceral fat pads removed upon completion of experiment (end W8) (n=9-10, t-test, **p<0.01 compared to controls).

(B) Leptin mRNA in WAT removed upon completion of experiment (end W8), (n=9, t-test, p>0.05). Dotted line indicates normalized mRNA expression to control values.

(C) ARC mRNA expression of NPY, AGRP and POMC at end of W8, (n=4-10, t-test, p>0.05). Dotted line indicates normalized mRNA expression to control values.

(D) TRH mRNA expression in the PVN at end of W8, (n=8, t-test, p>0.05). Dotted line indicates normalized mRNA expression to control values.

(E) Peripheral glucose levels at end of W8, (n=7, t-test, p>0.05).

(F) Correlation between DMH Fos expression and change in body temperature following leptin injections (2 mg/ml/ml) in control or fcHFHS animal at W7.

Supplementary Fig. 2, related to Figure 2 & 3. Validation of the role of intra-DMH leptin signaling on regulation of core body temperature.

(A) Shows an experimental scheme, where animals are food restricted for two days (10 g chow, day 1&2) and then subsequently injected with drugs on day 3.

(B) Core body temperature following two days of caloric restriction (n=3, t-test, *p<0.05 compared to ad-libitum state).

(C) Core body temperature following intra-DMH saline/leptin injection (bilateral/300 ηg/300ηl/30sec) on day 3 averaged over 2 hr, (n=3, t-test, * p<0.05 compared to saline condition).

(D) Core body temperature following i.p saline or leptin injections (2mg/Kg/ml) 15 min prior to bilateral intra-DMH injections of leptin antagonist (0.3 ηg/ηl, 300ηl bilaterally over 30 sec) on day 3 averaged over 2 hr. (n=6, Two way ANOVA, *p<0.05 compared to saline/saline condition).

Supplementary Fig. 3, related to Figure 3. Effects on prolonged intra-DMH leptin receptor antagonism by AAV leptin receptor overexpression on physiological parameters.

(A) Visceral fat pad weight at end of experiment in control and leptin antagonist treated animals (n=7-8, t-test, ***$p<0.001$).

(B) Locomotor activity during the whole experimental period averaged across the weeks (n=6-8, Two-way ANOVA, $p<0.05$, post hoc: not significant).

(C) Caloric intake in control, leptin antagonist ad-libitum (LepA ad libitum) and pair-fed (LepA pair fed) animals over the whole duration of the experiment (n=8, two-way ANOVA, **$p<0.01$, **<0.001, leptin antagonist ad-libitum vs controls θθθ $p<0.001$ leptin-antagonist pair-fed vs controls).

(D) Locomotor activity during the entire experimental period control, leptin Antagonist ad-libitum (LepA ad libitum) and pair fed (LepA pair fed) animals (n=8, two-way ANOVA, **$p<0.01$ leptin antagonist ad-libitum vs controls, θθ $p<0.01$ leptin-antagonist pair-fed vs controls)

(E) Digoxigenin labelled GFP in situ pseudocoloured green showing virus expression in the DMH.

Supplementary Fig. 4, related to Figure 4. Effects of specific activation of DMH-l/dl PAG neurons on physiological parameters

(A) Neurons in the DMH show immunofluorescence for mCherry (red) and PrRP (green). Lower panel shows digitally zoomed images with co-localizations (yellow arrows) identifying PrRP positive neurons projecting to the l/dl PAG.

(B) Locomotor activity over 240 min following activation of DMH-l/dl PAG projections by CNO (i.p. 0.3 mg/Kg/ml) injections, (n= 10, two-way ANOVA, $p<0.05$, post hoc not significant).

(C) Correlation between activity and core body temperature averaged across 240 min following CNO injections (n=10).

(D) Core body temperature following activation of DMH-l/dl PAG projections by CNO (i.p. 0.3 mg/Kg/ml) (n=3, two way ANOVA, **$p<0.01$).

(E) BAT temperature following activation of DMH-PAG connection by CNO (i.p. 0.3 mg/Kg/ml) injections (n=3, two way ANOVA, *$p<0.05$).

General Discussion

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Following the discovery of leptin in 1994 [1], in a groundbreaking publication, Ahima et al. proposed that leptin signaling is crucial for informing the brain about energy deficits rather than energy excess [2]. Drawing lessons from leptin deficient ob/ob mice, the authors conclude that the drop in leptin levels provoke a cascade of neurochemical changes in order to minimize energy expenditure and thus maximize chances of survival. We know that accumulation of fat is the only mechanism for long term storage of energy, since other forms (like glycogen storage) are rather limited. Thus, situations that threaten adiposity levels (such as decreased caloric intake in the form of starvation or dieting) can be expected to trigger compensatory neuro-humoral changes in order to counteract weight loss [2]. As outlined in the general introduction, decreased leptin levels as a result of caloric restriction during dieting are accompanied by a range of behavioral and physiological adaptations that hinder weight loss. The current thesis focuses on two effects of decreased leptin levels: increased motivation for food and decreased thermogenesis.

