# GETTING READY FOR DINNER:

## the role of ghrelin in food anticipatory activity

## COLOFON

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# GETTING READY FOR DINNER:

## the role of ghrelin in food anticipatory activity

## Klaar om te eten:

*de rol van ghrelin in voedsel gerelateerd anticipatie gedrag*

(met een samenvatting in het Nederlands)

## Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op

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## Myrte Merkestein

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# Chapter 1

**GENERAL INTRODUCTION**

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## CONTENTS

## Relevance for studying food anticipatory activity

Weight gain has increased tremendously over the past decades. In many Western societies, a majority of the adult population is considered overweight. A person is classified as overweight if the body mass index (BMI) is between 25.0 and 29.9 kg/m$^2$, a person with a BMI > 30 kg/m$^2$ is defined obese. The increased prevalence of obesity raises many important health issues, since it is associated with an increased risk of diabetes mellitus, cardiovascular diseases, and several cancers.[1]

Obesity occurs when energy intake exceeds energy expenditure over a period of time and excess energy is stored as fat. It is considered as a multifactorial disease to which both heritability and environmental factors contribute. The estimated heritability of BMI is between 50 and 90%.[2,3] Genome-wide association studies have identified multiple genetic loci implicated in obesity susceptibility.[4] However, the rapid rise in the prevalence of obesity cannot be attributed solely to genetic factors, since our genes have not changed considerably in this short time period. On the other hand, our environment has changed significantly. Nowadays, food is available abundantly, and physical activity levels in the population have dropped. Hence, the interaction of genetic predisposition with exposure to this obesogenic environment is likely to contribute to the onset of obesity.

In an environment with an overload of cheap palatable and energy dense food, the decision to eat or not to eat is only to a limited extent determined by hunger and satiety. The sight or smell of food triggers neural circuits that urge us to eat in the absence of hunger. On the other hand, staying lean is considered healthy and attractive. Thus, when confronted with palatable food one has to decide to take an immediate short-term reward by consuming the palatable food, or to suppress this and go for the delayed reward of staying lean. This important decision is made in the brain when we are confronted with food.

Not only obese people are faced with this dilemma, it is a crucial problem for patients suffering from eating disorders as Bulimia Nervosa, binge eating disorder and Anorexia Nervosa (AN) as well. Restricting-type Anorexia Nervosa (AN) patients are the extremes in refusing food in order to be lean. AN is characterized by several criteria as described in the DSM IV (American Psychiatric Association: Diagnostic and Statistical Manual of Mental disorders, fourth edition

(2000)); 1) refusal to maintain a normal body weight for age and height (body mass index (BMI) < 17.5 kg/m$^2$), 2) intense fear of gaining weight or becoming fat, 3) disturbances in body perception, 4) amenorrhea in women. AN occurs predominantly in females and has a strong genetic origin. The average prevalence of AN is around 1% in teenagers[5] and a high mortality rate (> 10%) has been reported[6,7]. Although not mentioned in the DSM IV criteria for AN, hyperactivity is often considered as a symptom of the disorder[8-12].

With regard to eating disorders and obesity, an important question is: What happens in the brain when we are triggered to think about food? This chapter focuses on a behaviour named food anticipatory activity (FAA) and its underlying mechanisms. FAA is expressed when a rodent has time-restricted access to food, i.e. restricted feeding schedule (RFS) or a palatable treat, i.e. palatable feeding schedule (PFS), and involves hyperactivity preceding meal-time.

A better understanding of the processes underlying FAA is clinically relevant to eating disorders as AN and obesity in several ways. First, the hyperactivity observed in these models reflects hyperactivity in AN patients, and might share common regulatory mechanisms. Furthermore, many studies have shown that metabolism and circadian rhythms are tightly coupled. Meal timing plays a pivotal role in integrating behavioural and physiological rhythms, and deficiencies in FAA could diminish this circadian organization and in this way hamper metabolic function. Conditioned cues can elicit feeding in sated rats[13] and humans[14]. Hence, entrainment to a daily treat in PFS models could lead to increased vulnerability to overconsume palatable food in a specific time window or in a specific context.

## Rat models of food anticipatory activity

### Restricted feeding schedule

Rats on a RFS have daily limited access to food at the same time point of their circadian phase. Due to this intervention, circadian rhythms alter and FAA occurs.[15-17] At first, rats on RFS will not be able to consume their baseline food intake during the two hours of food access. However, as the model continues, they gradually increase their food intake and after two weeks, food intake does not differ from baseline food intake anymore.[18] At the beginning of a RFS, rats

will loose weight, due to reduced food intake. Weight loss stabilizes at a certain moment[18], but it depends to a large extent on the amount of locomotor activity how much weight a rat will loose. When RFS rats have access to running wheels, hyperactivity will cause a rapid decline in body weight. On the other hand, when rats are housed in standard cages, body weight loss will not be that severe, and rats have time to adjust to the RFS. In response to a RFS, rats will alter their rhythms of locomotor activity and body temperature[18-20]. Anticipatory peaks of these parameters arise, and values of locomotor activity and body temperature reduce in the dark phase.[20] In general, FAA can be observed within 3 days of onset of RFS. Subsequently, the amount of FAA increases in amplitude and duration in the course of RFS.[15] Although FAA usually extincts when rats are provided with *ad libitum* food access, FAA re-establishes when rats are food deprived.[15,21]

## Palatable feeding schedule

The development of anticipatory locomotor activity is not limited to animal models of food deprivation. *Ad libitum* chow-fed rats were shown to anticipate a daily palatable treat in 1987.[22] Rats anticipating a small palatable treat will not show an anticipatory increase in body temperature. However, due to the relatively large meal ingested in the middle of the light period, they will show postprandial hyperthermia, potentially diet-induced thermogenesis. In response to the palatable feeding schedule, rats will not shift their circadian rhythm of locomotor activity, unlike rats on RFS. However, a small anticipatory peak in locomotor activity can be observed in the hour preceding availability of the palatable treat.[17,21,23,24]

The ability of a palatable meal to evoke FAA is dependent on several parameters. First, the palatable meal needs to have some nutritive value, since a palatable mash without caloric content did not induce anticipatory wheel running.[22] Studies in rats indicate that carbohydrates, but not fat, have properties to induce a phase shift in the circadian food-entrained clock.[25] Remarkably, FAA to a palatable treat is in mice only observed in the males, and only when given a high fat treat, and not a chocolate treat.[26] In rats, chocolate[21,23,24], a palatable mash consisting of chow, vegetable oil, chocolate syrup and icing sugar[22], chocolate Ensure[27], and sucrose[28] have been used to evoke palatable meal induced FAA. Second, palatable meal size has to be reasonably large. The anticipated palata-

ble meal is suggested to have to exceed a certain caloric threshold to be able to induce FAA, either in absolute values or relative to the total caloric intake.[29] A 32 % sucrose solution resulted in FAA in 85% food-deprived animals, but not in *ad libitum* chow fed rats.[28] A 4g palatable meal was able to induce FAA in only a minority of rats, whereas the majority of rats with a 2 hour-window access to the palatable food, of which they consumed on average 9 g, exhibited FAA.[22] This study used running wheel activity as a read-out parameter for FAA. Another study using this read-out parameter showed as well that only 37% of the rats anticipating a palatable treat exhibited FAA. However, studies that assessed FAA with general locomotor activity measurements, e.g. infrared motion sensors, did show that only 5 gr of chocolate is able to evoke FAA in rats.[21,23,24] Whereas FAA in rats on RFS model starts 2-3 hours prior to meal-time, palatable meal entrained rats showed a brief increase in anticipatory locomotor activity 15-30 minutes before access to the palatable snack.[21,24] Interestingly, once the palatable feeding schedule was discontinued and rats had *ad libitum* access to regular chow, FAA was still observed around palatable meal time for several days.[21]

Thus, FAA consists of hyperactivity preceding meal time and can be evoked by timed access to a food source in restricted and non-restricted rats. A RFS schedule, which involves restricted access to normal chow, probably reflects hunger and motivational aspects of anticipation. On the other hand, a PFS schedule, with limited access to a palatable meal on top of *ad libitum* access to chow, likely mainly represents the motivational component of FAA. More insight into the appetitive phase of food intake, in these models observed as FAA, is of great importance to understand potential causes of disturbances in food intake, e.g. obesity and anorexia nervosa. Therefore, it is imperative to examine the underlying mechanisms of FAA.

## Underlying mechanisms of food anticipatory activity

Under normal condition, daily circadian rhythms in all kinds of behaviours, such as locomotor activity, are entrained by the master clock of the brain; the suprachiasmatic nucleus (SCN). This hypothalamic area receives direct input from the retina and synchronizes other brain areas and peripheral tissues to the light:dark cycle via neuroendocrine and autonomic output pathways.[30-33] Rats are nocturnal animals, and their feeding behaviour is also coupled to the SCN-controlled

circadian rhythm and occurs, hence, mostly in the dark phase. Not only is food intake controlled by the circadian system, food intake can in return affect circadian rhythms. When access to food is restricted to a few hours in the light period, i.e. the normal resting phase for nocturnal animals, the circadian rhythm of behaviour is disengaged from the central clock and cycles in relation to feeding time. Animals will develop hyperactivity preceding feeding time, hence FAA, at a moment of day when they would normally not be active.[15-17] This FAA is reflected in several features, including general hyperactivity, exploratory behaviours, increased instrumental behaviours to obtain food, and food-bin directed behaviours. Moreover, the circadian rhythm of body temperature alters, as well as the rhythms of metabolic parameters, such as glucose, hormones and free fatty acids.[34,35] Numerous studies aimed to identify the food entrainable oscillator (FEO); the brain structure or signaling pathway that drives FAA. Both peripheral as well as central pathways have been examined as potential FEOs.

## Involvement of peripheral regulation of food anticipatory activity

The peripheral digestive system and the brain could communicate with each other to regulate FAA, as food intake might be a stimulus for this behaviour. However, transsection of the vagus nerve, which innervates the peripheral organs and sends sensory input to the brain about the state of the organs, does not prevent the corticosterone shift observed during FAA.[36] Additionally, subdiaphragmatic transsection of this nerve does not impact FAA measured by running wheel activity in SCN-lesioned rats.[37] Moreover, disruption of nonvagal visceral input to the brain by intraperitoneal capsaicin injections does not hamper the development of FAA either.[38] Altogether, neural communication is not likely to be the essential route of communication between the gut and the brain for the regulation of FAA. This implies that humoral signaling could play a role in the development of FAA. However, adrenalectomy, which prevents the secretion of corticosterone, does not attenuate FAA.[39] In addition, diabetic rats with destroyed insulin-producing cells[40] and rats with a mutated leptin receptor[41] still exhibit FAA. Hence, to date, the pathway via which the brain and the periphery communicate to regulate FAA remains to be elucidated. A potential candidate is ghrelin signaling, since ghrelin levels rise prior to meal-time[42-44] and ghrelin receptor knock out mice show attenuated FAA[42,45-47]. The role of ghrelin in FAA will be discussed in more detail in a later paragraph.

## Involvement of neural circuits in food anticipatory activity

The central regulation of food intake occurs at multiple levels in the brain. In general, the hindbrain has been suggested to mediate satiety, the hypothalamus is thought to serve as a homeostatic regulator, and corticolimbic areas have been implicated in reward-related regulation of food intake. These three levels have also been investigated with regard to the regulation of FAA.

### *Hindbrain*

Peripheral feeding-related input arrives at the brain at various locations. The gastrointestinal system sends information to the nucleus of the tractus solitarius (NTS) in the hindbrain. A blood brain barrier is absent at the nearby area postrema (AP), which enables AP to detect humoral signals. Both NTS and AP project to the parabrachial nucleus (PBN). Electrolytic and neurotoxin-induced lesions of the latter structure attenuated FAA as measured by food bin approach behaviour.[48] However, lesions of the AP[49] or NTS[50], the main inputs to the PBN, did not affect FAA. Additionally, none of these brain areas became Fos positive during FAA, but exhibited Fos activation after consumption of the anticipated meal.[51] Hence, of the investigated hindbrain areas, the PBN is the most prominent candidate to play a role in FAA. How-ever, which role the PBN has in the regulation of FAA remains to be elucidated.

### *Hypothalamus*

The hypothalamus has been implicated in the homeostatic regulation of energy balance and autonomic behaviours, and is hence a logical candidate for mediating FAA in rats on a RFS. It has been demonstrated that dorsomedial hypothalamus (DMH), lateral hypothalamus (LH), tuberomammilary nucleus (TMN), and perifornical area (PeF) showed increased Fos expression during FAA[20,52-56], and that Per1 rhythms shifted or changed in DMH, arcuate nucleus (Arc), PeF, paraventricular nucleus (PVN), ventromedial hypothalamus (VMH)[21,57-59] and Per2 rhythms shifted or changed in DMH, PVN, and VMH in rodents on RFS[27,57-59]. On the other hand, rats subjected to a palatable feeding schedule (PFS) with restricted access to palatable food in addition to *ad libitum* access to chow did not show any hypothalamic increase in Fos at the moment of FAA[23], and Per2 rhythms did not change in DMH[27]. Per1 rhythms have been shown to change in this paradigm in SCN, DMH and PeF.[21,24]

The SCN, which contains the light entrainable oscillator, is located within the hypothalamus. Already in 1979, Stephan and colleagues demonstrated that this brain area is not the food entrainable oscillator, as SCN lesions did not impair FAA.[39] Lesions of the PVN and LH did not attenuate FAA either.[60] Although PVN lesions decreased FAA in general locomotor activity measurements, anticipatory food bin approaches were still intact.[60] At first, ablations of the ventromedial hypothalamus (VMH) seemed to attenuate or even abolish FAA.[61,62] However, later studies revealed that this effect was only transient and that FAA recovered eventually.[63,64] Additionally, only a weak correlation between the size of VMH lesion and the reduction in FAA has been reported.[20] The arcuate nucleus (Arc) lacks a blood brain barrier, just as the AP. Lesions of the Arc induced by neonatal monosodium glutamate did not decrease, but rather increased FAA.[65]

A vivid discussion takes place in literature on the role of DMH in FAA.[20,66-70] Electrolytic lesions of this brain structure did not impair FAA as detected by general locomotor activity and food-bin directed behaviour.[67] In contrast, neurotoxin-induced lesions of the DMH, which spare passing fibres, were reported to attenuate FAA as measured by wakefulness, body temperature and general locomotor activity[20], which was also observed in mice with a large mediobasal hypothalamic lesion which included DMH[71]. Differences between these studies include parameters assessed, lesion type, and cage configuration (e.g. use of dark pipes in cage). Replication of all parameters from the Gooley study[20], apart from type of lesion, by Landry and colleagues[66] still revealed no impact of DMH lesion on FAA.[66] In two studies, FAA persisted after 48 hours of food deprivation in DMH-lesioned rodents, indicating that the food-entrainable rhythm is not impaired.[59,66] The discussion focuses on the correct way of assessing FAA and the best approach to lesion a brain area[68-70] and has yet to be resolved. Interestingly, a recent paper showed that DMH ablation diminished FAA. However, when in addition the SCN was lesioned, FAA returned. This suggests that the DMH has a role in silencing output from the SCN to permit FAA.[55] In line with this modulatory role of DMH in FAA, it was shown that DMH lesions might attenuate FAA to a daytime meal, but when food was provided at nighttime, FAA was intact.[72]
Orexin neurons are predominantly located in the LH, and orexin is known for its orexigenic and arousal-stimulating properties. Orexin neurons were activated during FAA in rats expecting a chow meal and those that anticipated a palatable meal.[73-76] Orexin knock out mice still exhibited FAA, although reduced or

with a delayed acquisition.[73,76-79] Interestingly, the effect of the lack of orexin signaling on FAA seems to be dependent on the circadian phase, since anticipation to RFS in the light period was impaired to a larger extent than anticipation to RFS in the dark phase.[73] However, specific ablation of orexin neurons in LH did not impair FAA.[80]

Altogether, the hypothalamus is activated during FAA, and is likely part of the network that regulates this behaviour. Although lesion studies have conflicting results, the VMH, DMH and orexin neurons within the LH could be important nodes in this network. The SCN, which is entrained by the light-dark cycle, probably needs to be inhibited by the DMH to allow FAA during the light phase.

*Corticolimbic areas*

Food intake is not just a fact of balancing energy intake with energy expenditure, motivational and rewarding aspects of food influence food intake as well. Dopamine is known for its involvement in the regulation of reward and motivation via the mesolimbic dopamine system.[81] This system originates in the dopaminergic neurons of the VTA, which project to the nucleus accumbens (NAc), which in turn projects to many other limbic areas. Ingestion of palatable food results in augmented dopamine release in the NAc[82,83], which attenuates at that time point following habituation and transfers subsequently to the food-signaling cue[84-87], hence in anticipation of the predicted reward. Dopamine is a potential candidate for the regulation of hyperactivity and FAA in RFS and PFS models, since local injections of dopamine in the NAc induced locomotor activity.[88] Moreover, disruption of dopaminergic neurotransmission in the NAc prevented nicotine and amphetamine induced increases in locomotor activity.[89] In addition, AN patients and people suffering from obesity show alterations in reward signaling, especially related to dopamine.[90-94] Obese individuals exhibit increased activation of brain areas involved in motivation and reward in response to pictures of high-caloric food.[94] In addition, decreases in dopamine release and striatal dopamine receptor 2 (D2R) density have been reported in obese individuals and rodents.[90-93]

Dopamine depletion reduced hyperactivity in restricted rats with periodic food access.[95] This is in line with the finding that dopamine antagonism reduced hyperactivity in the activity-based anorexia (ABA) model (a variant on RFS), although levels of FAA remained stable.[96] In addition, in ABA rats, dopamine re-

lease in the nucleus accumbens increased during food intake, but not during FAA.[97] This is in contrast to previously mentioned observations, that dopamine is initially released upon food intake, but transfers to the food-signaling cue, hence during anticipation. However, in these studies[96,97], the ABA-model lasted for 3-4 days, which might too short for this transfer to have taken place. Others showed as well, that dopamine antagonism did not affect FAA in restricted rats anticipating normal chow.[98] However, FAA was reduced by dopamine antagonism in rats anticipating a palatable meal[98-100].