The role of leptin, neuropeptide Y, melanocortins in motivation for food

Food restriction lowers circulating leptin levels and increases an animal´s motivation to obtain food [3-5]. Peripheral leptin injections can reverse this enhanced motivation (Appendix I and [6]), implying that leptin decreases motivation for food. One of the crucial sites of action for leptin is the arcuate nucleus of the hypothalamus (ARC). Here, leptin acts on neuropeptide Y (NPY) and pro-opiomelanocortine (POMC) neurons to decrease and increase their activity (and peptide expression), respectively [7]. Interestingly, lowered caloric consumption that increases motivation for food also alters NPY and POMC levels (NPY↑, POMC↓) [8, 9]. Similarly, despite the high leptin levels, diet-induced obesity causes leptin resistance in the ARC and increases an animal's motivation for food [10, 11]. This obese state is also accompanied by increased NPY and decreased POMC levels [11]. Thus, alteration in NPY and POMC levels, irrespective of the nutritional status (dieting or obesity) affects motivation for food. However, whether a causality exists between ARC neuropeptide changes and food motivation, remains unknown. Section I of the thesis is dedicated to understanding the role of NPY and POMC on motivation for palatable food. Since caloric deprivation, besides decreasing leptin levels, also triggers other neurochemical alterations [2], motivation for sucrose was determined following intracerebroventricular or local injections of NPY and the POMC products αMSH and γMSH in ad libitum fed rats. Next to motivation, we also studied whether the injections of these peptides affected food consumption (chow, sugar) independent of motivation.

Earlier studies already showed that NPY, administered into the lateral ventricle, increased motivation to respond for a food reward [12]. Here, we extended these findings by focusing on the region-specific role of NPY in motivation and food consumption. Chapter 4 shows that

infusion of NPY into three different brain areas, the lateral hypothalamus (LH), the ventral tegmental area (VTA) and the nucleus accumbens (NAC), has distinct effects on motivation for sugar and free-feeding of sugar [13]. NPY infusion into the VTA solely increased motivation for food, while NPY infusion into the LH solely affected free-feeding. In contrast, NPY infusion into the NAC increased both motivation and free-feeding. Furthermore, our data also show that NPY increases motivation when administered into the VTA by a dopamine-dependent mechanism. Since (a) NPY Y1 receptors are expressed in VTA dopaminergic neurons [14] and (b) increased firing of dopaminergic neurons is known to enhance motivation [15], it is plausible that NPY acts on dopamine neurons to increase motivation for food. In contrast to the expected, i.e. that NPY increases dopamine neuron firing, Korotkova and colleagues demonstrated the opposite [14]. However, with the advent of newer tracing technologies, researchers have challenged the existing dogma regarding the uniformity of dopaminergic neurons within the VTA. Instead, VTA dopaminergic neurons are shown to be projection specific and may contain other neurotransmitters (such as glutamate) next to dopamine [16, 17]. Thus, treating dopaminergic neurons as a uniform entity provides a restricted view on the role of these neurons in motivation. Instead, the next step would be to use viral based technologies (such as the rabies virus [18]) to delineate the role of specific dopaminergic projections, and thereby study the electrophysiological effects of NPY on those specific projections. A second possibility is that NPY acts via GABA neurons within the VTA [14]. It is known that GABA neurons express NPY receptors and are inhibited by NPY [14], suggesting that the motivational effects of NPY in the VTA could occur via inhibition of GABA neurons (and thus indirect activation of dopaminergic neurons).

Intriguingly, while NPY infusion into the VTA enhanced motivation and NPY administration into the LH increased free-feeding, the NPY infusion into the NAC increased both free-feeding of sugar and motivation for sugar. These results can be partially explained by a recent study in which NPY infusion into the NAC resulted in decreased neuronal firing and overconsumption of palatable food (however, with a preference for fat but not sugar in a choice paradigm) [20]. In that study, the authors propose that increased NPY signaling in the NAC underlies the increased motivation and overconsumption following a HFHS diet [20]. Our data are in line with these findings, showing that NPY signaling within the NAC increases consumption of palatable foods. We propose that NPY within the NAC increases the hedonic value of sucrose by which both free consumption and motivation are increased. However, the downstream pathway involved need to be identified. The increase in free-consumption of sucrose following NPY infusion into the LH could also be explained by an effect of NPY on orexin or melanocyte stimulating hormone (MCH) signaling, which lie downstream of NPY. However, as LH orexin neurons project to the VTA and increases in their activity enhances motivation for sucrose [21], one would expect that if NPY works via orexin neurons, NPY infusion into the LH would also increase motivation. However, we did

not observe any effects on motivation following NPY infusion into the LH. Thus, to gain more insights into the mechanism underlying NPY involvement in free feeding and not in motivation, studies should be conducted to determine whether NPY receptors are expressed in orexin/MCH neuronal populations that specifically project to the VTA or the NAC to regulate the motivation for sugar or free-feeding of sugar.