In line with this, corticolimbic areas involved in the reward-related effects of food intake have been shown to play a more important role in FAA in rats anticipating a palatable treat. During FAA in PFS rats, Fos activation was increased in several corticolimbic areas, including the paraventricular nucleus of the thalamus (PVT), central amygdala (CeA), nucleus accumbens (NAc) core, NAc shell and prefrontal cortex (PFC).[23,24] In rats on RFS, Fos levels increased as well in these brain areas, although to a lesser extent.[23,101,102] In addition, circadian rhythmicity of these brain areas changed during FAA in rats on PFS as well as on RFS, as observed by altered Per1 rhythms.[21,24,101]

Although these brain areas are activated during FAA, lesion studies did not implicate any corticolimbic area in the development of FAA. PFC has been suggested to organize adaptive autonomic alterations to an expected situation.[103] Although lesions of the infralimbic cortex, which is part of PFC, prevented anticipatory and postprandial rises in body temperature, FAA remained unaffected.[104] The PVT receives projections from the brainstem and hypothalamus and projects to corticolimbic and hypothalamic areas that play a role in the regulation of reward and arousal.[105,106] Lesioning of the PVT resulted in decreased FAA as measured by general locomotor activity[107]. However, when examining food bin-directed behaviour, rats with PVT ablations still showed robust FAA.[108] Large lesions covering the hippocampus, involved in learning and memory, and a large part of the amygdala, implicated in emotional memory, did not prevent the development of FAA either.[109] Discrepancy exists in the effects of NAc ablations on FAA. One study determined FAA as food-bin directed activity and reported no reduction in FAA[109], whereas another study looked at general locomotor behaviour and observed a reduction in FAA in NAc core-lesioned rats, but not in NAc shell lesioned rats[102].

In summary, dopamine is suggested to play a role in the hyperactivity observed in ABA and RFS models. Although its role in FAA is less clear, data indicate that dopamine signaling could be important for FAA, particularly in anticipation of a palatable food source. Dopamine signaling might also be implicated in the regulation of FAA by the orexigenic hormone ghrelin.

## Ghrelin

The orexigenic hormone ghrelin was discovered in 1999.[110] Preproghrelin mRNA is mainly expressed in X/A-cells in the oxyntic mucosa of the gastric fundus[111], but lesser amounts are also produced elsewhere in the gastro-intestinal tract and in other peripheral organs[112]. Preproghrelin is processed by cleavage of the signal peptide, which results in proghrelin and obestatin.[113] Proghrelin is further cleaved into desacylated ghrelin by the endoprotease prohormone convertase 13 in (rat) stomach.[114] In order to bind to its receptor, growth hormone secretagogue receptor 1a (GHS-R1a), ghrelin needs to be acylated.[110] However, only a minority of circulating ghrelin is acylated.[115-117] Active, acylated ghrelin consists of 28 amino acids, of which the serine-3 residue is n-octanoylated[110], a reaction catalyzed by ghrelin-O-acyl transferase (GOAT).[118-120]

Two different variants of GHS-R have been found, GHS-R1a and GHS-R1b, of which GHS-R1a is the fully functional $G_{aq}$-protein coupled receptor that binds acylated ghrelin. GHS-R1a is abundantly expressed in the hypothalamus and brainstem areas linked to energy balance, but also in other areas involved in reward (e.g. the ventral tegmental area, VTA) and emotion/cognition (e.g. hippocampus).[112,121,122] The extent to which activation of this receptor is dependent on the afferent gut-brain signal provided by ghrelin remains to be determined as this receptor appears to have constitutive activity[123,124] and potentially heterodimerizes with other receptors such as the dopamine receptor 1[125].

The first described effect of ghrelin is its capacity to induce GH release in a dose-related way.[126] Other effects of ghrelin include improvement of gastric motility and regulation of glucose and lipid metabolism.[127-129] However, we will focus ghrelin's orexigenic effect and its implication in FAA.

## Regulation of food intake and motivation for food

In humans and rodents plasma levels of ghrelin rise preprandially and fall postprandially, suggesting a role for ghrelin in meal initiation or meal anticipation.[43,44,130-135] Acute injections of ghrelin stimulate food intake in rats[136,137] and in humans[138,139]. In addition, chronic administration of ghrelin leads to increased body weight gain[136,140,141] and adiposity[140,142] in rodents. Both the feeding and adipogenic effects appear to be dependent on GHS-R1a as evidenced from studies using ghrelin antagonists.[143] Mice with knockout of ghrelin or its receptor have normal food intake when fed chow, and reductions as well as no alterations in body weight have been reported.[144-149] When fed a high-fat diet, GHS-R1a null mice decreased food intake and exhibited protection from diet-induced obesity.[148,149] Furthermore, acute and chronic administration of ghrelin have been reported to affect body temperature[150-152], and to increase respiratory quotient[140,153], indicating a metabolic switch from the utilization of fat to carbohydrates, while energy expenditure was not affected[136,140]. Consistent with this, ghrelin -/- mice decrease respiratory quotient when exposed to a high fat diet.[148]

The hypothalamus, a key area for the homeostatic regulation of appetite and food intake, is a well-established target for ghrelin, reflected not only by the abundance of GHS-R1a in discrete cell groups[121,122], but also by the clear Fos response to ghrelin injection in the arcuate nucleus and other hypothalamic areas[136,151,154-158], reflecting neuronal activation[159]. However, mesolimbic areas involved in reward may be especially important for ghrelin's effects on food intake by stimulating goal-directed behaviour for food.

Within the hypothalamus, one major target neuronal population for ghrelin is the orexigenic NPY neurones, that co-express another orexigenic peptide, agouti-related protein (AgRP). These neurons are activated by ghrelin as shown by stimulation of Fos expression[158,160,161], by electrophysiological studies[162,163], and by increased NPY and AgRP mRNA expression[136,141,164,165]. Ghrelin's feeding effects appear to require normal NPY/AgRP signalling as they are abolished in mice with deletions of both NPY and AgRP[164], in mice with ablations of AgRP neurons[166] and in rodents treated with NPY receptor antagonists[136]. Downstream targets include the anorexigenic pro-opiomelanocortin (POMC) system that becomes inhibited as ghrelin increases GABAergic signalling, from NPY/

AgRP to POMC neurons.[162] Ghrelin leads to an increase in the number of excitatory synapses on NPY/AgRP neuron [136] and in the number of inhibitory synapses on anorexigenic POMC neurons [163]. Consistent with this, ghrelin-induced food intake can be blocked by α-melanocyte-stimulating hormone (α-MSH, a product of the POMC gene).[167,168] Moreover, mice with deletions of melanocortin 3 receptor and melanocortin 4 receptor, the target brain receptors for α-MSH, do not show an orexigenic response to ghrelin.[164]

Ghrelin exerts its effects on food intake and food-oriented behaviours through multiple brain mechanisms and not only, as initially believed, through hypothalamic energy balance circuits (for a review of this emerging field see[169]). GHS-R1a is expressed in several nodes of the mesolimbic system, including VTA and NAc.[121,122,170,171] Ghrelin administration directly in either the VTA or NAc was found to have an orexigenic effect.[170,172] Furthermore, ghrelin specifically enhances the intake of palatable food[173], which is dependent on VTA ghrelin signalling.[174] Multiple studies revealed that ghrelin can increase the rewarding value of palatable food as measured by classic tests of reward (conditioned place preference)[174,175] and food motivation (progressive ratio)[175-178]. These effects are at least partly mediated by the VTA, while the NAc does not seem to play a role in ghrelin's effects on motivated behaviour.[176]

Dopamine is the main neurotransmitter involved in reward-related behaviours. More than 50% of dopaminergic VTA neurons co-express GHS-R1a. In addition, GABAergic VTA neurons that regulate activity of the dopaminergic neurons also express GHS-R1a.[170] Thus ghrelin might augment afferent reward signals via increased dopaminergic transmission from the VTA to the NAc.[171] In line with this idea, ghrelin administration resulted in increased dopaminergic levels in the NAc[170,179-181], which required GHS-R1a in the VTA. In addition to ghrelin's effect on dopaminergic neurons in the VTA, ghrelin may be able to modulate dopaminergic transmission at synaptic terminals both presynaptically[163] and postsynaptically via heterodimerization of GHS-R1a with dopamine receptor 1[125], although this needs to be confirmed in vivo. Interestingly, ghrelin can also amplify the effects of other reinforcing behaviours, such as drug- and alcohol-induced behaviours.[182-189]

Ghrelin interacts with other neuropeptides implicated in food intake and reward as well. Orexin acts downstream of ghrelin's effect on food intake. Ghre-

lin activates specifically orexin neurons in the LH.[151,190] Furthermore, blocking orexin signalling attenuated ghrelin-induced feeding[190] and motivation for a palatable reward[175]. In addition, ghrelin is likely to interact with the endocannabinoid system. Endocannabinoids have been reported to stimulate food intake, to increase the motivation to obtain food and to modulate dopaminergic signalling.[191,192] Interestingly, a cannabinoid receptor 1 antagonist blocked ghrelin-induced food intake and mice which lack this receptor showed an attenuated food response to ghrelin.[193,194]

### Ghrelin and food anticipatory activity

Based on ghrelin's effect on (palatable) food intake, motivation for food, and its capacity to amplify the effects of other reinforcing behaviours, ghrelin has been suggested to play a role in FAA. Indeed, ghrelin plasma levels showed entrainment to habitual meal patterns in humans and rats[43,44,131]. Plasma ghrelin levels were increased in ABA and RFS rats and correlated with FAA.[42,44,195] Central administration of ghrelin increased FAA in RFS rats[46], whereas a GHS-R1a antagonist reduced anticipatory locomotor activity in ABA rats[42]. Moreover, GHS-R1a -/- mice showed attenuated FAA in RFS and ABA models, without an effect on general locomotor activity.[42,45-47] In contrary, ghrelin -/- mice exhibited normal levels of FAA[78,196], which could suggest that ghrelin signaling does not play a pivotal role in FAA and that a still unknown ligand of GHS-R1a is able to modulate FAA via this receptor.

Altogether, ghrelin plays an important role in the homeostatic regulation of energy balance by modifying food intake and altering the incentive value of food. In addition, ghrelin signalling might be involved in the regulation of FAA.

## Scope and outline of this thesis

Deciphering the underlying mechanisms of FAA might shed light on the processes that regulate our drive to eat, which is crucial to understand how eating disorders, such as Anorexia Nervosa and obesity, develop. Despite many lesion studies[50], the neuronal substrate of FAA has not been found yet, favoring the existence of a distributed network that controls this behaviour, rather than a single brain area. In addition, how the gastro-intestinal system communicated

with our brain to elicit FAA, is to date still unknown. The orexigenic hormone ghrelin[197] has been implicated in the regulation of FAA, as its plasma levels peak preprandially[43,44], it stimulates FAA[46], and knockdown of its receptor GHS-R1a attenuates FAA[42,45-47]. Therefore, the aim of this thesis is to examine the underlying neuronal and molecular substrates that are involved in two animal models of FAA, i.e. RFS and PFS, and more specifically, the role of ghrelin signalling in FAA will be investigated.

In **chapter 2**, neuronal, hormonal and physiological changes in response to RFS, PFS and a random RFS are examined. The latter group is included as it suffers from a negative energy balance, just as RFS, but cannot anticipate a daily meal. Hence, it serves as an extra control group. The physiological changes in food intake, body weight gain, locomotor activity and body temperature are described. Using the neuronal activity marker Fos, brain areas that are activated during FAA are investigated. In addition, plasma levels of the anorexigenic hormone leptin and orexigenic hormone ghrelin are measured.

As ghrelin signaling mediates FAA in rodents subjected to a RFS and as ghrelin is known to increase the intake and motivational value of palatable food[171,174,175], the involvement of ghrelin in anticipation to a PFS was investigated. In **chapter 3**, the effects of central injections of ghrelin and an antagonist of its receptor, GHS-R1a, on FAA are described.

Fos studies have shown activation of the hypothalamus during FAA in rats on RFS. We were interested whether Fos activation of the DMH/VMH area during FAA is also reflected in increased firing frequency, and whether neurons activated during FAA are responsive to either leptin or ghrelin. Hence, an *in vivo* electrophysiology study was conducted in awake, behaving rats. **Chapter 4** describes the responses of these neurons during a cue that signals food availability. In addition, the changes in firing frequency upon administration of leptin and ghrelin were recorded.

As ghrelin and the hypothalamus have both been suggested to mediate FAA. We sought to examine the role of hypothalamic GHS-R1a in FAA. AAV containing shRNA directed against GHS-R1a were constructed and injected in the VMH and DMH. The responses of rats during *ad libitum* feeding and during RFS are described in **chapter 5.**

The main findings and implication of the experiments reported in chapters 2-5 will be summarized and discussed in **chapter 6.**

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# Chapter 2

## PHYSIOLOGICAL AND NEURONAL CHANGES IN RAT MODELS OF FOOD ANTICIPATORY ACTIVITY

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## ABSTRACT

With the increasing prevalence of obesity, the need to investigate the regulation of appetitive behaviour increases. Appetitive behaviour is reflected as increased activity and arousal preceding meal-time in rodent models of food anticipatory activity (FAA). Rats with timed restricted access to chow or limited access to palatable food with chow available *ad libitum* display FAA. This study aimed to identify changes in locomotor activity, body temperature, ghrelin levels, and Fos activation that occur in these two experimental models of FAA compared to rats that had *ad libitum* access to chow or random restricted access to chow. Analysis of daily locomotor activity and body temperature revealed that food restriction shifted the circadian rhythms of these parameters. FAA developed faster in restricted than in non-restricted rats. Only time-restricted rats showed an anticipatory rise in body temperature. Rats anticipating a palatable treat did not show increased levels of hypothalamic or accumbal Fos activity, in contrast to food-restricted rats. Co-localization studies revealed that activation of the anorexigenic cell population in arcuate nucleus was decreased in both restricted groups and in the palatable treat group. Ghrelin levels increased in anticipation of an expected treat and meal, but also in the random restricted group. This study reveals that rats anticipating a palatable treat show anticipatory locomotor activity and a rise in ghrelin levels, but lack many of the other physiological changes induced in restricted, anticipating rats. Random restricted access to chow evokes many of these physiological changes as well, indicating that the phenotype observed in rats anticipating a restricted meal is due to a combination of negative energy balance and anticipation.

## INTRODUCTION

The prevalence of obesity has grown tremendously over the past decades. There-fore, the need to investigate the mechanisms underlying appetitive behaviour has increased as well. Animal models of food anticipatory activity (FAA) induce increased arousal and activity prior to the expected meal-time, thereby reflecting appetitive behaviour. In a restricted feeding schedule (RFS), food intake is limited to a fixed period within the light phase. This is associated with a change in the engagement of the circadian rhythms of locomotor activity and body temperature from the light-dark cycle to the period of food availability.[1-7] Additionally, if something palatable is available for a restricted period with normal chow available *ad libitum* (i.e. palatable feeding schedule (PFS)), rats show FAA as well, although to a lesser extent.[8-10] These observations have led to the concept of a food entrainable oscillator (FEO) that could regulate FAA.[1,11-13]

Various studies aimed to identify the FEO by lesioning brain areas[14] or by disabling neural connectivity between the gut and the brain[15-17]. However, results have been inconclusive, and FAA is likely to be mediated by a network of brain areas rather than one single brain area. Collectively, studies indicate that the hypothalamus and corticolimbic areas are part of this network.

The hypothalamus has been implicated in the homeostatic regulation of energy balance and autonomic behaviours, and is therefore a logical candidate contributing to RFS-induced FAA. Various hypothalamic areas showed increased expression of the neuronal activation marker Fos during FAA[6,18-21]. In contrast, rats subjected to a PFS did not exhibit any hypothalamic activation during FAA[8]. This suggests that the hypothalamus is involved in the regulation of FAA in RFS rats, but probably not in PFS rats.

Corticolimbic areas involved in the reward-related effects of food intake have been demonstrated to play a more important role in FAA in PFS rats. During FAA, Fos was increased in various corticolimbic areas, including nucleus accumbens (NAc).[8,22-24] Corticolimbic areas were also activated during FAA in RFS rats[5,25,26], although to a lesser extent than in PFS rats[8]. Hence, corticolimbic areas appear to play a role in the regulation of FAA in PFS rats, and to a lesser extent in RFS rats.

This study aimed to further investigate the neuronal and physiological changes that occur during FAA in rats on both RFS and PFS regimens. In contrast to previous studies, we included an extra control group on a random RFS (R-RFS) in which food was available at random time-points during the light phase. This group was in the same negative energy balance as RFS rats, but could not anticipate their daily meal.

## MATERIAL AND METHODS

*Animals*

Male Wistar rats (n=22, Charles River, Germany) weighing 175-225 grams upon arrival were individually housed in a temperature (21±2°C) and humidity controlled room on a 12h/12h light-dark cycle (lights on at 7.00AM). Animals had *ad libitum* access to water and food, unless mentioned otherwise. All described procedures were approved by the ethical committee on use and care of animals of Utrecht University, the Netherlands. For ethical reasons, the experiment had to be terminated when rats lost more than 20% of their initial body weight (BW).

*Surgical procedures*

Rats were left to habituate for one week after arrival. Subsequently, rats received telemetric transmitter probes (TA10TA-F40, Data Sciences International, St. Paul, Minnesota, USA) in the abdominal cavity as described previously.[27] Rats were allowed to recover for two weeks.

*Experimental set-up*

Rats were assigned to an experimental group based on baseline measurements of BW, food intake and water intake in the week before onset of the feeding schedules. The *ad libitum* group (AL) (n=5) had *ad libitum* access to chow throughout the experiment. The PFS group (n=6) had *ad libitum* access to chow and received in addition 5 grams of milk chocolate (Droste® Nederland B.V, Vaassen, the Netherlands, per 100 gr 562 kCal, 35.4 gr fat, 6.1 gr protein, and 54.7 gr carbohydrates) at zeitgeber time (ZT) 6. The RFS group (n=6) had access to chow for two hours from ZT6-ZT8. The last group, the R-RFS group (n=5), had access to chow for two hours in the light period as well, but at a different time-point

every day. The daily intervals between the moments of food access ranged from 18–30 hours and were on average 24 hours.