Chapters 5 and 6 focused on the disentanglement of the role of melanocortin 3 and 4 receptors (MC3R and MC4R) in the motivation for sugar and free-feeding of sugar. In chapter 5, the central effects of MC4R stimulation were investigated by infusing the MC4R agonist αMSH. As hypothesized, infusion of αMSH decreased motivation for sucrose, independent of sucrose consumption, indicating that the anorexigenic effects of MC4R stimulation are at least in part the result of decreasing the motivation of animals to respond for palatable food. These data are in line with other studies in which infusion of the MC4R inverse agonist Agouti related peptide (AGRP) increased the motivation for food [22]. Furthermore, in this chapter, the NAC was identified as a principle site of melanocortin action. Firstly, we show that the ARC sends dense POMC projections to the shell region of the NAC. Secondly, infusion of αMSH into the NAC mimicked its central effects on motivation, indicating that MC4R stimulation within the NAC is sufficient to decrease the motivation for food. Based on the findings of a previous publication in which αMSH was shown to decrease the firing of dopamine D1 receptor-expressing neurons [23], we speculate that the motivational effects of melanocortins within the NAC are mediated by these neurons. Chapter 6 aimed to identify the contribution of MC3Rs on the motivation for food. Here, animals received a combined infusion of αMSH together with a MC4R antagonist. αMSH is an endogenous agonist of the MC4R, but it also has a low affinity for the MC3R. Thus, combined αMSH and MC4R antagonist infusions, would unmask the effects of MC3R stimulation on motivation for food. Interestingly, in contrast to the central effects of αMSH discussed in Chapter 5, a combination treatment of αMSH and a MC4R antagonist increased motivation to respond for food, indicating that melanocortins can increase motivation via MC3Rs. In agreement with these findings, central infusion of the MC3R agonist γMSH also increased the motivation for sucrose. Furthermore, direct infusion of γMSH into the VTA was found to mimic its central effects. Chapter 6 also shows that MC3Rs are expressed in a subset of VTA dopaminergic cells that may project to the NAC. In summary, this chapter weaves together the pieces of existing information on MC3R and its role in food intake to provide a concrete picture on the role of MC3Rs in food reward. In fact, to our knowledge, this study is the first to report that administration of a melanocortinergic peptide increases food motivation.

Based on the findings in section I, we can theorize on how both dieting/hungry and obese states are accompanied by heightened motivation for food, as both these situations lead to increased ARC NPY levels that increase motivation for food. Indeed, the studies in section I

demonstrate that alterations in ARC neuropeptide levels affect motivation for palatable food rewards, implying that leptin, besides directly influencing motivation within the VTA [24], also regulates the motivation for food via NPY and POMC neurons. Nevertheless, summarizing all findings from this section also brings forward some interesting discrepancies. Firstly, comparing results from chapters 4 and 5 showed that infusion of NPY into the NAC affected both motivation and free-feeding, while infusion of αMSH solely influenced motivation for sugar but not free feeding. This discrepancy becomes more evident if we take into account that both MC4Rs and Y1 receptors are expressed in both dopamine D1- and D2-expressing populations of medium spiny neurons in the NAC [20, 23, 25]. It may therefore be that different neuronal subsets within the NAC express either NPY- or MC4 receptors, i.e. that NPY and αMSH modulate separate neuronal subsets (and thus projections) from the NAC. Secondly, whether other NPY receptor subtypes are present within these two neuronal populations remains to be determined; expression of different NPY receptors might underlie this discrepancy.

Secondly, lateral ventricle infusions of αMSH predominantly decreased motivation for sucrose while local injections of αMSH into the NAC and VTA decreased and increased motivation, respectively. This discrepancy could lie in the fact that the placement of the ICV cannulas was closer to the NAC compared to the VTA, providing a higher drug concentration at the NAC as compared to the VTA. Nevertheless, the possibility that αMSH decreases motivation through another area, besides the NAC, cannot be excluded. As a result, the decreased motivation upon ICV αMSH infusion could be a synergistic effect on the motivational reducing effects of αMSH in the NAC and other brain area(s) (and thus the predominant effect) upon ICV injections.

Finally, the fact that two peptidergic products of POMC gene, i.e. αMSH and γMSH, had opposite effects on motivation for sugar, was surprising at first glance. However, since melanocortin receptors show region specificity, the motivational effects (↑or↓) of melanocortins may depend on receptor expression and the neuronal types expressing these receptors rather than the peptides themselves. Evidence to support this notion comes from the fact that both αMSH and γMSH bind to MC3R and MC4R, but with different affinities [26]. Pondering on the physiological role of MC3R-dopamine system interaction, one might find the effects of γMSH on food motivation surprising, especially since melanocortins in the historical perspective are considered as anorexigenic peptides. However, in light of evolutionary biology, an organism although satiated, should maintain its intrinsic motivation to seek out food-sources and consume them in order to increase the chances of survival, and MC3R signaling within the VTA might be the crucial element driving this motivational behavior even at times of energy abundance. If we look into earlier studies showing that a subset of NPY neurons within the ARC are GABAergic and project to POMC neurons to

inhibit them, and our own findings that even 'anorexogenic' neurons such as POMC neurons can increase an animal's motivation for feeding, it becomes clear that in general the factors that promote food intake dominate over factors that curb food intake. Thus, the drive to overeat and thus save 'fat' for the future may be tremendous. Interestingly, not only γMSH but also β-endorphin, another peptidergic product of the POMC gene increases food intake [27, 28]. Besides, it is also important to take into account that in the current thesis, we have focused on the role of ARC peptides (NPY, POMC) on the motivation for food. However, the motivation lowering effects of peripheral leptin injections in food-deprived animals (Appendix I), can also be mediated by other areas besides the ARC. Leptin receptors are widely expressed in brain areas such as the ventral tegmental area (VTA) and the lateral hypothalamus (LH), where leptin has been shown to influence motivation [24, 29]. It is also important to note that in the current thesis, we speak about the POMC neurons within the ARC. Next to the ARC that harbors the leptin sensitive ARC POMC neurons [30], the nucleus of the solitary tract also contain POMC neurons, but they are known to devoid of leptin receptors [31]. Similarly, NPY immunoreactivity has been reported in diverse brain nuclei [32], while in the current thesis we have considered alterations in ARC NPY levels (during starvation and obesity) as the starting point for experiments conducted in chapter 4.