After 3 experimental weeks, rats were sacrificed at ZT6.5 without receiving their expected meal. The previous day, R-RFS rats had received their meal from ZT6-ZT8, just as the RFS group. Rats were injected intraperitoneally with an overdose of sodium pentobarbital. Blood samples were taken directly from the right atrium, collected into heparin-coated tubes with 83 µmol EDTA and 1 mg aprotinin and kept on ice until centrifugation (20', 3000 rotations per minute, 4°C). Plasma was stored at -20°C until subsequent analysis. In addition, the left epididymal fat pad was dissected and weighed as an indication of fat mass. Subsequently, rats were perfused transcardially with 150 ml 0.9% sodium chloride solution followed by 250 ml 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were removed, post-fixated in 4% paraformaldehyde in PBS overnight at 4°C, cryoprotected in 30% sucrose solution, and stored at -80°C until brain sectioning.

*Immunohistochemistry*

Coronal sections of 40 µm were cut on a freezing microtome (Micron HM 500, Germany). Sections were stored free-floating in anti-freeze solution (30% glycerol, 40% PBS, 30% ethylene glycol) at -20°C. Fos immunohistochemistry was carried out as previously described.[28] In brief, following pre-incubation, quenching and blocking, sections were incubated with Fos antibody (polyclonal rabbit c-fos (1:2000), sc-52, Santa Cruz Biotechnology, Santa Cruz, California, USA). After incubation with secondary antibody (biotinylated donkey anti-rabbit (1:200), Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA), sections were incubated with avidin- and biotin complex (ABC, 1:200). The peroxidase reaction was visualized in 3,3-diaminobenzidine solution containing 7.5% nickel ammonium sulphate. Sections were rinsed, mounted onto glass slides and dehydrated with ethanol, cleared with xylene and coverslipped with Entallan (Merck, Darmstadt, Germany). Double immunohistochemistry for Fos and α-melanocyte stimulating hormone (α-MSH) was performed as described above with the following changes. At day 1, polyclonal sheep α-MSH antibody (1:2000, a kind gift from Jeffrey B. Tatro) was added together with the Fos antibody. Following day 2, sections were incubated with another secondary antibody (biotinylated donkey anti-sheep, 1:200, Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA), followed by rinsing and ABC incubation as

mentioned above. The second peroxidase reaction was visualized in 3,3-diaminobenzidine solution. Finally, sections were processed as described above. Using this procedure, Fos-positive cells were detectable by a dark nuclear staining, whereas α-MSH-positive cells had a brown cytoplasmatic staining.

*Quantification of Fos-positive cells*

Sections of all rats were matched using a stereotaxic brain atlas (Paxinos and Watson (1998)) and were analyzed at 20x magnification using a computerized image analysis system (Leica Qwin V3, Rijswijk, the Netherlands). Rat numbers were blinded and Fos-positive cells were counted unilaterally in various coronal sections. For the hypothalamus, sections of rostrocaudal levels bregma -1.6, -2.8, and -3.3 mm were used. Using the fornix as a reference, the hypothalamus was subdivided into different quadrants including SCN, paraventricular nucleus (PVN), anterior hypothalamic area (AHA), dorsal and ventral lateral hypothalamus (dLH and vLH), Arc, dorsomedial hypothalamus (DMH), and ventromedial hypothalamus (VMH). The number of α-MSH positive cells and Fos/α-MSH double labelled cells were counted unilaterally in the Arc in sections equivalent to bregma -2.8 mm and -3.3 mm. For NAc, divided in core and shell, sections at bregma +1.2 mm and +2.2 mm were used.

*Plasma analysis*

Plasma levels of total ghrelin (Phoenix Pharmaceuticals, Belmont, California, USA) and leptin (Millipore, Billerica, Missouri, USA) were measured by radio immunoassay kits according to manufacturer's protocol. For total ghrelin measurements, each plasma sample was diluted 2, 5 or 10 times (for AL, PFS and RFS/R-RFS rats respectively) in assay buffer to obtain levels in the optimal range of the kit. All measurements were done in duplicate. Unfortunately, we were unable to collect blood samples from 2 rats (1 from PFS group, 1 from RFS group). One RFS-rat was considered an outlier and excluded from the analysis.

*Data analysis*

All data are expressed as mean ± standard error (SEM). BW, food intake and water intake were measured daily. Food intake data were also collected by Scales (Department Biomedical Engineering, UMC Utrecht, the Netherlands), which recorded the weight of food hoppers automatically every 12 seconds. A meal was defined as an episode with a minimal consumption of 0.3 g chow and a

minimal intermeal interval of 5 min.[29] Measurements of core body temperature (°C) and locomotor activity (arbitrary units) were sent by the transmitters to receiver plates below the home cages via radio frequency signals. These data were automatically recorded every 10 minutes using DSI software (Data Science International, St. Paul, Minnesota, USA) and averaged per hour for statistical analysis. FAA was defined as the amount of locomotor activity in the 3-hour period preceding ZT6, normalized for total locomotor activity. SPSS 15.0 for Windows software was used for statistical analysis. Outliers were defined using a boxplot analysis. Data-points which exceeded three interquartile ranges from the edge of the box were considered outliers and removed from the analysis. Correlations between Fos activation and other parameters were investigated using Pearson's bivariate correlational analysis. Differences in parameters at different time points of the experiment were measured using repeated measures ANOVA with feeding schedule as a between groups factor. F-values and p-values for these analyses are depicted in Table S1. In case of a significant time*group interaction, data were analyzed per group applying repeated measures ANOVA with a predefined simple contrast comparing all time points to baseline. Differences between groups per time-point were analyzed using a (multivariate) ANOVA with Tukey HSD *post hoc* testing. Statistical significance was set at $p<0.05$.

## RESULTS

*BW gain, caloric intake and meal patterns*

AL rats and PFS rats gained an equal amount of BW during the experiment, whereas RFS rats and R-RFS rats lost weight. (Figure 1A) Although R-RFS rats lost more BW than RFS rats, these groups did not differ significantly from each other at any time point. The differences in BW gain were also reflected in the amount of epididymal fat ($F=10.539$, $p<0.001$) (Table S2).

During baseline measurements, groups were indistinguishable regarding caloric intake ($F=0.158$, $p=0.923$) (Figure 1B), meal frequency ($F=1.298$, $p=0.306$) (Figure 1C) and meal size ($F=0.777$, $p=0.522$) (Figure 1D). AL rats did not alter their caloric intake ($F=0.660$, $p=0.592$), meal frequency ($F=1.873$, $p=0.180$) or meal size ($F=1.410$, $p=0.279$) over the course of the experiment. PFS rats ($F=26.662$, $p<0.001$) initially increased their caloric intake in weeks 1 and 2, but total ca-

loric intake was not different from baseline levels in week 3. PFS rats exhibited reduced chow intake compared to baseline in weeks 1, 2 and 3 ($F=150.691$, $p<0.01$), due to a decrease in meal frequency ($F=36.055$, $p<0.001$), while meal size did not change ($F=1.410$, $p=0.279$). R-RFS ($F=149.675$, $p<0.001$) and RFS rats ($F=105.707$, $p<0.001$) exhibited an initial decrease in chow intake in week 1,

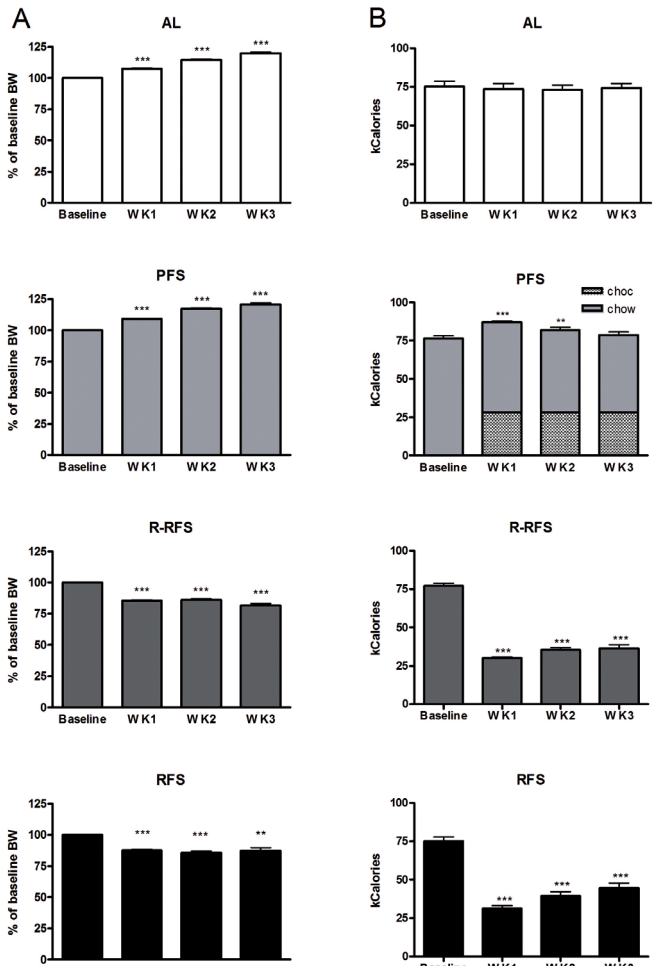



**Figure 1**

Relative body weight gain (A), total caloric intake (B), meal frequency (C) and average meal size (D) of AL, PFS, R-RFS and RFS rats during baseline measurements and experimental weeks 1, 2 and 3. Values represent mean ± SEM per group. Data were analyzed using repeated measures ANOVA with feeding schedule as a between groups factor. In case of a significant time*group interaction, data were analyzed per group applying repeated measures ANOVA with a predefined simple contrast comparing all time points to baseline. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

| C | Meal frequency (number/24h) | | | |
|---|---|---|---|---|
| | *Baseline* | *WK1* | *WK2* | *WK3* |
| **AL** | 13.9 ± 0.68 | 13.7 ± 0.77 | 14.7 ± 0.60 | 13.9 ± 0.93 |
| **PFS** | 15.0 ± 0.72 | 11.6 ± 0.42** | 10.8 ± 0.42** | 9.8 ± 0.31** |
| **R-RFS** | 13.4 ± 0.70 | 1.3 ± 0.12*** | 2.2 ± 0.44*** | 2.1 ± 0.25*** |
| **RFS** | 13.6 ± 0.47 | 1.5 ± 0.18*** | 1.6 ± 0.24*** | 1.7 ± 0.25*** |

| D | Meal size (grams of chow) | | | |
|---|---|---|---|---|
| | *Baseline* | *WK1* | *WK2* | *WK3* |
| **AL** | 0.81 ± 0.04 | 0.79 ± 0.05 | 0.74 ± 0.03 | 0.80 ± 0.05 |
| **PFS** | 0.77 ± 0.05 | 0.73 ± 0.03 | 0.73 ± 0.04 | 0.73 ± 0.04 |
| **R-RFS** | 0.87 ± 0.06 | 3.35 ± 0.46** | 2.56 ± 0.48* | 2.66 ± 0.36** |
| **RFS** | 0.83 ± 0.04 | 3.97 ± 1.17* | 3.95 ± 0.65** | 5.45 ± 1.16* |

which subsequently increased in weeks 2 and 3. Since these two groups were only allowed to eat for 2 hours a day, meal frequency was consequentially reduced compared to baseline measurements (RFS: $F=353.500$, $p<0.001$, R-RFS: $F=235.882$, $p<0.001$). Average meal size was increased (RFS: $F=14.755$, $p<0.001$, R-RFS: $F=14.755$, $p<0.001$). RFS rats consumed more than R-RFS rats, especially in weeks 2 and 3, although this did not reach statistical significance. This difference could be attributed to an increased meal size of RFS rats compared to R-RFS, which was statistically significant by week 3 ($p<0.05$).

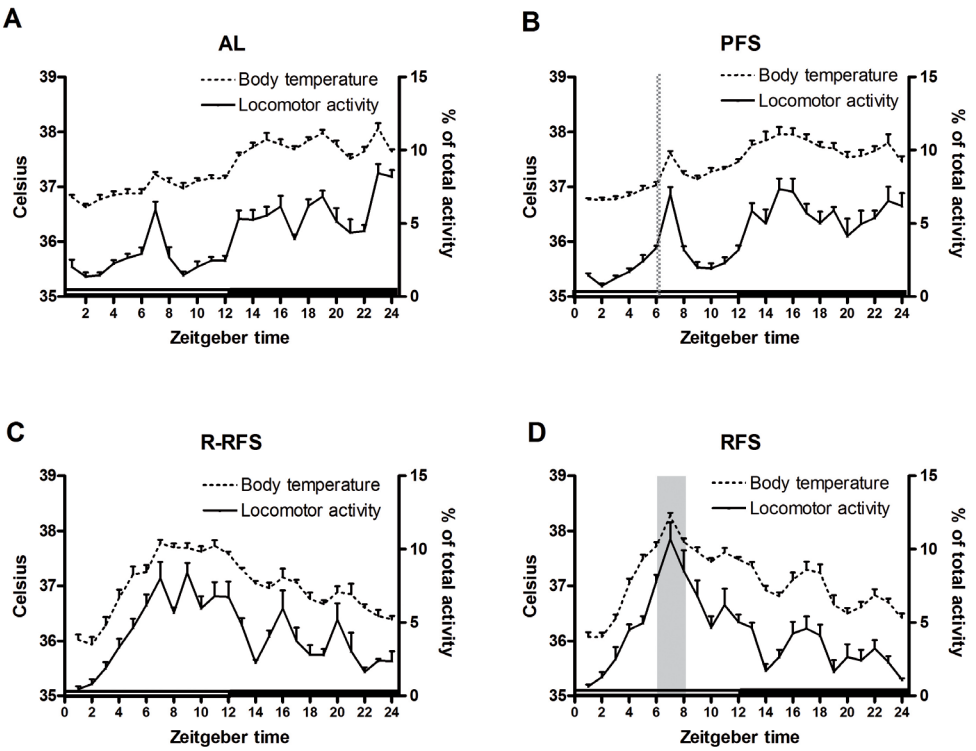
*Locomotor activity and body temperature*

In response to the feeding schedules, RFS and R-RFS rats shifted their circadian rhythms of locomotor activity and body temperature, as illustrated in Figure 2. During baseline measurements, all groups exhibited 20-30% of their daily locomotor activity during the light period ($F=1.778$, $p=0.187$). After 3 weeks, RFS and R-RFS rats exhibited most of their daily locomotor activity during this normally inactive period, whereas AL and PFS rats still showed locomotor activity rhythms comparable to baseline measurements ($F=72.806$, $p<0.001$). The small peak at ZT7 in AL rats in Figure 2A can be explained by the fact that all rats were housed together and weighed at this time-point. Table S3 represents

the average percentages of locomotor activity per 3-hour and 12-hour periods for all groups throughout the experiment.

During baseline measurements, body temperature values peaked in the dark period in all groups (F=2.141, p=0.131) and had a nadir at the beginning of the light period (F=0.197, p=0.897). In the third week, all groups still had a nadir in body temperature early in the light phase (F=0.480, p=0.700), but body temperature peaked in the middle of the light phase in RFS and R-RFS groups (F=37.272, p<0.001). Interestingly, nadir values of body temperature in week 3 were reduced in RFS and R-RFS rats compared to AL and PFS rats (F=28.315, p<0.001), while peak values were only decreased in R-RFS rats (F=7.965, P=0.001). An overview of the times and values of peak and nadir of body temperature is provided in Table S4.

Alterations in FAA and anticipatory body temperature are depicted in Figures 2E and 2F. In AL rats, FAA (F=2.669. p=0.095) and anticipatory body tempera-



| E | FAA (% of total activity) | | | |
|---|---|---|---|---|
| | *Baseline* | *WK1* | *WK2* | *WK3* |
| **AL** | 6.05 ± 0.91 | 6.70 ± 0.47 | 7.38 ± 0.42 | 8.15 ± 0.69 |
| **PFS** | 4.39 ± 0.74 | 4.53 ± 0.58 | 6.44 ± 0.84~ | 7.92 ± 0.31** |
| **R-RFS** | 4.18 ± 0.65 | 8.83 ± 0.81* | 12.64 ± 1.37* | 13.90 ± 1.27** |
| **RFS** | 3.87 ± 0.41 | 11.73 ± 0.40*** | 15.24 ± 0.66*** | 18.32 ± 0.82*** |

| F | Anticipatory body temperature (°Celsius) | | | |
|---|---|---|---|---|
| | *Baseline* | *WK1* | *WK2* | *WK3* |
| **AL** | 36.86 ± 0.04 | 36.88 ± 0.04 | 36.91 ± 0.04 | 36.87 ± 0.07 |
| **PFS** | 36.88 ± 0.05 | 36.90 ± 0.03 | 36.94 ± 0.06 | 36.94 ± 0.06 |
| **R-RFS** | 36.88 ± 0.07 | 36.94 ± 0.08 | 37.05 ± 0.10 | 37.08 ± 0.14 |
| **RFS** | 36.90 ± 0.05 | 37.07 ± 0.05* | 37.31 ± 0.06* | 37.42 ± 0.08** |

**Figure 2**

Circadian rhythms of locomotor activity and body temperature in the third week of the imposed feeding schedules in AL (A), PFS (B), R-RFS (C) and RFS (D) rats. The vertical bar in Figure 2B represents the moment of access to chocolate at ZT6. RFS rats had access to chow from ZT6 – ZT8 daily, indicated by the light grey vertical bar in Figure 2D. R-RFS rats (C) had 2 hours access to chow daily at random time points throughout the light period. Data presented are averaged on an hourly basis for the measurement period and represent the mean ± SEM.

Average values of anticipatory locomotor activity (normalized for total locomotor activity) (E) and body temperature from ZT3-ZT6 (F) per week (baseline weeks 1, 2 and 3) of AL, PFS, R-RFS and RFS rats. Data represent the mean ± SEM per group per time point. Data were analyzed using repeated measures ANOVA with feeding schedule as a between groups factor. In case of a significant time * group interaction, data were analyzed per group applying repeated measures ANOVA with a predefined simple contrast comparing all time points to baseline. ~ $p<0.10$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

ture ($F=1.146$, $p=0.370$) remained similar to baseline measurements. RFS rats had increased FAA ($F=126.374$, $p<0.001$) and anticipatory body temperature ($F=15.991$. $p<0.001$) from week 1 onwards. R-RFS rats did not exhibit increased body temperature during ZT3-ZT6 over time ($F=1.428$, $p=0.283$), whereas LMA increased during ZT3-ZT6 ($F=18.004$. $p<0.001$) from week 1 onwards. PFS rats showed increased FAA ($F=17.348$, $p=0.009$) in the third week, but failed to increase anticipatory body temperature ($F=1.887$, $p=0.228$).

*Leptin and ghrelin*

Analysis of plasma samples revealed significant differences in plasma leptin ($F=25.264$, $p<0.001$) and ghrelin ($F=12.942$, $p<0.001$) levels during FAA. PFS rats had significantly higher leptin levels than all other groups. RFS and R-RFS rats had decreased leptin levels compared to AL rats, but were indistinguishable from each other (Figure 3A). PFS rats had significantly increased ghrelin levels as compared to AL rats. RFS and R-RFS rats exhibited similar ghrelin levels, but had significantly higher ghrelin levels than AL rats. RFS rats showed a trend towards increased ghrelin levels compared to PFS rats (Figure 3B).

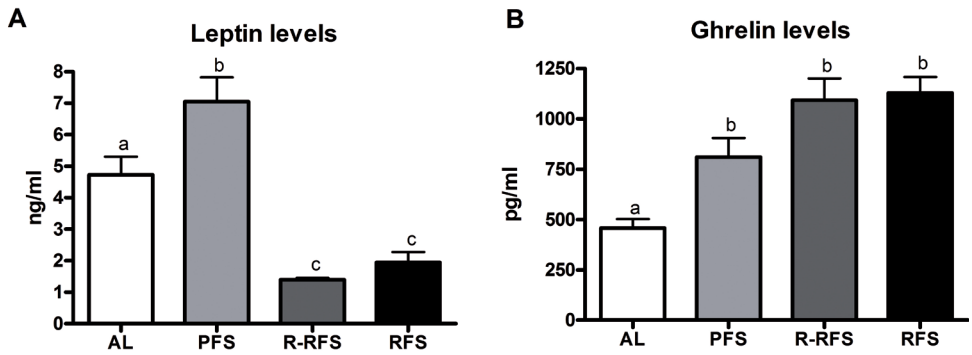



**Figure 3**

Plasma samples, obtained at ZT6.5 after 3 weeks on the feeding schedules, were analyzed for levels of leptin (A) and total ghrelin (B). Differences between groups were calculated using (multivariate) ANOVA with Tukey HSD post hoc testing. Significance was set at $p<0.05$. Groups that did not differ significantly from each other are indicated with the same character.

*Hypothalamic Fos expression*

Fos activation in the hypothalamus during FAA was examined. Arc ($F=0.662$, $p=0.593$) and SCN ($F=0.388$, $p=0.763$) did not show any differences in the amount of Fos-positive cells between groups, whereas in the PVN ($F=7.526$, $p<0.01$), AHA ($F=6.750$, $p<0.01$), LHd ($F=6.291$, $p<0.01$), LHv ($F=7.832$, $p<0.01$), DMH ($F=16.160$, $p<0.001$), and VMH ($F=6.182$, $p<0.01$) differences were found between groups (Figure 4A). PFS rats did not differ in Fos activity from AL rats in any of the hypothalamic areas. RFS rats showed an increase in Fos-positive cells compared to AL rats in DMH and LHd, although the latter remained a trend ($p=0.079$).

R-RFS rats exhibited higher levels of Fos in PVN, DMH, LHd, LHv, and AHA compared to AL rats. RFS and R-RFS rats did not differ significantly from each other in any hypothalamic area apart from the PVN, where the R-RFS group showed an increased number of Fos-positive cells. Typical examples of Fos stainings in each group are shown in Figure S1.

Anticipatory LMA and Fos-positive cells correlated significantly with each other in RFS rats in VMH (Pearson 0.975, p<0.001), DMH (Pearson 0.918, p=0.010), and Arc (Pearson 0.861, p=0.028) (Figure S2), but in none of the other groups.

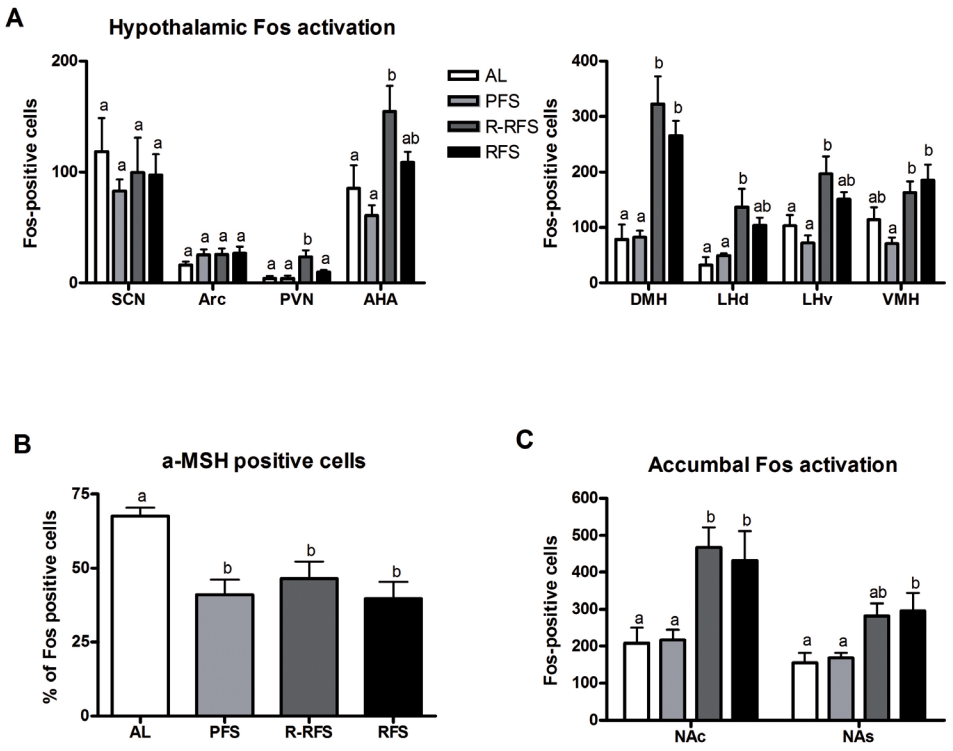



**Figure 4**

Total number of Fos-positive cells per brain area was analyzed in the hypothalamus (A) and nucleus accumbens (C). In addition, the number of α-MSH positive cells as a percentage of Fos positive cells in the arcuate nucleus was examined per group (B). Values represent mean ± SEM per group. Differences between groups were calculated using (multivariate) ANOVA with Tukey HSD post hoc testing. Significance was set at p<0.05. Groups that did not differ from each other significantly are indicated with the same character.

*Co-localization Fos and α-MSH*

No statistically significant differences in the number of Fos- (F=0.721, p=0.553), α-MSH- (F=0.930, p=0.448) or double-labelled cells (F=1.337, p=0.295) were found between groups. However, the percentage of Fos-positive cells that was also positive for α-MSH did differ between groups (F=5.128, p=0.010): in RFS, PFS and R-RFS rats, the population of α-MSH neurons amongst Fos-positive cells during FAA was found to be smaller as compared to AL rats (Figure 4B). An example of Fos and α-MSH doublestaining is depicted in Figure S3.

*Accumbal Fos expression*

To investigate whether NAc plays a role in FAA, Fos-positive cells were counted in NAc core and shell. A significant difference between groups was detected in both NAc core (F=6.041, p=0.005) and shell (F=4.721, p=0.013): RFS rats exhibited significantly increased Fos activation in NAc core and shell as compared to AL rats, whereas PFS rats showed Fos expression levels similar to AL rats. Fos expression in NAc shell was not significantly altered in R-RFS rats as compared to AL rats. However, Nac core and shell Fos activation in R-RFS and RFS rats was not statistically significantly different from each other (Figure 4C).

## DISCUSSION

This study revealed that negative energy balance rather than FAA increased Fos expression in hypothalamic and accumbal areas of food-restricted rats, since R-RFS rats showed similar Fos activation as RFS rats. However, only in RFS rats, anticipatory locomotor activity correlated with Fos expression in the DMH, VMH and Arc. No differences in Fos activation were found in PFS rats versus AL rats in the hypothalamus and NAc. This challenges earlier studies that reported increased Fos activation in corticolimbic areas during FAA in PFS rats.[8,22-24] Furthermore, the anorexigenic neuronal population in the Arc was less activated in PFS, RFS, and R-RFS rats, probably reflecting the drive to eat. In line with this, plasma ghrelin levels were increased in PFS, RFS, and R-RFS rats.

*Locomotor activity and body temperature*

Circadian rhythms of locomotor activity and body temperature remained syn-

chronized to the light-dark cycle in PFS rats, whereas they clearly uncoupled in RFS and R-RFS rats. This indicates that restricted feeding in the light period disrupts normal circadian rhythms, even when food availability is at random. The reduction in nadir of body temperature in restricted rats is most likely due to a compensatory decrease in basal metabolic rate, induced by food restriction.
RFS and R-RFS rats exhibited an increase in locomotor activity during ZT3-ZT6 already in the first week. In R-RFS rats, this is probably due to the shift of locomotor activity from dark to light phase in general. R-RFS rats could not predict the exact meal-time, but were always fed in the light phase. As a consequence, they might have associated the light period with food access. As their information about meal-time was less accurate than for RFS rats, increases in locomotor activity were distributed over the entire light period, rather than the time-specific FAA as observed in RFS rats prior to ZT6. RFS rats showed more FAA than R-RFS rats, which suggests that anticipatory locomotor activity in RFS rats is a combination of a shift in locomotor activity and time-specific anticipation to meal-time. In contrast, an anticipatory rise in body temperature was unique for the RFS group. In PFS rats, FAA could only be detected in the third week. PFS rats showed a decreased amplitude and delayed onset of FAA compared to RFS rats and lacked an anticipatory rise in body temperature. Taken together, this indicates that food restriction is an important component of the development of FAA, but not crucial.

*BW gain, food intake and hormonal levels*

As a consequent of body weight loss, RFS and R-RFS rats had lower leptin levels than AL rats. The decreased meal size of R-RFS rats compared to RFS rats might reflect that they are less prepared to eat a large amount as food availability is less predictable in R-RFS rats. Compared to baseline measurements, both RFS and R-RFS rats increased average meal size, indicating decreased satiation.[30,31]
PFS rats compensated chocolate intake by reducing the frequency of chow meals, which is thought to reflect meal initiation[32] and suggests that PFS rats are less hungry. Intermittent access to a palatable food source has been reported to decrease the reinforcing properties of chow[33], which agrees with the observed reduction in chow intake in PFS rats. Despite their initial overconsumption, BW gain of PFS rats was similar to AL rats thoughout the experiment. Remarkably, PFS rats had increased leptin levels compared to AL rats. Ghrelin is considered a "hunger" hormone, and its levels rise in anticipation of expected meals in

humans and in rats.[34;35] Indeed, ghrelin levels of PFS, RFS, and R-RFS rats were higher than those of AL rats, indicating that these rats are prepared to eat. Although not statistically significant, RFS rats showed a trend towards increased ghrelin levels as compared to PFS rats, indicating that their internal drive to eat is increased, which is also reflected in the amplitude and onset of FAA. As ghrelin and leptin levels were only measured at a single time-point in this study, additional studies could elucidate whether the circadian rhythms of these hormone levels were altered or whether plasma levels were tonically elevated or decreased.

*Hypothalamic Fos activation*

In line with previous observations[8,9], PFS did not increase Fos activity in hypothalamic areas. In RFS rats, increased Fos activation was found in DMH and LHd, as has been demonstrated before.[6,8,36] In the present study, an extra R-RFS group was included to control for possible effects of negative energy balance on Fos activation. Both DMH and LHd were found to be activated in R-RFS as well. Interestingly, PVN was the only hypothalamic brain area that exhibited increased Fos activation in R-RFS rats as compared to RFS rats. In RFS rats, Fos activation in PVN was reported to occur after food access, not during FAA.[6,21] The unpredictable feeding schedule in R-RFS rats could lead to more stress and, as a consequence, to activation of PVN, whose CRH neurons are known to be activated by stress[37]. Taken together, the observed increase in hypothalamic Fos expression in RFS rats cannot be solely attributed to anticipation as R-RFS rats showed similar activation.

FAA correlated exclusively in the RFS group with Fos activation in DMH, VMH and Arc. This correlation was found previously only in the DMH[38] and suggests that coordinated activation of hypothalamic nuclei during anticipation, as observed in RFS rats, is lost in R-RFS rats. Although the role of DMH in FAA is still unclear, it has been attributed to be involved in the regulation of circadian rhythms and FAA in a vast number of studies.[19,39-43] Lesioning VMH attenuated FAA, which eventually recovered.[44-47] It has also been reported that VMH was the first hypothalamic area to be activated during FAA in response to a shift in meal-time.[48]

The anorexigenic population of Arc neurons was less activated at ZT6 in RFS, R-RFS and PFS rats. Fos and a-MSH showed less co-localization in RFS rats in a

previous study as well.[21] Unfortunately, we were not able to examine Fos and NPY co-localization, which represents the orexigenic population of the Arc. We could not detect cytoplasmatic staining for NPY in the Arc, a problem encountered before.[21] It is known that ghrelin not only activates NPY/AgRP neurons, but also inhibits POMC neurons.[49] Therefore, a decrease in activation of the anorexigenic neuronal population fits with the increased ghrelin levels observed in PFS, RFS and R-RFS rats in the present study. Since RFS and R-RFS rats exhibited the same amount of co-localization, this probably reflects the orexigenic drive rather than anticipation.

*Accumbal Fos activation*

RFS and R-RFS rats showed more Fos activation in NAc. Corticolimbic areas were shown to be activated during FAA in RFS rats before.[5,25] This is the first study to reveal that a R-RFS induced Fos activation in NAc to the same extent as a RFS, indicating that food restriction itself is the major cause for this activation. The rhythm of expression of Per2 was also disrupted by both RFS[26] and R-RFS[50] during the light phase in dorsal striatum and limbic areas. In contrast to a previous study[8], PFS rats failed to exhibit Fos activation in NAc. The experimental design of the mentioned study[8] was similar to our study. However, we could only detect FAA in PFS rats in the third week, whereas in the previous study, FAA was already visible after 1 week[8]. In another study, no changes in Fos activation in various limbic areas were found in PFS rats with access to palatable food at ZT4.[26]

*Conclusion*

In conclusion, FAA in RFS rats involved hypothalamic and accumbal neuronal activation. VMH, DMH, and Arc could be potential nodes in the network regulating FAA in RFS rats, as Fos expression in these areas correlated with anticipatory locomotor activity in RFS rats exclusively. Since increases in ghrelin levels were not limited to the restricted groups, but also appeared in PFS rats, this hormone could be a mediator of FAA. Many of the physiological, hormonal and neuronal changes that occurred in RFS rats, also occurred in R-RFS rats. Therefore, we advise to include a control group with comparable negative energy balance and completely unpredictable meal-timing in order to dissociate anticipation from starvation-induced changes when studying mechanisms underlying FAA.