Leptin and energy expenditure

Next to increasing motivation for food, decreases in leptin levels are also associated with changes in energy expenditure [33]. Since thermogenesis is one important aspect of energy expenditure, section II of the thesis studied the relationship between leptin and thermogenesis. These studies were performed in a context of dieting, i.e. inducing a state of relative leptin deficiency. It is a well known fact that dieting is one of the most commonly employed strategies to lose excess body weight, but it has high failure rates [34]. This high failure rate has often been attributed to low leptin levels, that trigger both physiological and behavioral adaptations to limit energy expenditure [35, 36]. By introducing a novel model of dieting in which animals are withdrawn from an obesogenic diet, Chapter 8 shows that the dieting phase is accompanied by lower body temperature and higher adiposity. Here, animals were exposed to high fat high sugar (HFHS) diet for four weeks (obesogenic phase), where next to regular chow, animals receive a 30% sucrose solution and lard. Following the obesogenic phase, the fat and the sugar components of the diet were removed, leaving access to chow only (dieting phase). The HFHS diet has been used successfully before as a model for diet-induced obesity [10, 11]. The choice of fat and sugar that the animals received mimics in part the energy-dense food-rich environment we are exposed to in the Western world. Furthermore, animals exposed to the HFHS diet have shown to be hyperphagic and leptin resistant at as early one week following diet exposure, making it a suitable model for studying diet-induced obesity [10, 11]. The goal of chapter 8 was to study if the obese phenotype observed after the obesogenic phase could be reversed upon returning the

animals to a 'healthy' diet (i.e. chow only) with the idea that withdrawal from the fat and sugar components of the diet would mimic the human situation of dieting, in which obese individuals reduce their caloric intake in an attempt to lose body weight. This decrease in caloric intake is mostly in the form of avoiding sugar- and fat-containing products. A downside of the model is, however, that compared to the human situation, laboratory experimental conditions are very controlled. For example, the external food-associated stimuli that humans are exposed to during dieting, which can curb diet compliance and evoke resumption of unhealthy eating patterns, are absent in this model. Furthermore, in contrast to the human situation of opting for a healthier diet, in the laboratory setting animals are ´forced´ to choose a healthy diet.

To our surprise, returning animals to chow-only diet for four weeks, failed to alleviate the obese phenotype as determined by higher adiposity and persistent leptin resistance. However, eight weeks of withdrawal did restore the leptin sensitivity and normalized adiposity (Appendix I) indicating that a prolonged period of withdrawal from a high fat diet does lead to restoration of leptin sensitivity, a conclusion drawn also by other authors [37].

Another important observation of the study was the hypothermia observed in the HFHS-withdrawn animals. The hypothermia that persisted despite the normalization of caloric intake to pre-obese levels, could be reversed by peripheral leptin replacement. Interestingly, leptin replacement in withdrawn animals also activated leptin receptors in the dorsomedial hypothalamus (DMH). The chapter further demonstrated that besides being sufficient, intra-DMH leptin replacement is necessary to mediate the thermogenic actions of leptin. Moreover, intra-DMH leptin receptor antagonism was shown to increase adiposity, independent of food intake. Next to decreased thermogenesis, the dieting phase was also accompanied by decreased heart rate and brown adipose tissue temperature, suggesting an attenuated sympathetic nervous system (SNS) tone. The relationship between leptin and the SNS is well known; this has been extensively discussed in Chapter 7. In short, leptin is known to increase thermogenesis, by increasing the SNS tone to the brown adipose tissue. Our data strongly suggest that the drop in leptin levels during dieting, leads to a decrease in thermogenesis and sympathetic nervous system activity and is a possible mechanism to conserve energy in the time of energy scarcity.

 Chapter 8 also establishes the role of leptin sensitive DMH neurons that project to the peri-aqueductal grey area of the hindbrain in regulation of thermogenesis. The findings of this section are also supported by a series of recently published articles on this topic [38-41]. Thus, Rezai-Zadeh and colleagues confirmed that pharmacogenetic activation of leptin-sensitive neurons within the DMH increases BAT thermogenesis, probably via the raphe pallidus [38, 40]. Dodd et al. identified these leptin receptor positive neurons to be Prolactin

Releasing peptide (PrRP) neurons that mediate the thermogenic actions of leptin [41]. To summarize, our data suggests that under physiological conditions, leptin maintains thermogenesis via the DMH. However, under conditions of lower caloric intake, a state of relative leptin deficiency is developed, that ultimately decreases energy expenditure by decreasing thermogenesis.

Concluding remarks

Despite the progress in the understanding of neurobiology of food intake in the past decade, treatment options for obesity using non-surgical methods remain minimal, if not absent. Thus, shedding more light on the neurobiology of body weight regulation was a logical next step taken in the hope of discovering new drug targets affecting different realms of feeding behavior. Another question of societal relevance addressed in this thesis is the issue of dieting or reducing one's caloric intake. Previous research has shown that decreased caloric intake in humans and rodents is associated with a range of neuro-humoral adaptations. One such consequence of decreased caloric intake is lowered leptin levels. In the current thesis, we have studied two of the consequences of decreased leptin levels on behavior (motivation for food) and physiology (thermogenesis). It has been suggested before, based on both rodent [2] and human [35] studies, that when calorie-restricted, the body tries to conserve energy by limiting energy expenditure. At the same time, the internal motivation of an animal to obtain food is extremely high [4, 42-45], and given the possibility to work for food, food-deprived animals are willing to work more.

Interestingly, leptin resistance (i.e. failure to decrease food intake upon leptin injections) that can be seen as an evolutionary adaptation to amass energy in times of energy abundance, and spend it economically in times of energy shortage, acts against us in today's Western environment. Our eating habits have drastically transformed from the times of man as hunter-gatherers to today's modern world. Unfortunately, our genetic makeup is slower in adapting to the fast changing world and eating habits. Thus, the weight cycling that is observed in seasonal mammals where periods of leptin sensitivity alternates with periods of leptin resistance, would probably also been seen in humans if we still lived as hunter-gatherers. The present Western World provides us with eternal summers where food is abundant both in the summer months and in the winter, making overconsumption easy. In contrast, attempts to cut back calories in order to lose weight is still perceived by the brain as a threat to survival, that leads to transitioning into an energy-saving mode, thus hindering weight loss. This evolutionary pressure to maximize energy stores at times in energy abundance has also been termed as the 'thrifty genotype' [46-48]. In short, limiting caloric intake in order to lose weight comes in direct conflict with the evolutionary pressure to store energy.

The current thesis provides a mechanistic explanation to the underlying neurobiology of increased motivation during negative energy balance. Low leptin levels due to caloric restriction, results in alterations in ARC NPY and POMC levels that possibly contribute to the heightened motivation to obtain food. Extrapolating these findings to the human dieting situation, it is safe to assume that the constant food stimuli in our surroundings and easy accessibility of food, combined with a higher biological drive to consume food, remains the biggest obstacle for weight loss. Hence, next to focusing at the individual level, the obesity problem can also be addressed by working at a macroscopic level in which individuals, companies, policy makers and the government, discuss and promote healthy eating habits. However, such macroscopic approaches to curb the obesity epidemic are gradual and take years to develop. Therefore, the investigating the underlying biology is crucial for discovering newer drug targets for reversing the physiological changes triggered by dieting. It is also important to note that our own studies (Appendix I) and studies from other labs [37] show that prolonged abstinence from a high fat diet reverses leptin resistance and adiposity in rodents, implying that dieting in humans could also be successful in reversing or counteracting metabolic effects of obesity, if given sufficient time and endurance. But as indicated before, despite the physiological parallels between rodent and human physiology, laboratory setups are entirely different from the real situations which dieters are subjected to, constant stimulation by food-associated cues, which makes dieting even more challenging.

The fact that relative leptin deficiency during dieting triggers a series of behavioral and physiological changes that resist weight loss, opens the possibility to use leptin replacement therapy in dieting individuals. This has been experimentally shown by Leibel and colleagues who demonstrated that leptin replacement in part reverses the physiological adaptations to decreased caloric load [49]. However, until now, such studies have only been conducted in small number of participants, so this approach awaits rigorous testing in the form of large clinical trials [50, 51]. On the other hand, replacement of leptin, as easy as it may seem, can also have its drawbacks. Within a couple of years of leptin discovery, its role in cardiovascular regulation also became evident [52-54]. Furthermore, recently it was shown that high circulating leptin levels during obesity underlies hypertension in obese subjects [55]. Thus, treating dieting individuals with leptin in order to maintain thermogenesis could counterproductively aggravate leptin's hypertensive effects. This is the result of the fact that leptin, via the sympathetic nervous system (SNS) (Chapters 7 and 8) regulates both the cardiovascular system and thermogenesis. Importantly, even within a single brain nucleus, such as the dorsomedial hypothalamus, leptin can affect both processes. Thus, future research should identify the neuronal populations that separately mediate the thermogenic and cardiovascular effects of leptin, if they exist. Thus, studies should be also directed in identifying leptin-sensitive neuronal projections from the hypothalamic nuclei to hindbrain

structures. Singling out these neuronal projections and confirming their phenotypical identities would provide better chances for drug development.

Nevertheless, we also know that the current therapy for hypertension includes blocking of adrenergic receptors on the heart and the blood vessels. Thus, treatment of dieting individuals with leptin to sustain thermogenesis, while co-administrating adrenergic antagonists to minimize leptin's cardiovascular effects, can also be considered in the human population. The fact that caloric restriction decreases core body temperature in humans has been reported by other groups [56], but this has yet to be replicated and verified. As indicated earlier, a recent review also indicates that the number of studies with leptin replacement in weight reducing obese subjects, is fairly limited [50]. Therefore, such studies could be considered in order to establish the validity of our data and data from other labs [35, 36, 56, 57]. Similarly, whether by eating smaller meal portions, dieting individuals can maintain a high basal metabolic rate and thus prevent the drop in leptin levels and thermogenesis, has not yet been systematically addressed and remains an inviting topic to investigate. Here, I would also like to stress that the current thesis focuses at an individual aspect of obesity and dieting, i.e. the biology behind it. However, their societal and organizational factors are also crucial when we speak about a healthy society. In the future, we should take a more cohesive approach where information from studies providing a biological explanation to a known phenomenon, such as in the current one, can be readily implemented into practice.