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## SUPPLEMENTAL DATA

| | Group | | Time | | Interaction | |
|---|---|---|---|---|---|---|
| | F | p | F | p | F | p |
| Caloric intake | 3631.75 | <0.001 | 166.21 | <0.001 | 77.85 | <0.001 |
| Body weight | 236.55 | <0.001 | 27.92 | <0.001 | 133.98 | <0.001 |
| Meal frequency | 142.84 | <0.001 | 382.62 | <0.001 | 47.81 | <0.001 |
| Meal size | 13.85 | <0.001 | 11.58 | <0.001 | 5.72 | <0.001 |
| Anticipatory LMA | 40.52 | <0.001 | 93.43 | <0.001 | 14.94 | <0.001 |
| Anticipatory BT | 7.16 | <0.01 | 12.44 | <0.001 | 4.22 | <0.01 |

**Table S1 Statistical parameters**
Overview of the F-values and p-values of the group, time and group*time interaction effects of the repeated measures ANOVA for several experimental parameters comparing baseline measurements and measurements in weeks 1, 2 and 3 of the feeding schedules.

| Epididymal fat mass (% of total BW) | |
|---|---|
| AL | 0.81 ± 0.07a |
| PFS | 0.91 ± 0.05a |
| R-RFS | 0.57 ± 0.03b |
| RFS | 0.60 ± 0.05b |

**Table S2 Epididymal fat mass**
At the end of the experiment, RFS and R-RFS rats had decreased epididymal fat mass (expressed as percentage of total body weight) compared to AL and PFS rats. Values represent mean ± SEM per group. Differences between groups were calculated using (multivariate) ANOVA with Tukey HSD post hoc testing. Significance was set at $p<0.05$. Groups that did not differ from each other significantly are indicated with the same character.

| | AL | Baseline | SEM | WK1 | SEM | WK2 | SEM | WK3 | SEM |
|---|---|---|---|---|---|---|---|---|---|
| Light | ZT01 - ZT03 | 5.95 | 0.80 | 4.47 | 0.29 | 5.22 | 0.71 | 4.85 | 0.42 |
| | ZT04 - ZT06 | 6.08 | 0.88 | 6.73 | 0.46 | 7.47 | 0.40 | 7.86 | 0.48 |
| | ZT07 - ZT09 | 8.70 | 0.71 | 8.85 | 0.87 | 9.33 | 0.76 | 10.08 | 0.57 |
| | ZT10 - ZT12 | 7.19 | 0.97 | 9.24 | 0.99 | 7.19 | 0.72 | 6.97 | 0.84 |
| Dark | ZT13 - ZT15 | 17.19 | 1.61 | 17.49 | 0.69 | 16.85 | 1.22 | 16.10 | 0.95 |
| | ZT16 - ZT18 | 17.33 | 1.13 | 14.83 | 1.01 | 17.33 | 1.96 | 16.27 | 1.30 |
| | ZT19 - ZT21 | 14.71 | 1.25 | 16.92 | 1.80 | 16.62 | 0.70 | 16.36 | 1.65 |
| | ZT22 - ZT24 | 23.25 | 1.24 | 21.46 | 1.44 | 19.98 | 1.40 | 21.12 | 1.28 |
| Light | ZT01 - ZT12 | 27.52 | 2.86 | 29.29 | 1.80 | 29.21 | 2.21 | 30.15 | 1.61 |
| Dark | ZT13 - ZT24 | 72.48 | 2.86 | 70.71 | 1.80 | 70.79 | 2.21 | 69.85 | 1.61 |

| | RFS | Baseline | SEM | WK1 | SEM | WK2 | SEM | WK3 | SEM |
|---|---|---|---|---|---|---|---|---|---|
| Light | ZT01 - ZT03 | 4.71 | 0.23 | 4.12 | 0.72 | 4.43 | 1.23 | 4.43 | 0.78 |
| | ZT04 - ZT06 | 3.81 | 0.42 | 11.57 | 0.42 | 15.05 | 0.58 | 17.28 | 0.59 |
| | ZT07 - ZT09 | 6.69 | 0.54 | 14.09 | 1.55 | 20.91 | 2.81 | 26.01 | 2.48 |
| | ZT10 - ZT12 | 8.00 | 0.93 | 14.29 | 1.01 | 15.44 | 1.89 | 15.88 | 1.55 |
| Dark | ZT13 - ZT15 | 17.96 | 0.91 | 16.71 | 1.70 | 12.08 | 1.44 | 9.01 | 0.66 |
| | ZT16 - ZT18 | 20.50 | 1.44 | 15.50 | 1.49 | 14.94 | 1.60 | 12.98 | 1.44 |
| | ZT19 - ZT21 | 15.14 | 1.08 | 13.80 | 1.32 | 9.57 | 1.35 | 6.71 | 0.71 |
| | ZT22 - ZT24 | 23.19 | 0.69 | 9.92 | 0.71 | 7.58 | 0.85 | 6.62 | 0.70 |
| Light | ZT01 - ZT12 | 23.22 | 1.83 | 44.07 | 2.98 | 55.82 | 3.61 | 64.68 | 2.10 |
| Dark | ZT13 - ZT24 | 76.78 | 1.83 | 55.93 | 2.98 | 44.18 | 3.61 | 35.32 | 2.10 |

| | R-RFS | Baseline | SEM | WK1 | SEM | WK2 | SEM | WK3 | SEM |
|---|---|---|---|---|---|---|---|---|---|
| Light | ZT01 - ZT03 | 4.32 | 1.10 | 2.95 | 0.49 | 2.73 | 0.38 | 3.16 | 0.85 |
| | ZT04 - ZT06 | 4.17 | 0.67 | 8.64 | 0.78 | 12.47 | 1.45 | 14.15 | 1.04 |
| | ZT07 - ZT09 | 6.50 | 0.64 | 12.42 | 1.15 | 18.79 | 1.52 | 22.12 | 1.42 |
| | ZT10 - ZT12 | 5.03 | 0.76 | 15.00 | 1.63 | 21.04 | 1.09 | 19.50 | 2.08 |
| Dark | ZT13 - ZT15 | 17.50 | 1.66 | 18.27 | 0.72 | 13.77 | 1.14 | 11.14 | 0.75 |
| | ZT16 - ZT18 | 19.16 | 1.17 | 17.20 | 1.32 | 13.70 | 1.62 | 12.52 | 1.05 |
| | ZT19 - ZT21 | 18.76 | 1.90 | 14.36 | 1.83 | 10.92 | 1.30 | 11.07 | 1.55 |
| | ZT22 - ZT24 | 24.56 | 0.89 | 11.16 | 0.86 | 6.58 | 1.08 | 6.40 | 0.88 |
| Light | ZT01 - ZT12 | 20.03 | 2.55 | 39.01 | 3.69 | 55.02 | 3.14 | 58.87 | 3.39 |
| Dark | ZT13 - ZT24 | 79.97 | 2.55 | 60.99 | 3.69 | 44.98 | 3.14 | 41.13 | 3.39 |

| | PFS | Baseline | SEM | WK1 | SEM | WK2 | SEM | WK3 | SEM |
|---|---|---|---|---|---|---|---|---|---|
| Light | ZT01 - ZT03 | 3.96 | 0.46 | 3.73 | 0.28 | 3.65 | 0.39 | 3.42 | 0.43 |
| | ZT04 - ZT06 | 4.33 | 0.75 | 4.54 | 0.57 | 6.49 | 0.85 | 7.42 | 0.35 |
| | ZT07 - ZT09 | 8.26 | 0.83 | 9.71 | 0.25 | 10.22 | 0.91 | 12.16 | 0.39 |
| | ZT10 - ZT12 | 7.15 | 0.61 | 8.76 | 0.67 | 6.60 | 1.03 | 7.42 | 0.88 |
| Dark | ZT13 - ZT15 | 18.06 | 1.21 | 18.99 | 1.09 | 18.17 | 1.63 | 18.23 | 1.33 |
| | ZT16 - ZT18 | 18.82 | 0.56 | 17.73 | 1.09 | 20.11 | 0.76 | 17.90 | 1.33 |
| | ZT19 - ZT21 | 17.86 | 0.80 | 19.12 | 0.61 | 15.24 | 0.84 | 14.98 | 1.90 |
| | ZT22 - ZT24 | 21.56 | 1.10 | 17.43 | 1.32 | 19.52 | 1.37 | 18.11 | 1.96 |
| Light | ZT01 - ZT12 | 23.70 | 1.69 | 26.73 | 1.26 | 26.96 | 2.20 | 30.79 | 1.22 |
| Dark | ZT13 - ZT24 | 76.30 | 1.69 | 73.27 | 1.26 | 73.04 | 2.20 | 69.21 | 1.22 |

**Table S3 Normalized locomotor activity per week**

For each week (baseline, weeks 1, 2 and 3), locomotor activity data was averaged per 3 hour-period and normalized to total locomotor activity per rat. In addition, normalized locomotor activity per light and dark period per week are expressed. Values represent mean ± SEM per 3 or 12 hour-period.

| PEAK | Baseline | | WK1 | | WK2 | | WK3 | |
|---|---|---|---|---|---|---|---|---|
| | ZT | SEM | ZT | SEM | ZT | SEM | ZT | SEM |
| AL | 21.20 | 1.56 | 19.60 | 1.89 | 19.20 | 1.69 | 19.80 | 1.96 |
| RFS | 18.17 | 1.54 | 11.50 | 2.03 | 7.00 | 0.00 | 7.00 | 0.00 |
| R-RFS | 22.80 | 0.20 | 14.40 | 0.81 | 11.80 | 1.07 | 8.00 | 0.77 |
| PFS | 20.83 | 1.33 | 14.67 | 0.33 | 15.17 | 0.48 | 15.33 | 0.49 |

| | Baseline | | WK1 | | WK2 | | WK3 | |
|---|---|---|---|---|---|---|---|---|
| | Peak | SEM | Peak | SEM | Peak | SEM | Peak | SEM |
| AL | 38.16 | 0.07 | 38.17 | 0.07 | 38.18 | 0.03 | 38.12 | 0.06 |
| RFS | 38.12 | 0.04 | 38.06 | 0.05 | 38.14 | 0.05 | 38.27 | 0.06 |
| R-RFS | 38.15 | 0.06 | 37.90 | 0.11 | 37.74 | 0.08 | 37.80 | 0.08 |
| PFS | 38.09 | 0.06 | 38.23 | 0.07 | 38.20 | 0.07 | 38.22 | 0.08 |

| NADIR | Baseline | | WK1 | | WK2 | | WK3 | |
|---|---|---|---|---|---|---|---|---|
| | ZT | SEM | ZT | SEM | ZT | SEM | ZT | SEM |
| AL | 3.00 | 0.63 | 2.20 | 0.58 | 2.20 | 0.20 | 2.00 | 0.00 |
| RFS | 3.17 | 0.31 | 2.17 | 0.31 | 2.00 | 0.00 | 1.50 | 0.22 |
| R-RFS | 2.60 | 0.40 | 2.20 | 0.20 | 1.80 | 0.20 | 1.80 | 0.20 |
| PFS | 3.00 | 0.68 | 2.50 | 0.43 | 2.67 | 0.61 | 1.83 | 0.48 |

| | Baseline | | WK1 | | WK2 | | WK3 | |
|---|---|---|---|---|---|---|---|---|
| | Nadir | SEM | Nadir | SEM | Nadir | SEM | Nadir | SEM |
| AL | 36.74 | 0.07 | 36.74 | 0.06 | 36.68 | 0.05 | 36.63 | 0.06 |
| RFS | 36.80 | 0.04 | 36.10 | 0.09 | 35.96 | 0.06 | 36.04 | 0.09 |
| R-RFS | 36.74 | 0.07 | 36.08 | 0.05 | 35.98 | 0.11 | 35.89 | 0.11 |
| PFS | 36.75 | 0.03 | 36.78 | 0.04 | 36.73 | 0.02 | 36.70 | 0.03 |

**Table S4 Peak and nadir of body temperature per week**

Overview of the times and values of peak and nadir of body temperature for each week (baseline, weeks 1, 2 and 3) of the experiment per group (AL, PFS, R-RFS and RFS). Values represent mean ± SEM.

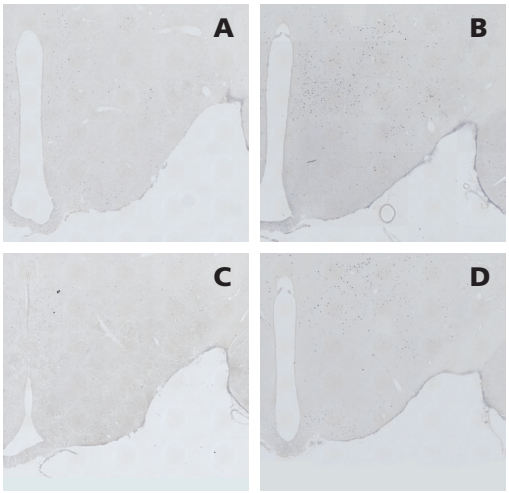


**Figure S1**
Typical examples of Fos immunohistochemistry in sections approximately 2.80 mm posterior to Bregma in ad libitum fed rats (A), in rats on a random restricted feeding schedule (B), in rats on a palatable feeding schedule (C) and in rats on a restricted feeding schedule (D).

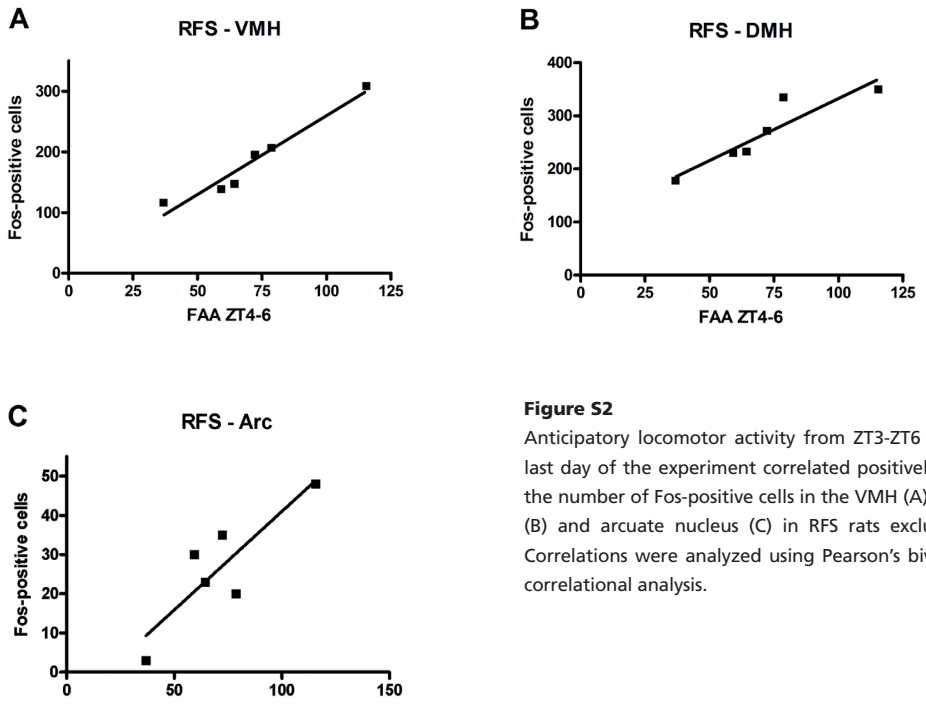


**Figure S2**
Anticipatory locomotor activity from ZT3-ZT6 at the last day of the experiment correlated positively with the number of Fos-positive cells in the VMH (A), DMH (B) and arcuate nucleus (C) in RFS rats exclusively. Correlations were analyzed using Pearson's bivariate correlational analysis.

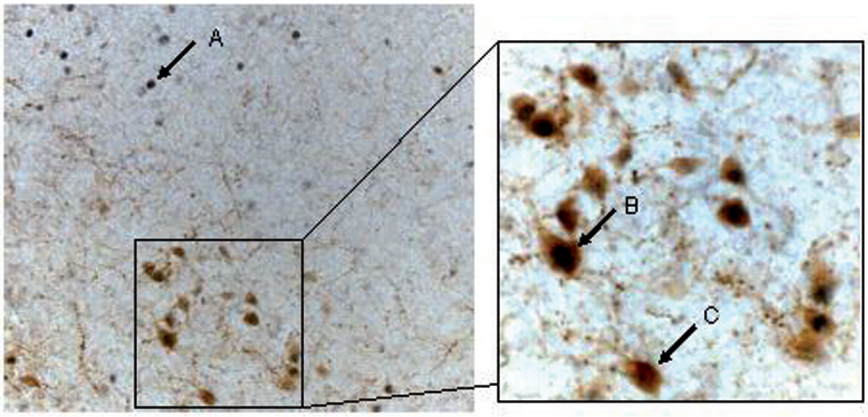


**Figure S3**

An example of Fos and α-MSH doublestaining. The dark nuclear staining represents Fos and α-MSH is visible as brown cytoplasmatic staining. Cells expressing Fos (A), expressing both Fos and α-MSH (B) and cells expressing α-MSH can be distinguished.

# Chapter 3

## GHRELIN MEDIATES ANTICIPATION TO A PALATABLE MEAL IN RATS

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## ABSTRACT

Food anticipatory activity is displayed in rats when access to food is restricted to a specific time frame of their circadian phase, a behaviour thought to reflect both hunger and the motivation to eat. Rats also display food anticipatory activity in a feeding schedule with *ad libitum* access to normal chow, but limited availability of a palatable meal, which is thought to involve mainly motivational aspects. The orexigenic hormone ghrelin has been implicated in food anticipatory activity in rodents with restricted access to chow. Since ghrelin plays an important role not only in the control of food intake, but also in reward, we sought to determine the role of ghrelin in anticipation to a palatable meal. Plasma ghrelin levels of non-restricted rats that anticipated chocolate correlated positively with food anticipatory activity and were increased compared to chow fed control rats. Furthermore, centrally injected ghrelin increased, whereas an antagonist of the ghrelin receptor decreased, the anticipation to chocolate. Therefore, we hypothesize that central ghrelin signaling is able to mediate the motivational drive to eat.

## INTRODUCTION

Many daily activities, including feeding, display a circadian rhythm. In the case of feeding, physiological processes are organized in such a way that they prepare us to digest and metabolize an anticipated meal and, at the same time, increase our appetite.[1] During this so-called "cephalic phase", gastrointestinal function and endocrine activity are enhanced. Notably, there is an increased secretion of a number of appetite-regulating gut-brain signals that include both anorexigenic hormones (e.g. insulin and glucagon like peptide-1)[1,2], and the orexigenic hormone, ghrelin[3,4].

Circadian rhythms in the body are driven by the master clock in the suprachiasmatic nucleus (SCN) in the hypothalamus. SCN is entrained by the day/night cycle and is able to direct the phase of peripheral clocks and the expression of clock genes in neurons in other brain areas, and can hence regulate the expression of circadian behaviours, like feeding (see [5] for review).

Interestingly, under restricted feeding schedules (RFS), in which rodents have limited access to food at a fixed time point of their circadian phase, a phase shift takes place as many behaviours and physiological processes normally coupled to the light/dark cycle are instead entrained by food availability, potentially via a food entrainable oscillator. This results in a behaviour called "food anticipatory activity" (FAA) which is characterized by increased arousal, increased locomotor activity and an elevated body temperature in anticipation of the predicted meal[6-9]. Hypothalamic areas, controlling food intake, as well as corticolimbic areas, involved in non-homeostatic control of food intake, show phase shifts of the expression of clock genes and neuronal activity marker Fos upon exposure to RFS[10-13].

Anticipatory behaviour for food is not limited to situations of restricted food access. Rats with *ad libitum* access to normal chow, but restricted access to a palatable food source also show FAA, albeit to a lesser extent[12,14-16]. Interestingly, this feeding paradigm results in phase shifts in expression of Fos protein[14] and clock protein Per1[12] in corticolimbic areas involved in reward and motivation, but not in hypothalamic areas. Entrainment of circadian rhythms of metabolic parameters such as serum concentrations of glucose and free fatty acids occur

in rats on RFS, but not in *ad libitum* chow fed rats that have restricted access to chocolate[8,14]. Collectively these findings suggest that anticipation to a palatable meal in a non-restricted setting may reflect just the motivational drive to eat, whereas anticipation in a RFS has a "hunger" component as well.