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Appendix I

Fig. 1: Leptin suppresses the motivation for palatable food. Increased motivation for sucrose following food restriction (FR+Saline) is reversed upon peripheral leptin treatment (i.p. 2 mg/Kg/ml; FR+leptin). *indicates significant difference from FR+saline condition, paired t-test. Dashed line indicates motivation in an ad libitum state (n=7). Details on operant testing can be found in Chapter 3 [1].

Fig 2: Leptin sensitivity following 8 weeks of withdrawal from a HFHS diet: Decrease in food intake after leptin injections as compared to saline injections. *indicates significantly different from saline condition, paired t-test. Dashed line indicates food intake following saline injection normalized to 100% (n=6-7).

Fig.3: Abdominal fat-pads following 8 weeks of withdrawal from a HFHS diet (n=7-8):
Briefly, intra-abdominal fat-pats were removed following sacrifice.

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Nederlandse Samenvatting

Obesitas is een groeiend probleem in de westerse samenleving. In Nederland komt overgewicht vaak voor, ruim boven de 40% bij mannen en 30% bij vrouwen. Hoewel obesitas niet als een ziekte wordt beschouwd, is het toch een belangrijke oorzaak voor verschillende aandoeningen zoals hart- en vaatziektes, suikerziekte en sommige vormen van kanker. Dus, de zorgwekkende groei van obesitas gaat gepaard met een toegenomen incidentie van de bovengenoemde ziektes. Binnen de EU werd de afgelopen decennia obesitas erkend als een groot gezondheidsrisico voor de samenleving. Dit leidde tot toekenning van verschillende beurzen om de fundamentele biologische processen van obesitas te kunnen onderzoeken. Alleen door onderzoek naar de onderliggende mechanismen van obesitas kunnen we betere behandelingsmethoden en geneesmiddelen ontwikkelen om deze epidemie te bestrijden. Dit is het uitgangspunt van dit proefschrift.

 proefschrift gaat over de rol van leptine bij obesitas. Leptine, een hormoon dat pas in 1994 werd ontdekt, wordt aangemaakt in vetcellen en wordt afgegeven aan de bloedbaan. De hoeveelheid leptine die in het bloed voorkomt, is afhankelijk van de vetmassa van een individu - naarmate de vetmassa toeneemt, stijgt de leptinespiegel. Vanuit het bloed wordt leptine vervoerd naar de hersenen, waar de voedselinname en eetlust worden geremd, maar het energieverbruik wordt gestimuleerd. In het brein werkt leptine via leptinereceptoren, die zich in verschillende hersengebieden (o.a. in de hypothalamus) bevinden. Onderzoekers hebben aangetoond dat er in het zogenaamde arcuate nucleus gebied in de hypothalamus twee belangrijke soorten neuronen zijn: de neuropeptide Y (NPY) en de POMC neuronen. NPY is een bekend eetluststimulerend peptide (eiwit), terwijl de effecten van de POMC afgeleide melanocortine peptiden eetlustremmend zijn. Verder is ook aangetoond dat leptine de neuronale activiteit van de NPY neuronen remt, terwijl die van de POMC neuronen juist wordt gestimuleerd. Op deze manier wordt voedselinname beïnvloed door leptine. Oftewel, bij proefdieren (ratten en muizen) nemen de NPY-niveaus toe en nemen de POMC-niveaus af als het proefdier geen voedsel krijgt. Dus, een daling van de leptinespiegels wordt door het brein waargenomen als een alarmsignaal, waardoor verschillende neuro-hormonale processen worden aangezet met het doel de energieopslag op een nuttige manier te gebruiken. Omdat vetopslag de enige manier is waarop we energie voor de toekomst kunnen opslaan, zal iedere bedreiging voor de vetmassa door de hersenen worden waargenomen als een bedreiging voor het leven. Hierdoor worden compenserende mechanismen in werking gezet om energie te kunnen besparen. Maar wat zijn deze compenserende mechanismen? Dat is het onderwerp van dit proefschrift.

De regulatie van lichaamsgewicht is een complex proces. Om het simpeler te kunnen maken, wordt in dit proefschrift lichaamsgewicht als een product van voedselinname en energieverbruik beschouwd. Voedselinname wordt verder ingedeeld in homeostatische inname, m.a.w. het voedsel dat we eten om te kunnen groeien en te kunnen voortplanten, en

hedonische inname (of overeten), m.a.w. het voedsel dat we wel eten maar dat we niet per se nodig hebben (het laatste stuk taart of dat zakje chips).

Het doel van dit proefschrift is de gedragsgerelateerde veranderingen zoals de fysiologische veranderingen die veroorzaakt worden door leptinetekort, in kaart te brengen. Het is ook interessant op te merken dat er bij overgewicht sprake is van meer vetmassa en daardoor ook hogere leptinespiegels. Dit zou moeten betekenen dat er bij overgewicht een sterkere remming van voedselinname is. Maar in werkelijkheid is dat niet het geval. Dit komt door een verschijnsel dat leptine-ongevoeligheid wordt genoemd. Ratten die langdurig blootgesteld werden aan een vet- en suikerrijk dieet, vertonen verschillende overeenkomsten met een dier dat hongerig is. Ondanks de hogere leptinespiegels (door de verhoogde vetmassa) is er sprake van hogere NPY en lagere POMC spiegels; net als bij dieren op voedselrestrictie. Dit komt door het feit dat deze neuronen in het arcuate nucleus gebied ongevoelig zijn geworden, waardoor ze de leptinespiegels niet kunnen registeren. Het is bekend dat voedselrestrictie de leptinespiegels doet dalen. Daarnaast verhoogt het de intrinsieke motivatie voor voedselbeloningen. In de natuur betekent dit: als er minder voedsel in de omgeving beschikbaar is, dan zijn de dieren extra gemotiveerd om nieuwe voedselbronnen te gaan zoeken. Of deze extra motivatie door de verhoogde NPY of gedaalde POMC niveaus komt, is nog onbekend. Dat is het onderwerp van het eerste onderdeel (Hoofdstukken 1 t/m 6).

In dit eerste onderdeel wordt de rol van leptine en de rechtstreekse doelwitten NPY en POMC peptiden op motivatie voor voedselbeloning onderzocht. Motivatie werd gemeten als de bereidwilligheid van een dier om op een pedaal te drukken die een voedselbeloning (suiker) levert, waarbij de rat na elke beloning vaker moet drukken om zijn volgende beloning te krijgen. Dus een hoger aantal pedaaldrukken wordt vertaald als een hogere motivatie. Hoofdstukken 1 t/m 3 zijn gepresenteerd als literatuuronderzoek. Hierin zijn de effecten van leptine en leptine-ongevoeligheid op motivatie en hedonische consumptie (Hoofdstuk 1) en de rol van POMC peptiden en NP Y op de motivatie voor voedselbeloningen beschreven (Hoofdstuk 2 en 3).

In hoofdstuk 4 worden de belonende effecten van NPY in drie verschillende hersengebieden onderzocht. Hiervoor werd NPY direct toegediend in drie verschillende hersengebieden: de nucleus accumbens (NAC), de ventral tegmental area (VTA) en de laterale hypothalamus (LH). Deze bovengenoemde hersengebieden vallen onder het zogenoemde 'limbisch systeem', dat te maken heeft met intrinsieke motivatie en natuurlijke beloningen zoals seks. Bij deze neuronale circuits speelt dopamine (een neurotransmitter die betrokken is bij verslaving en depressie) een belangrijke rol. We hebben aangetoond dat NPY toediening direct in de VTA, waar dopaminerge neuronen zitten, de motivatie voor suikerbeloning

verhoogt. Dit impliceert dat de leptinespiegeldaling die tijdens het vasten tot een verhoging van NPY leidt, tevens de motivatie voor voedselbeloningen verhoogt, waarschijnlijk via het VTA gebied.

In de twee daaropvolgende hoofstukken wordt de rol van POMC (melanocortine) peptiden op motivatie onderzocht. POMC peptiden werken via de MC3 en MC4 receptoren, die in verschillende hersengebieden voorkomen. Hier laten we zien dat de toediening van POMC peptiden direct in het brein tot verlaagde motivatie leidt (Hoofdstuk 5). Deze motivatieremmende effecten van de POMC peptiden vinden plaats via de MC4 receptoren in het NAC gebied. De stimulering van MC3 receptoren door POMC peptiden in het VTA gebied leidt daarentegen tot een motivatieverhoging (Hoofdstuk 6). Hiermee wordt aangetoond dat er niet alleen verschillen tussen de effecten van NPY en POMC peptiden op motivatie bestaan, maar dat de effecten van POMC peptiden zelf ook verschillend zijn, afhankelijk van de receptortypen.

Het tweede onderdeel van dit proefschrift gaat over de fysiologische effecten van leptinetekort. Hiervoor gebruiken wij een dierenmodel voor afvallen, of lichaamsgewichtverlies als gevolg van verminderde voedselinname. Ratten die naast hun gewone voedsel blootgesteld worden aan extra vet- en suiker, worden dik. Na een periode van vet- en suikerrijk dieet, worden het vet en de suiker van het dieet weggehaald om het proces van het afvallen na te bootsen. Hierbij mogen de dieren nog steeds zo veel van het gewone voedsel eten als ze willen. Ze aten minder calorieën dan op het vet en suiker dieet. Hierdoor werd niet alleen hun voedselinname verminderd, maar ook de lichaamstemperatuur. Daarnaast was er sprake van een lage leptinespiegel bij deze ratten. Omdat lichaamstemperatuur op peil wordt gehouden door energieverbruik, suggereerden deze resultaten dat de daling in leptinespiegels de hypothermie bij deze ratten veroorzaakt. Deze hypothese werd verder bevestigd door leptinetoediening aan deze dieren. Zoals verwacht, corrigeerde leptinetoediening de hypothermie bij deze dieren. Bovendien was het dorsomedial hypothalamus (DMH) gebied in het brein geactiveerd na de leptinetoediening. Het DMH gebied is een onderdeel van de hypothalamus dat een rol speelt bij de warmtehuishouding. Leptinetoediening direct in dit gebied bootste perifere effecten van leptine na. Dit betekent dat het herstel van leptinesignalering in de DMH genoeg is om de lichaamstemperatuur te handhaven in een dieetsituatie.