The peptide ghrelin is secreted by the oxyntic cells of the stomach and its acetylated form has a potent appetite-stimulating effect[17], including the intake of palatable food[18-20]. It exerts its actions via growth hormone secretagogue receptor 1A (GHS-R1A)[21], located predominantly in the arcuate nucleus and ventromedial hypothalamus, but also in other brain areas such as the ventral tegmental area (VTA)[22]. Plasma ghrelin levels are known to rise in anticipation of expected meals in both humans[4] and rodents[3,23]. In rodents, ghrelin levels correlate with the amount of anticipatory running wheel activity during a restricted feeding paradigm[24]. Interestingly, clock proteins Per1 and Per2, as well as ghrelin production in the oxyntic cells of the stomach are entrained to a scheduled meal[25]. Furthermore, mice with GHS-R1A deletion display decreased FAA and Fos activation in hypothalamic areas prior to food presentation when exposed to RFS[23-26]. In addition, administration of ghrelin increases [25], whereas GHS-R1A antagonism suppresses[24] FAA in rats. Taken together, these data indicate that ghrelin plays a role in meal anticipation in a restricted feeding paradigm. The findings that ghrelin can specifically stimulate the intake of palatable food[19,20], that it increases the rewarding properties of palatable food[18,27,28] and that its receptor GHS-R1A is expressed in brain areas known to be involved in reward signaling[22], implicates the central ghrelin signaling system in the anticipation to a palatable meal in a non-restricted feeding paradigm.

In this study, we sought to determine whether plasma ghrelin levels correlate with anticipatory behavioural responses for palatable food and whether inhibition of ghrelin signalling by a GHS-R1A antagonist suppresses this anticipatory behaviour.

## MATERIAL AND METHODS

*Animals*

Male outbred Wistar rats, weighing 250-275 g, were purchased from Charles River Laboratories (Crl-Wu, Germany). Upon arrival, rats were individually housed in a temperature (21°C ± 2°C) and humidity controlled room under a 12-hour dark-light cycle (lights on at 7 am = zeitgeber time (ZT) 0). All experimental procedures were approved by the Ethical Committee for Animal Experimentation of Utrecht University.

*Procedures*

Animals were allowed to acclimatize to the animal facility for one week. To be able to measure core body temperature and locomotor activity continuously, all rats were implanted with a transmitter (TA10TA-F40, Data Sciences International, St. Paul, Minnesota, USA) in the abdominal cavity under fentanyl/fluanisone (0.2 mg/kg fentanyl, 10 mg/kg fluanisone, i.m., Hypnorm®, Janssen Pharmaceutica, Beerse, Belgium) and midazolam (2.5 mg/kg, i.p., Dormicum®, Roche, Woerden, The Netherlands) anesthesia. In addition, rats received saline (6 ml s.c.) post-operatively and carprofen (5 mg/kg, s.c., Rimadyl®, Pfizer Animal Health, Capelle a/d Ijssel, the Netherlands) as pain medication pre-operatively and once daily post-operatively for 2 days. After surgery, rats were allowed to recover for at least 2 weeks. Following recovery, basal measurements of locomotor activity, core body temperature, body weight, food intake and water intake were undertaken. Subsequently animals were placed on 1) *ad libitum* access to normal chow and water (AL), 2) a palatable feeding schedule (PFS) receiving milk chocolate (Droste®, Vaassen, the Netherlands, per 100 gr 562 kcal, 35.4 gr fat, 6.1 gr protein, and 54.7 gr carbohydrates) at ZT6 in the middle of the light phase that was supplementary to *ad libitum* access to normal chow and water or 3) a RFS with *ad libitum* access to water, and limited access to normal chow from ZT6-ZT8. Animals that lost more than 20% of their initial body weight during RFS were terminated and excluded from the experiments.

*Experiment 1*

Male Wistar rats (n=12) bearing chronically implanted transmitters were after one week of baseline measurements subjected to a PFS with restricted access to chocolate for 15 minutes each day at ZT6 for 5 weeks. The first 4 weeks of

the PFS could be considered a habituation phase during which the rats became adjusted to the PFS. Unless otherwise stated all data described in the "Results" section were from the last week of the feeding schedule. Locomotor activity and core body temperature were measured every 10 minutes during the whole experiment as these have been shown previously to increase in anticipation of a scheduled meal[6-9]. In our experiment, FAA was defined as locomotor activity in the 3 hours preceding access to chocolate. On the final day of the experiment, rats were decapitated at ZT6 (the time point of the anticipated palatable meal). Trunk blood for subsequent plasma ghrelin measurement was collected into heparin tubes containing 83 µmol EDTA and 1 mg aprotonin for on average 3-5 ml blood and stored on ice until centrifugation for 20 minutes at 3000 rpm at 4°C. Plasma was stored at -20°C until used.

*Experiment 2*

Here we sought to determine whether rats showing FAA for a palatable food have increased total plasma ghrelin levels at ZT6 (i.e. the moment just before access to chocolate) compared to *ad libitum* fed rats. Given our hypothesis that increased ghrelin may be of importance for FAA for palatable food, for comparisons, we included an additional food anticipatory group, namely a restricted food group, for which there are already indications that the increased anticipatory locomotor activity for normal chow is ghrelin dependent[24]. Male rats (n=17) were assigned to one of three groups; controls that had *ad libitum* access to chow (AL) (n=5), PFS (n=6) or RFS (n=6). Rats on RFS had restricted access to chow from ZT6-ZT8 and were deprived from any kind of food for the rest of the day. Rats on PFS had *ad libitum* access to chow and were, supplied with 5 grams of chocolate at ZT6. In contrast to experiment 1, in which rats had 15 minutes access to chocolate, only 5 grams of chocolate was used here, in order to eliminate possible variations in the amount of chocolate eaten. Others have shown that a palatable feeding schedule with 5 grams of chocolate a day is capable of eliciting FAA[12,14]. In addition, in experiment 1, rats were able to eat large amounts of chocolate within 15 minutes, thereby consuming the majority of their daily caloric intake in this short time period (see "Results"). In a PFS with 5 grams of chocolate, rats only depend on chocolate for a minority of their daily calories (see "Results"). The goal of this study is to demonstrate that ghrelin is involved in the motivation-driven anticipation to palatable food, in addition to the already known implication of ghrelin in more hunger-driven anticipation to

a restricted meal[23-26]. Hence, a PFS with 5 grams of chocolate will have a strong motivational component, whereas a PFS with 15 minutes access to chocolate could have a substantial hunger-component as well.

After recovery of the implantation of the transmitter and baseline measurements for 1 week, rats were put on feeding schedules for 3 weeks. In this case a shorter habituation period was used than in experiment 1, since rats on RFS decrease in body weight during the experiment and would loose more than the ethically approved 20% of their initial body weight. Otherwise, the protocol was similar to that described for experiment 1. Briefly, experimental data were collected during the last week of the feeding schedule; locomotor and core body temperature measurements took place every 10 minutes during the whole experiment and FAA was determined during the 3 hour period preceding ZT6. As AL rats showed increased locomotor activity in the 3 hour period preceding ZT6 during baseline measurements compared to RFS and PFS rats, we chose to express FAA as locomotor activity in the 3 hour period preceding ZT6 in the last week on the feeding schedule as a percentage of locomotor activity in that same period during baseline measurements. Unless mentioned otherwise, data presented is from the last week of the feeding schedules. The protocol for euthanization, plasma collection for ghrelin assay was identical to experiment 1. Unfortunately, we were unable to collect blood from 2 rats (1 from PFS group, 1 from RFS group).

*Experiment 3*

The purpose of this experiment was to determine whether central administration of ghrelin can enhance FAA to a palatable meal. Thus, in addition to the placement of the transmitter in the abdominal cavity, rats (n=24) were chronically implanted with an intracerebroventricular (i.c.v.) cannula placed into the lateral ventricle (coordinates 1.0 mm posterior from bregma, 1.0 mm lateral from midline, 5.0 mm below the surface of the brain) that was fixed in place with two small screws and dental cement. After recovery from surgery rats were subjected to a PFS with 5 grams of chocolate at ZT6 for 5 weeks. As before, locomotor activity and core body temperature were measured every 10 minutes and FAA was measured during the 3 hour period preceding access to chocolate.

After an habituation phase of 3 weeks of PFS, when animals had established anticipation to chocolate, animals received at ZT3 i.c.v. injections of saline or

ghrelin (Tocris, Ellisville, USA, 0.3 nmol / 3 µl i.c.v.) using a latin square design, such that each rat received injections of saline and ghrelin, thereby serving as its own control. This time point corresponded to the time when FAA was due to commence. The selected dose of ghrelin (0.3 nmol ~ 1 µg) has been used previously to demonstrate effects on food intake, without effects on general locomotor activity in rats.[29] In total, each rat received a maximum of 4 i.c.v. injections which were always at least three days apart. The protocol enabled investigation of the effect of central administration of ghrelin (versus saline) on FAA, core body temperature, chow intake and chocolate intake. Two rats had to be excluded from the experiment, since they lost more than 20% of their body weight after an i.c.v. injection of saline.

At the end of the experiment, rats were euthanized at ZT6 without receiving the anticipated chocolate. For the localization of i.c.v. injections, brain sections (40 µm) were stained with Cresyl violet. Cannula placement was defined appropriate when positioned in the lateral ventricle, based on a brain atlas (Paxinos and Watson, 1998). Rats with incorrect cannula placements were removed from further analysis.

*Experiment 4*

Here we sought to determine whether decreasing central ghrelin signaling, by central administration of a GHS-R1A antagonist, could diminish FAA in rats on a PFS. Rats (n=24) were implanted with the transmitter and an i.c.v. cannula as described previously. All protocols were identical to experiment 3. However, in this case, at ZT3 rats were injected i.c.v. with either a GHS-R1A antagonist (4, 7 and 12 µg of JMV2959[30], kindly provided by Æterna-Zentaris GmbH) or an equal volume (3 µl) of saline vehicle. Rats were injected according to a latin square design in which each rat received all doses in a randomized order. I.c.v. injections were always at least 3 days apart. To be able to detect a decrease in FAA only rats showing a minimum of FAA of 50 arbitrary units (a.u.) during ZT3-6 of saline injection were included in the analysis. One rat had to be excluded from the analysis, due to technical issues which interfered with data recording of locomotor activity and core body temperature.

As a control experiment, rats (n=24) received also injections of saline and one concentration (4, 7 or 12 µg in 3 µl) of GHS-R1A antagonist at the start of the dark phase in the last week of the experiment to investigate the effect on locomotor activity, core body temperature, and chow intake at a time point in the day when there was no anticipation to palatable food. Unfortunately, 3 rats

had to be excluded from this analysis, as we could not get information about their chow intake due to technical issues with their automated food weighing system. In total, each rat received 6 i.c.v. injections, which were always at least three days apart.

*Plasma analysis*

Plasma levels of total ghrelin were measured by a commercially available radio-immuno-assay kit (Phoenix Pharmaceuticals, Belmont, California, USA) according to manufacturer's protocol. Each plasma sample was diluted 2, 5 or 10 times (for AL rats, PFS rats, and RFS rats respectively) in assay buffer before measurements to obtain levels in the optimal range of the kit. All measurements were done in duplicate.

*Data analysis*

All data are expressed as mean ± standard error (SEM). Body weight, food intake and water intake were measured daily. Food intake data were also collected by Scales (Department Biomedical Engineering, UMC Utrecht, The Netherlands). This program records the weight of food hoppers in the home cage automatically every 12 seconds.

Measurements of core body temperature (°C) and locomotor activity (a.u.) were sent by the transmitters to a receiver plate below the home cage via radio frequency signals. These data were automatically recorded every 10 minutes using DSI software (Data Science International, St. Paul, Minnesota, USA) and averaged per hour for statistical analysis.

SPSS 15.0 for Windows software was used for statistical analysis. In experiment 1 and 2, differences between baseline and PFS were calculated using paired-samples t-test. Correlations between plasma ghrelin levels and other parameters were investigated using Pearson's bivariate correlational analysis. Differences between groups were calculated using (multivariate) ANOVA with Tukey HSD post hoc testing. In experiment 3, differences between saline and ghrelin were calculated using paired t-tests. In experiment 4, differences between saline and different doses or drugs were calculated using repeated measure ANOVA with predefined simple contrasts with saline as reference. In addition, differences in effect of injections at ZT12 were investigated using paired t-tests comparing saline and dose of JMV2959. Datapoints were considered outliers and were excluded from the analysis when they exceeded group average plus three times the standard deviation. Statistical significance was set at $p<0.05$.

## RESULTS

*Experiment 1: Correlation between plasma ghrelin levels and FAA, chocolate intake and body temperature in PFS rats.*

In *experiment 1*, rats (n=12) were allowed 15 minutes access to a palatable food (chocolate) at ZT6. In the last 6 days of PFS, the rats consumed on average 45.2 ± 3.9 calories from chocolate and 33.7 ± 4.3 calories from chow per day. Thus, only 42.7 ± 4.5 % of their daily caloric intake was in the form of chow. Average daily caloric intake did not differ between baseline and PFS (last 6 days of baseline: 76.4 ± 1.6, last 6 days on PFS: 78.9 ± 4.9 calories, t=0.615, p=0.551).

During the course of the experiment rats developed FAA in the hours preceding chocolate access compared to baseline measures. At week 5 of the PFS rats displayed increased activity at ZT4 (i.e. data from ZT3-ZT4) and at ZT6 compared to baseline (figure 1A). Interestingly, the increased anticipatory locomotor activity preceding chocolate access was prolonged beyond the short period of chocolate access such that the activity level was increased also in ZT7 (figure 1A). Furthermore, locomotor activity at ZT13, ZT16-ZT17 during the dark phase was also increased following PFS compared to baseline (figure 1A).

Anticipation of the chocolate meal was also reflected by an increase in core body temperature (figure 1B). In the fifth week of PFS, rats showed significantly increased core body temperature in the hour preceding chocolate access (ZT6), during chocolate access (ZT7) and well beyond chocolate access (ZT8-ZT12) as well as during part of the dark phase (ZT15-ZT17, ZT23- ZT24).

Plasma ghrelin levels of samples taken at the end of the experiment at ZT6 correlated positively with anticipatory locomotor activity during ZT3-ZT6 ($r=0.733$, $p<0.01$) and also with the average core body temperature at ZT3-ZT6 ($r=0.794$, $p=<0.01$), and average chocolate intake ($r=0.757$, $p=<0.01$) of the last experimental day (figures 2A- 2C). No correlation could be found between plasma ghrelin levels and total locomotor activity of the last 24 hours ($r=0.162$, $p=0.614$). Average plasma ghrelin level of samples taken at the end of the experiment at ZT6, i.e. the moment rats would normally have access to chocolate, was 1107 ± 130 pg/ml.

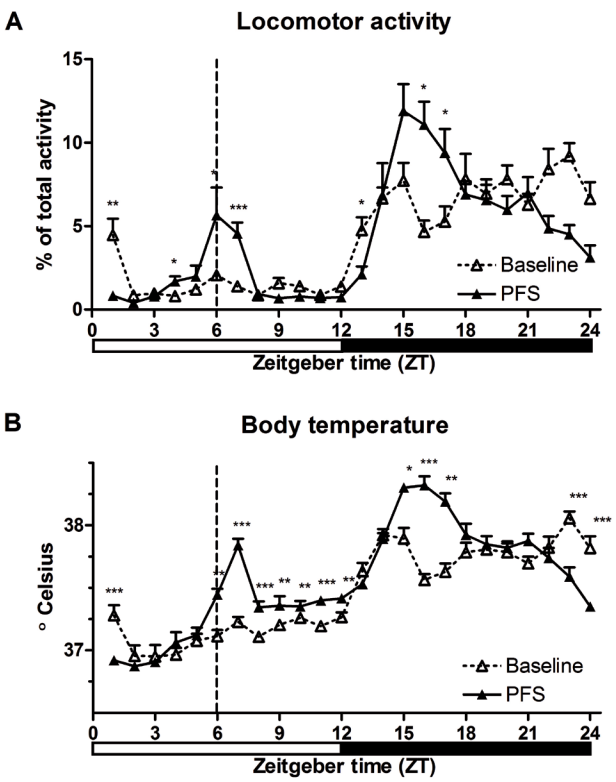


**Figure 1**

Rats on a palatable feeding schedule (PFS) display increased locomotor activity (A) and core body temperature (B) in anticipation to a palatable meal. In the last week of the PFS locomotor activity and core body temperature were significantly increased in the hours preceding (locomotor activity: ZT4, ZT6; core body temperature: ZT6) and the hours following (locomotor activity: ZT7; core body temperature: ZT7 - ZT12) chocolate access as well as during part of the dark phase (locomotor activity: ZT13, ZT16, ZT17; core body temperature: ZT15, ZT16, ZT17, ZT23, and ZT24) compared to baseline measurements. The dotted vertical line indicates the onset of the 15 minutes of chocolate availability. Values presented are averaged on an hourly basis for the 7 day measurement periods and represent the mean ± SEM. n=12 for both baseline and PFS, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Differences between baseline and PFS measurements were analyzed using paired t-tests.

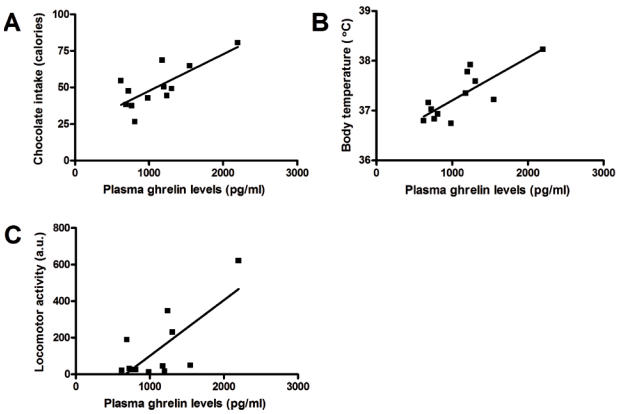


**Figure 2**

Plasma ghrelin levels at ZT6 correlate positively with chocolate intake (A), anticipatory body temperature (B), and anticipatory locomotor activity (C) in rats (n=12) on a palatable feeding schedule (PFS). Average body temperature (°C) and anticipatory locomotor activity (arbitrary units) during ZT3-6 of the last experimental day of the PFS and chocolate intake data from the previous day was used for the correlations. Correlations were analyzed using Pearson's bivariate correlational analysis.

*Experiment 2: Increased plasma ghrelin levels in PFS rats.*

Experiment 1 showed that plasma ghrelin levels at ZT6 correlate with the amount of FAA, anticipatory core body temperature, and average chocolate intake. To control for the variation in chocolate intake between rats (as observed in experiment 1), rats in experiment 2 were given an equal amount of limited chocolate (5 grams) as opposed to 15 minutes access to chocolate at ZT6 in experiment 1. In this way, we were able to address whether plasma ghrelin levels are increased at ZT6 in rats on PFS independently from the amount of chocolate

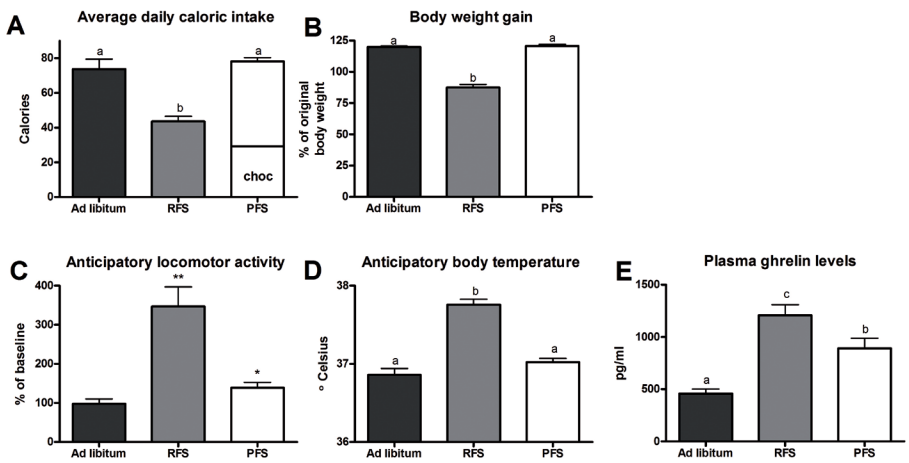


**Figure 3**

Rats were divided in three groups, a control group with *ad libitum* access to chow *(ad libitum)*(n=5), a group on a restricted feeding schedule (RFS) with access to chow from ZT6-ZT8 (n=6), and a group on a palatable feeding schedule (PFS) with *ad libitum* access to chow and 5 grams of chocolate at ZT6 (n=6). Measurements were taken in the last week of the feeding schedules, unless mentioned otherwise. Average daily caloric intake (a) did not differ between *ad libitum* rats and PFS rats, whereas RFS rats had a significantly reduced caloric intake. The lower part of the bar in PFS rats represents chocolate intake, the upper part chow intake. RFS rats also showed a decreased body weight gain at the end of the experiment (as percentage of original body weight before onset of the feeding schedules) (b), compared to *ad libitum* rats and PFS rats, which did not differ from each other in body weight gain. Increases in average anticipatory locomotor activity (c) during ZT3-6 were observed in both RFS rats and PFS rats, although to a lesser extent in the last group. Average anticipatory body temperature (° Celsius) (d) during ZT3-6 was only increased in the RFS group. Plasma ghrelin levels (pg/ml) (e) of blood samples taken at the end of the experiment at ZT6 were elevated in both RFS rats and PFS rats, although again to a lesser extent in the PFS rats. Data represent mean ± SEM per group. In Figures 3a, 3b, 3d, and 3e, groups that do not differ from each other are indicated with the same character. Differences between groups were calculated using (multivariate) ANOVA with Tukey HSD post hoc testing. In Figure 3c, asterisks indicate the statistically significant difference of each group compared to its own baseline (* $p<0.05$, ** $p<0.01$). Differences between baseline and PFS locomotor activity during ZT3 – ZT6 were calculated using paired t-tests. Data are expressed as percentage of baseline measurements.

eaten. Furthermore, a group of rats on RFS was included as a positive control, as these will have increased plasma ghrelin levels [23]. PFS (n=6), RFS (n=6) and AL control (n=5) rats were put on a feeding schedule for 3 weeks.
PFS rats ate on average 50.0 ± 2.2 calories of chow per day, which was 63.7 ± 2.1 % of total daily caloric intake of 78.1 ± 2.2 calories. Average daily caloric intake was not different between PFS and AL rats, but RFS rats had a decreased caloric intake (AL: 3.6 ± 5.8, RFS: 43.5 ± 3.0, PFS: 78.1 ± 2.2 calories, $F=50.865$, $p<0.001$) (figure 3A). After 3 weeks on the feeding schedules AL and PFS rats had gained an equal amount of body weight, whereas RFS rats had lost weight (AL: 119.9 ± 1.7, RFS: 87.4 ± 2.4, PFS: 120.8 ± 1.3 % of original body weight, $F=123.294$, $p<0.001$) (figure 3B).

Both PFS and RFS rats showed FAA preceding access to food / chocolate compared to AL animals. AL rats did not increase locomotor activity in the last week of the experiment in the 3 hour period preceding ZT6 in comparison to the same period during baseline measurements, whereas PFS and rats did (AL: 97.61 ± 12.52 ($t=0.109$, $p=0.918$), RFS: 347.30 ± 49.95 ($t=-5.453$, $p<0.01$), PFS: 138.98 ± 13.99 ($t=-2.581$, $p<0.05$) % of baseline) (figure 3C). Only RFS rats showed an anticipatory increase in core body temperature (AL: 36.86 ± 0.08, RFS: 37.76 ± 0.07, PFS: 37.02 ± 0.05 °C on average from ZT3-ZT6, $F=19.461$, $p<0.001$) (figure 3D).

Plasma ghrelin levels of PFS, RFS rats and AL rats differed significantly from each other (AL: 458 ± 43 pg/ml, RFS: 1208 ± 102 pg/ml, PFS: 810 ± 95 pg/ml, $F= 19.765$, $p<0.001$) (figure 3E). Plasma ghrelin levels were increased in anticipating rats compared to AL rats, and RFS rats showed higher levels of plasma ghrelin than PFS rats.

*Experiment 3: Ghrelin stimulates anticipation to chocolate.*
To determine whether ghrelin can increase FAA, rats (n=22) trained on a PFS with 5 grams of chocolate at ZT6, received i.c.v. injections of ghrelin (0.3 nmol i.c.v.) and saline as a control at ZT3. In the last week on PFS rats ate, on average, 41.0 ± 1.4 calories of chow per day, which was 58.0 ± 0.9% of their total caloric intake of 69.4 ± 1.4 calories per day (data from days following injections excluded).
Ghrelin administration at ZT3 significantly increased FAA for chocolate, compared to saline (saline: 84.81 ± 19.06, ghrelin: 141.07 ± 18.18 a.u., $t=-2.339$, $p<0.05$) (figure 4A). Administration of ghrelin at ZT3 had no effect on subsequent dark

phase chow intake (saline: 10.51 ± 0.69, ghrelin: 11.69 ± 0.99 grams, t=-0.768, p=0.452). In addition, central administration of ghrelin resulted in a decrease in anticipatory body temperature from ZT3-ZT6 (saline: 37.62 ± 0.058, ghrelin: 37.25 ± 0.094 ° Celsius, t=4.327, p<0.001)(figure 4B).

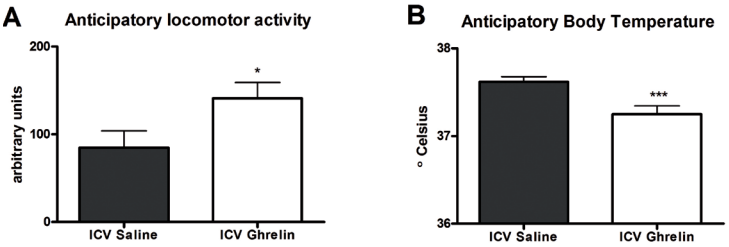


**Figure 4**

Rats (n=22) on a palatable feeding schedule (PFS) with 5 grams of chocolate at ZT6 were injected i.c.v. with saline and 0.3 nmol ghrelin at ZT3. Anticipatory locomotor activity (arbitrary units) during ZT3-6 was significantly increased after an injection with ghrelin (A), whereas anticipatory body temperature was significantly decreased. Data represent mean ± SEM per injection, * p<0.05, *** p<0.001. Differences between saline and ghrelin injections were analyzed using paired t-tests.

*Experiment 4: Antagonism of GHS-R1A reduces anticipation to chocolate.*

To investigate whether inhibition of ghrelin signaling could decrease anticipation to a palatable meal, rats (n=24) that were trained on a PFS with 5 grams of chocolate at ZT6 received i.c.v. injections of different doses (4, 7 or 12 µg) of GHS-R1A antagonist JMV2959 or saline at ZT3.

Rats on PFS developed FAA; however, as some rats showed only a limited amount of FAA, only rats that showed at least a FAA of 50 a.u. during ZT3-6 after saline injection were included in the analysis to be able to detect a decrease in FAA (n=10). During the last week on PFS these rats ate on average 53.8 ± 3.0 calories of chow per day, which was 64.0 ± 1.7 % of their total caloric intake of 81.9 ± 3.0 calories per day.

Administration of 7 and 12 µg JMV2959 significantly reduced FAA during ZT3-6, (saline: 106.04 ± 13.71, 4 µg: 78.94 ± 20.32, 7 µg: 41.88 ± .31, 12 µg: 59.87 ± 13.57 a.u., F=4.291, p<0.05) (figure 5A). GHS-R1A antagonism had no effect on chocolate intake; chocolate was always consumed within 10 minutes. In addition, no effect on subsequent dark phase chow intake was observed (saline: 16.76 ± 1.39,

4 µg: 16.41 ± 0.79, 7 µg: 15.79 ± 0.71, 12 µg: 14.76 ± 1.67 grams chow during dark phase, F=0.129, p=0.939).

As a control experiment, all 24 rats received in the last week on PFS injections with saline and either 4, 7 or 12 _g JMV2959 at the onset of dark phase (ZT12) to investigate the effect of JMV2959 on general locomotor activity. There were no significant effects of either dose of JMV2959 on locomotor activity in the dark phase. However, there was a trend towards increased locomotor activity in the first 3 hours of dark phase (4 _g: 280.36 ± 73.65 (t=-2.187, p=0.071), 7 _g: 164.82 ± 30.29 (t=-2.140, p=0.070), 12 _g: 135.91 ± 17.99 (t=-1.996, p=0.086) % compared to saline injection). I.c.v. administration at the onset of dark phase of 7 and 12 _g JMV2959 reduced total dark phase chow intake, although the effect of the highest dose was just not significant (4 _g: 107.14 ± 9.14 (t=-0.607, p=0.556), 7 _g: 89.91 ± 3.94 (t=2.337, p<0.05), 12 _g: 79.58 ± 7.81 (t=2.518, p=0.053) % compared to saline injection) (figure 5B).

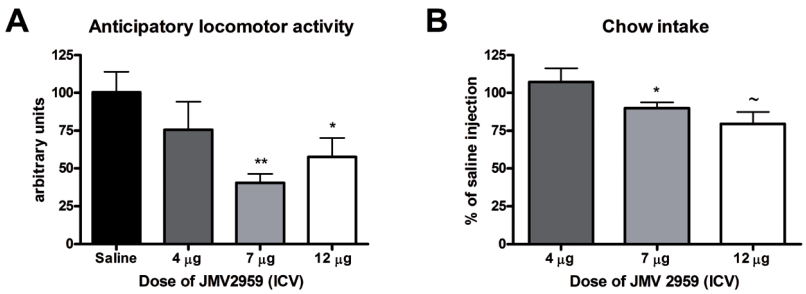


**Figure 5**

Rats on a palatable feeding schedule (PFS) with 5 grams of chocolate at ZT6 were injected i.c.v. with saline, 4 µg, 7 µg, and 12 µg JMV2959 (GHS-R1A antagonist) at ZT3. Rats that showed a minimum of 50 arbitrary units locomotor activity during ZT3-6 after a saline injection were included in the analysis (n=10). The two highest doses significantly decreased anticipatory locomotor activity during ZT3-6 (a). Data represent mean ± SEM per injection, * p<0.05. Differences between saline and different doses or drugs were calculated using repeated measure ANOVA with predefined simple contrasts with saline as reference. As a control experiment, rats (n=21) were injected i.c.v. with saline and either 4 µg (n=7), 7 µg (n=8), or 12 µg (n=6) JMV2959 at ZT12, the onset of the dark phase. Subsequent chow intake (expressed as a percentage of chow intake after saline injection) during the dark phase was diminished with the two highest doses of JMV2959. Data represent mean ± SEM per group, * p<0.05, ~ p<0.10. Differences in effect of injections at ZT12 were investigated using paired t-tests comparing saline and dose of JMV2959.

## DISCUSSION

In this study, we show that plasma ghrelin levels are increased during and correlate positively with FAA that precedes a palatable meal. In addition, we show that ghrelin increases and inhibition of ghrelin signaling decreases this anticipatory activity.

The aim of *experiment 1* was to examine whether plasma ghrelin levels correlate with several parameters related to anticipation to a palatable meal. Indeed, total ghrelin levels correlated with chocolate intake, anticipatory locomotor activity and anticipatory body temperature when rats were given 15 minutes daily access to chocolate *(experiment 1)*. In this paradigm, rats consumed the majority of their daily caloric intake from chocolate, although total caloric intake did not differ from baseline measurements. Since all rats in *experiment 1* were subjected to a PFS, we could not compare the observed correlations with plasma ghrelin levels to AL rats, and changes in ghrelin levels could be due not to the PFS, but due to aging of the rats. However, plasma ghrelin levels observed in *experiment 1* are higher than those of adult AL rats in literature[3] and in *experiment 2*. Furthermore, others have reported that the preprandial, anticipatory rise in ghrelin in RFS rats is not observed at the same moment in the AL control group.[3] Altogether, this indicates that the changes in plasma ghrelin levels at ZT6 in PFS rats are due to the expectation of the palatable meal, and not due to time-dependent changes.

In *experiment 1*, one rat exhibited high levels of plasma ghrelin and anticipatory locomotor activity. Although this rat did not fulfill our criteria to be considered an outlier, the correlation between ghrelin levels and FAA seems to be driven to a large extent by this particular rat. The correlation between ghrelin and chocolate intake appears to be more independent of this rat. As rats did not increase their total caloric intake, high chocolate intake was compensated by a reduction of chow intake. Hence, rats with a high levels of chocolate intake were probably more hungry at ZT6 than rats with a relatively low intake of chocolate. Plasma ghrelin levels have been shown previously to correlate with subjective hunger ratings.[31]

In order to test whether plasma ghrelin levels rise to a similar extent as in rats subjected to a RFS, we compared plasma ghrelin levels at ZT6 from AL control

rats, rats on PFS, and rats on RFS. Given that rats allowed 15 minutes access to chocolate can eat a substantial part of their day's caloric intake during this short period *(experiment 1)*, to show anticipatory responses to palatable food (and not only food), in this protocol we restricted the amount of chocolate to only 5 grams per day *(experiment 2)*. Plasma ghrelin levels were increased just before the scheduled feeding in both PFS and RFS rats compared to control animals. Thus, although the PFS group only received a minority of their daily calories from the chocolate, they still displayed an increase in plasma ghrelin levels. This finding strongly implicates ghrelin in the anticipation of palatable foods under normocaloric conditions. However, in the paradigm with only 5 grams of chocolate, the anticipatory rise in body temperature could not be detected anymore, indicating that this form of anticipation depends on the caloric content of the anticipated meal. Indeed, others have shown that anticipation to a palatable meal is depended on the caloric value of the palatable meal[16]. In response to the palatable meal, a prolonged increase in body temperature was observed, which might be caused by diet-induced thermogenesis.

Previous studies have also shown an increase in plasma ghrelin levels during food anticipation[23] and that correlated with anticipatory running wheel activity[24]. The increase in plasma ghrelin levels during anticipation in rats on a PFS is not as high as in rats on a RFS, but nonetheless implicates ghrelin signaling in some common pathway which regulates anticipatory locomotor activity in both paradigms. In concert with the smaller increase in plasma ghrelin levels, rats on PFS also show less FAA than rats on RFS.

Studies on the role of ghrelin signaling in locomotor activity are somewhat contradictory. Central administration of ghrelin has been reported to decrease[29] as well as increase[32] spontaneous locomotor activity in *ad libitum* fed rodents, and ghrelin administration increases FAA in rats on a restricted feeding schedule[25].
The increase in spontaneous locomotor activity is observed acutely after injection of ghrelin[32], whereas the decrease seems to be more of a late-onset (or chronic) effect[29]. It may be that ghrelin has bimodal effects on locomotor activity, depending on the motivational and nutrional state. Interestingly, it has been shown that peripheral ghrelin also has bimodal effects on dopamine release in nucleus accumbens depending on food-consumptive state, with increasing dopamine levels when food is available, and decreasing dopamine levels when food is removed.[33]

Central administration of 1 µg of ghrelin has been reported to induce both an increase[34] as well as a decrease[35] in body temperature. The decrease, which is also observed with a lower dose, seems to be an early-onset effect, whereas the reported hyperthermic effect occurs later, and is observed with higher doses as well. Interestingly, an acute hypothermic response to ghrelin was found in this study in the period preceding access to chocolate in which rats were more active and did not consume much chow. Hence, this study confirms previous findings[35] that ghrelin has an acute hypothermic effect, despite the increase in locomotor activity.

Both locomotor activity and body temperature show an anticipatory increase in response to RFS, as shown by *experiment 2*. In rats on a PFS, ghrelin increases FAA, but in contrast, reduces body temperature at the same moment, suggesting that anticipatory locomotor activity and body temperature are differentially regulated. Indeed, others have reported dissociated effects on these anticipatory parameters.[36]
In this study central injection of GHS-R1A antagonist (7 and 12 µg JMV2959) significantly decreased FAA to a palatable meal, whereas no effect on general locomotor activity could be detected. In addition, central injection of ghrelin increased FAA when injected at ZT3. The effect of the GHS-R1A antagonist on FAA could only be detected in rats that showed a certain level of anticipation. It is known from this and other studies that anticipation on a PFS is not as strong as anticipation on RFS[14,15], and that not all rats show clear FAA on PFS[15].