Verder tonen we in het tweede onderdeel aan dat de blokkering van leptinesignalering in de DMH tot een verlaagde lichaamstemperatuur en verhoogde vetmassa leidt. Dit onderstreept het belang van leptinesignalering in dit hersengebied om euthermie (of normale lichaamstemperatuur) te handhaven. Hieruit blijkt dat het wegvallen van leptinesignalering in dit gebied tijdens het lijnen tot hypothermie kan leiden. Kortom, uit deze data kunnen we

afleiden dat de verlaagde lichaamstemperatuur (in ons rattenmodel) tijdens het afvallen een belangrijke manier is waarmee het lichaam energieverbruik (voor het opwarmen van het lichaam) beperkt. Dit is heel normaal in de natuur, vooral bij seizoensgebonden dieren (zoals bij winterslaap). Indien er een tekort aan voedsel is, moeten de dieren hun energieverbruik beperken om te overleven. Deze situatie is dus vergelijkbaar met het lijnen in onze maatschappij, waar we minder eten om te kunnen afvallen. Maar de negatieve terugkoppeling van leptine die bedoeld is om perioden met voedseltekort te overleven, is juist een drempel tegen afvallen.

Samenvattend kan dit proefschrift beschouwd worden als een kleine poging om de rol van leptine (en leptinetekort) in kaart te brengen. De resultaten tonen aan dat het wegvallen van leptinesignalering (door minder voedselinname, m.a.w. lijnen, of vanwege leptineongevoeligheid bij obesitas) tot verhoogde motivatie en verlaagde lichaamstemperatuur leidt. Verschillende hersengebieden spelen hierbij een rol . Hoewel de experimenten bij ratten zijn uitgevoerd, kunnen deze resultaten geëxtrapoleerd worden naar mensen, zij het in een beperkte mate. Deze resultaten dienen echter als goede basis voor translationeel onderzoek. In feite is dat de bedoeling van de uitgevoerde experimenten: om een klein stapje in de richting van verder onderzoek en behandeling van obesitas te kunnen zetten.

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Sweety darling (**Marjolein**), remember the party where we met at Koekoekstraat? It feels like yesterday, right? Even though time has just flown by. From then till now we have experienced life and grown, both as individuals and as friends. Grown, as in 'no' to Saturday-night rooster making craze and to snoring 'super toffe buurman" at camping sites. And 'yes'

to following our hearts and going to 'after-parties'. As Bridget would say and we would agree, 'It is a truth universally acknowledged that when one part of your life starts going okay, another falls spectacularly into pieces'. Despite the odd relatedness of the statement, I am so grateful for your positive insights on life. #live

My dear kitty kat, **Angelica**, it's like a meant to be story: a fateful Dutch class, us sitting next to each other and us becoming friends. It reminds me of the song from First Aid Kit, 'We are gonna be friends'. I am so grateful to have you in my life. Whether it's about going to some obscure concert with our Subba card, panting for breath in the half moon pose at a Bikram class (gulp), discussing serious AG issues or forging for pancakes at a very wet theater festival, we have seen and done a lot. Even if we are miles apart at the moment, I always feel connected to you. I also think that we are both in the right direction in life. That been said, no more Zara totes ;-)

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Iliana, your cheerfulness and zest for life makes me believe that things will be ok. ;-) **Maria**, you are a symbol of how to combine dedication with fun. I still need the recipe for that.

Paris, remember us running to catch the night train back? Yes, it did happen. And no, it's no Jamais vu. So did the South Park style mediation course happen? Bright city lights, heart-shaped glasses, suitcases and the eternally haunting question 'do you see yourself…?'. It's what I call great times! Shoshanna would agree.

Yultje, be it hiccups at a party in the wee hours of the morning or the house concert with strangers or the song dedicated by a stranger, there is so much randomness involved. But, with you, it all has one single keyword: Amazing! Here´s a purr for our friendship.

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Curriculum Vitae

Rahul Pandit was born on 15th December, 1983 in Cuttack, India. He completed his high school (2000) and pre-university studies (2002) at St. Paul's Boarding and Day school and New Alipore Collge, Kolkata, India respectively. He obtained his MD Physician (Cum Laude) title from St. Petersburg State Medical Academy named after I.I.Mechnikov (2008), St. Petersburg, Russia. Following this, he obtained his Masters degree (Cum Laude) in Neuroscience and Cognition from Utrecht University (2010). The years following thereafter were focused on his PhD project on the role of leptin in body weight regulation at the department of Translational Neuroscience, University medical center, Utrecht. At the moment he is working as a junior university lecturer (pharmacology) in the Department of Translational Neuroscience.

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List of publications

1. Deficient leptin action in the dorsomedial hypothalamus underlies dieting-induced hypothermia.

R Pandit, A Omrani, M Matheny, MCM Luijendijk, S Hoek, L Eggels , V de Vrind, K Neijs, AJ Van Rozen, PJ Scarpace, SE la Fleur , RAH Adan. (submitted).

2. Melanocortin 3 receptor signaling in midbrain dopaminergic neurons increases motivation for food reward.

R Pandit, A Omrani, MCM Luijendijk, VA de Vrind, AJ Van Rozen, RJA Oude Ophuis, LJMJ Vanderschuren,SE la Fleur, RAH Adan. (manuscript in preparation).

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