The doses of the GHS-R1A antagonist used in this study did not reduce locomotor activity when injected at the onset of dark phase and, if anything, increased locomotor activity. Furthermore, the dose of ghrelin that was used in this study (0.3 nmol ~ 1.0 µg) did not result in a significant effect on spontaneous locomotor activity in a previous study in rats[29]. Hence, in addition to the effects that ghrelin might have on locomotor activity in general, it also seems to be involved specifically in the modulation of anticipatory locomotor activity.

The GHS-R1A antagonist JMV2959 is known to reduce ghrelin-, hexarelin- and fasting-induced food intake in acute studies[30,37]. GHS-R1A knockout mice and mice treated daily with peripheral injections of JMV2959 showed reduced intake of palatable food, but not of normal chow in a free choice diet[19]. We did,

however, observe a dose dependent decrease in dark phase spontaneous food intake upon administration of JMV2959, but in contrast to previous studies[19,20], we did not observe an effect of GHS-R1A antagonism on palatable food intake; all rats ate their 5 grams of chocolate, although we were not able to measure the time it took to eat the chocolate. This might be due to the restricted access to chocolate, which differs from the free choice diet mentioned above. Others have found that JMV2959 could decrease food intake in *ad libitum* fed rats, but not in rats with restricted access to food[24], indicating that the control of food intake by ghrelin depends on the motivational as well as the nutritional state.

In this study, central injection of the GHS-R1A antagonist decreased anticipatory behaviour for a palatable meal, thereby strongly implicating the central ghrelin signalling system in this anticipatory phase of appetitive behaviour for palatable foods. Consistent with this, it now seems clear that this system, involving ghrelin signalling within reward nodes such as the VTA, increases the intake of (and preference for) palatable food[19], as well as the motivation for palatable food, including sucrose [27,28] and fat[18]. As recently reviewed[38], the target systems for ghrelin for reward behaviour, that includes motivated behaviour for food as well as artificial rewards, likely includes the midbrain mesoaccumbal dopamine system. Given that this dopamine system becomes activated in response to or in anticipation of rewards (that can be food, drugs of abuse or social rewards[39]), it will be interesting to discover whether ghrelin's well-documented effects on this system[32,40] are also important for anticipatory behaviour for a palatable meal.

In conclusion, we show that ghrelin mediates not only anticipation to a RFS, but also to a PFS, and is therefore a good candidate for the modulation of the motivational drive to eat. Nowadays, in our Western society where our hedonic signals seem to overrule our homeostatic control, modulation of the motivational drive to eat might be a good tactic to prevent obesity.

## ACKNOWLEDGEMENTS

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# Chapter 4

## FOOD CUES AND GHRELIN RECRUIT THE SAME NEURONAL CIRCUITRY

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## ABSTRACT

Cues that are associated with the availability of food are known to trigger food anticipatory activity (FAA). This activity is expressed as increased locomotor activity and enables an animal to prepare for maximal utilization of nutritional resources. Although the exact neural network that mediates FAA is still unknown, several studies have revealed that both the dorsomedial- and ventromedial hypothalamus (DMH/VMH) participate in the behavioural expression of FAA. Moreover, both areas respond to leptin and ghrelin which have been shown to modulate FAA. However, how FAA is regulated by neuronal activity in the DMH/VMH and how leptin and ghrelin modulate this activity is still poorly understood. In order to examine this, we recorded neuronal activity in the DMH/VMH in freely-moving rats kept on a random feeding schedule, in which a light cue signalled upcoming access to food. To assess a possible modulatory role of ghrelin and leptin on neuronal activity both hormones were administered systemically following the behavioural paradigm. Results indicate that the food-predictive cue induced FAA as well as a significant increase in neural activity in the DMH/VMH neuronal population. Similarly, the actual presence of food as well as ghrelin, but not leptin, increased population activation. Most importantly, the results show that a subpopulation of DMH/VMH neurons displays highly correlated responses to both ghrelin and FAA, suggesting that these neurons are part of the network that regulates FAA.

Taken together, this study reveals a role for ghrelin, but not leptin, signalling within DMH/VMH in FAA on both a population level as well as in single cells. These data extent the previous findings by identifying a subset of neurons onto which cue information and ghrelin signalling converge, possibly to drive hypothalamic FAA.

## INTRODUCTION

The sight or smell of food triggers anticipatory responses that prepare us to eat. Unravelling the neural circuit mediating these food anticipatory responses is essential to enhance our understanding of the mechanisms underlying eating disorders and obesity.

In rodents, a timed restricted feeding schedule (RFS) elicits hyperactivity preceding access to food, i.e food anticipatory activity (FAA)[1] which is thought to reflect the drive to eat. Even when food is only available during the light phase, which is the inactive period of rats, the rhythm of locomotor activity shifts and peaks prior to meal-time. Similarly, cues predictive of food availability evoke FAA[2-4].

Numerous studies have sought to identify key brain areas responsible for driving FAA, but no single area has thus far been identified that is both sufficient and necessary for the expression of FAA[5], suggesting a network of brain areas rather than a single site. Important nodes within this network appear to be the dorsomedial hypothalamus (DMH) and ventromedial hypothalamus (VMH). These brain areas are activated during FAA, as assessed by increased expression of Fos following FAA.[6-10] Furthermore, lesions of either area have been shown to attenuate FAA in both the DMH[6,11,12], but see [13-15] and VMH[16,17], but see [18,19].

The DMH and VMH receive, and integrate, information about the metabolic state of the body via receptors for anorexigenic factors, such as leptin[20], and orexigenic factors like ghrelin[21]. Ghrelin as well as leptin have been implicated in the expression of FAA. Whereas reduced ghrelin signalling attenuates FAA[22-25], decreased levels of leptin have been associated with augmented expression of FAA[26-28]. Previous studies showed that ghrelin as well as the number of Fos-positive neurons in both DMH and VMH are correlated with FAA in the hours preceding access to food in rats exposed to scheduled feeding. Importantly, from a functional perspective it has been shown that both the DMH and the VMH could drive FAA through projections to brain regions involved in arousal and locomotion.[29-31] As such, these areas are ideally located to serve as a node in the network that regulates food-directed locomotor activity.

Although rodent studies have identified brain areas underlying FAA, thus far these studies have failed to directly relate neuronal activity to anticipatory behaviour. We assessed the relation of neuronal activity in the DMH/VMH with the anticipation of food and subsequent consumption. In addition, we examined the effect of the circulating (an)orexigenic 'feeding-hormones' ghrelin and leptin on neuronal activity in these areas. Despite existing data on brain areas and possible hormonal factors that are involved in FAA, current knowledge lacks longitudinal measurements of individual neurons within the DMH/VMH during FAA and the manner in which circulating factors drive this behaviour. Rather than lesioning the DMH/VMH or quantifying markers of neuronal activity we have directly measured neuronal activity in freely moving animals.

This study aimed to assess the relation between both individual neuronal activity and population response to cue-signalled food availability in awake, behaving rats using *in vivo* electrophysiology. To assess anticipatory responses, food was not presented at a fixed time-point during the day, but at a random time-point, signalled by a light cue. This allowed us to induce FAA immediately and for minutes, rather than hours, and relate neuronal firing to behaviour. Furthermore, in order to relate these neuronal responses to hormonal signalling, rats received intraperitoneal (i.p.) injections of ghrelin, leptin and saline following access to food. In this way, we aimed to unravel the role of DMH/VMH neurons in FAA, and the effects of the hormones leptin and ghrelin on these neurons.

## MATERIALS AND METHODS

All experiments were approved by the Animal Experimentation Committee of the VU University (Amsterdam) and were carried out in agreement with Dutch Law (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

*Subjects*

Data were collected from 14 recording sessions, obtained from 6 male Wistar rats (Harlan CPB; Horst, The Netherlands), weighing on average 326.83 ± 10.92 g at the time of surgery. At arrival, the animals were housed in filter top cages (Scanbur Technology A/S, Karslunde Denmark), weighed and handled daily, and kept

under a reversed day/night cycle (white light from 7 pm until 7 am) for a period of two weeks during which food (standard rat chow; Hope Farms, The Netherlands) and water was available *ad libitum*. Following acclimatization, the animals were transferred to the experimental cages (see below) where they remained for the duration of the experiment. From the time of individual housing on, animals were food restricted (16gr/animal/day) according to a random feeding schedule (see below). Water was available *ad libitum* throughout the experiment.

*Apparatus*

Throughout the experiment, animals were housed in locally constructed Perspex test-chambers. Each chamber was visually shielded from other cages and equipped with a trial light (Med Associates, Sanddown Scientific, Middlesex, UK) that was used to signal the arrival of food. Food was delivered to the chamber via an automated system that was operated with Med-pc software (Med Associates, Sanddown Scientific, Middlesex, UK). In short, a 100 ml syringe, of which the top was cut off, was filled with a pre-weighed amount of chow. The syringe was subsequently mounted onto a drug delivery pump (PHM-100, Med-ass.), placed above the cage, and emptied at pre-set time points. Recordings were made in the same chamber at which time it was shielded off from electrical interference by means of 30 µm aluminum foil (VWR International B.V.).

*Surgery and electrophysiology*

Animals were anaesthetized with 5% isoflurane in a 30%:70% $N_2O$:$O_2$ mixture. Following induction of anaesthesia, the isoflurane level was progressively reduced and maintained at 1.75%. The animals were mounted in a Kopf stereotaxic frame. After incision of the skin, additional local anaesthetic (Xylocaine spray; 10%, Astra) was applied to the skull. Body temperature was monitored and maintained at 37.5 °C using a heating pad (Bioseb). Eyedrops were applied to prevent dehydration and s.c. saline was given during surgery to maintain fluid balance. After exposure of the cranium, 9 small holes were drilled into the cranium to accommodate surgical screws, one of which served as ground. Another larger hole was drilled over the medial hypothalamus in the right hemisphere (center of the hole 2.6 mm posterior, 1.2 mm lateral to bregma according to Paxinos and Watson, 2005). The dura was opened and the bundle of tetrodes, extending ~4 mm from the drive, was lowered at an angle of 5° and implanted in the brain. The drive was then anchored to the screws with dental cement. To protect the

brain from the dental cement, and prevent blood from entering the tubing of the tetrode drive, the drill hole was first filled with mineral oil (Sigma).

Immediately after surgery, all tetrodes were further lowered into the target area (DV coordinate, ~9.3 mm). Following surgery, the animals were given at least 7 days of recovery before recordings started. Electrophysiological recordings were performed using a Multineuron Acquisition Processor (MAP) recording system (Plexon, Dallas, Texas, USA). Signals from the individual leads of the tetrodes (Palm Coast NiChrome microwire, 0.005", Sandvik, Germany) were passed through a unity-gain amplifier (20x) and amplified a second time with a Plexon 16-channel preamplifier (PBX, 16 s-pr). Subsequently, the signal was fed into the MAP unit for filtering and further amplification of individual channels. Digitized output (40 kHz) was then stored on a Windows XP workstation. A 1 ms data sample was stored whenever the signal crossed a pre-set voltage threshold so that the width of recorded spikes was 40 data points.

*Behavioural procedure*

Random-feeding schedule.

Two weeks prior to surgery, the animals were transferred to individual chambers and were placed on a random-feeding schedule. At varying time-intervals, ranging from 18 hours to 31 hours, food was delivered to the home-cage.[32] On average, animals received 7 meals per week with a mean interval of 24 hrs. To avoid interval-dependent anticipation, no identical intervals were repeated on consecutive days and food was never delivered after 24 hours (see table 1). To ensure that deprivation state of the animals could be controlled for, all animals received their first recording following 18 hrs of deprivation and their second measurement after 21 hrs of deprivation. In case of a third recording, this was conducted following 18 hrs of deprivation.

Cued-presentation

To invoke cued anticipation to food, the delivery of chow was preceded by the presentation of a light cue. Fifteen minutes prior to food presentation a small signal light was illuminated for a duration of 5 minutes. Subsequently, the light was switched off and a 10 min period of waiting commenced, food presentation followed the waiting period. To ensure that the animals were familiar with the cued feeding procedure, they were trained prior to surgery and during the recovery period.

example rat 1

| day of the week | Monday | Tuesday | Wednesday | Thursday | Friday | Saturday | Sunday | Monday |
|---|---|---|---|---|---|---|---|---|
| actual feeding time | 12:00 PM | 3:00 PM | 12:00 PM | 7:00 PM | 2:00 PM | 5:00 PM | 6:00 PM | 12:00 PM |
| time since last meal (h) | 18 | 27 | 21 | 31 | 19 | 27 | 25 | 18 |

**Table 1**

Example feeding schedule of a single subject. Day of the week (top row), actual feeding time (middle row) and delay since the previous meal is indicated. This schedule was shifted per subject such that each animal was fed at a different moment of the day.

## Experimental procedure

At the start of an experimental day (~8:00 AM), a single animal was connected to the recording device. Upon connection, the signal was visually inspected for neuronal activity. In case no distinguishable spiking activity was observed, individual tetrodes were lowered with increments of 40 µm until new neurons were found. Once the tetrodes were lowered, the appropriate referencing channels were chosen and the animal was left until unit activity stabilized.

Each recording day started with the recording of base-line activity for at least 30 min. Subsequently, the experimental procedure started and animals were presented with the light cue, waiting period, food presentation and hormone injections (see *Pharmacological intervention* for a detailed description). To ensure that new neurons were recorded in each session, the tetrodes were lowered at least 40 µm at the end of the session. The total number of recording sessions per animal varied between 1 and 3, depending on the capacity to record neuronal activity. During the recording sessions, the animals were in a separate room, only for hormone/vehicle injections the experimenters entered to recording area.

## Pharmacological intervention

To assess responses of VMH neurons to satiety, hormones ghrelin and leptin as well as vehicle were injected i.p. following cued-feeding. Concentrations of both hormones (ghrelin 250 µg/kg (Tocris), leptin 1 mg/kg (NHPP) were previously shown to affect food intake and induce hypothalamic Fos activation.[33-38] Saline was injected in a similar volume (0.1 ml/100 g) to control for neuronal responses to the injection. Injections were always administered in the same order; saline, ghrelin followed by leptin, each injection spaced 1 hr apart. Table 2 depicts an overview of the entire experimental procedure for a single measuring day.

**Experimental session**

| 8:00 AM | 11:15 AM | 11:45 AM | 11:50 AM | 12:00 PM | 1:00 PM | 2:00 PM | 3:00 PM | 3:30 PM |
|---|---|---|---|---|---|---|---|---|
| connecting animal<br>signal testing<br>adjusting tetrodes | base-line recording | cue presentation<br>(trial light) | wait period<br>(no light) | food presentation | saline injection<br>1 ml/kg | ghrelin injection<br>250 µg/kg/ml | leptin injection<br>1 mg/kg/ml | end of experiment<br>adjusting tetrodes |

**Table 2**
Overview of a single experimental session. Experimental procedures (bottom) are presented in chronological order; time-indications (top row) show the length and specific moment at which each of the procedures is executed.

*Data analysis*
All analyses were performed using SPSS for Windows (version 15.0) unless stated otherwise.

*Behavioural data*
To assess locomotor activity during the recording sessions, the animals were recorded using a CinePlex video tracking system (Plexon, Dallas, USA). For the purpose of quantifying anticipatory behaviour the total distance the animals moved during base-line and behavioural events was calculated. Data were analyzed by means of a repeated-measures ANOVA. In case an effect of time was observed a one-way ANOVA with simple-first contrast was used to compare behavioural activity during cue presentation, wait and first five minutes of food access with base-line locomotor activity.

*Electrophysiological data*
Single units were isolated by offline cluster cutting procedures (Offline Sorter version 3, Plexon, Dallas, USA). Before a cluster of spikes was accepted as belonging to a single unit, several parameters had been checked visually, namely the averaged waveforms across the four leads of each tetrode, the cluster plots showing spike parameter distributions such as peak amplitudes across the four dimensions and principal components, the autocorrelogram and the spike interval histogram. Since the absence of spike activity during the refractory period is indicative of good isolation, units of which the autocorrelogram and the spike interval histogram revealed activity during this period (<1500 µs) were removed

from the analysis. Cells with a base-line firing frequency less than 0.1 Hz were not considered for analysis, as were cells with frequencies more than 3 standard deviations from the mean (e.g. van Duuren et al., 2007[39]). To exclude effects on neuronal activity of handling the animals during hormone/vehicle administration, data from 10 sec prior to, until 10 sec following hormone/drug administration were not included in the analyses. Correlations between firing rate of individual neurons and events in the task were analyzed in several steps (see below).

*Population analysis*

To answer the question how DMH/VMH neurons respond as a group to events and hormones first a population analysis was performed.

Events and drug/vehicle

First, overall population responses were analyzed for the events cue presentation, wait, and food presentation and hormone/vehicle administration. Firing activity during each of the events was first calculated as the average frequency per 1 minute time-bin. Subsequently the response of each individual neuron was expressed as a percentage of its own base-line activity. Comparisons were then made between 5 min of averaged base-line activity (i.e. 5 bins) preceding the event and 5 min following the event. For cue presentation and wait period, firing frequency of the entire period was compared with base-line (one-way ANOVA, $p < 0.05$). The effects of hormone injection, as well as food presentation, were analyzed by comparing the average acute neuronal response in the 5 min following injection and over a sub-acute period of 30 minutes (one-way ANOVA, $p < 0.05$).

Locomotor activitiy

To examine the relation between locomotor activity (see above) and neuronal firing, the correlation between these factors were analyzed. For each animal population firing activity was compared to locomotor activity over a period of 10 minutes (1 min bins) prior to saline injection. To exclude possible interaction with events, the time-periods chosen did not contain any feeding behaviour or activity prior to food presentation. To adjust for individual differences, data were normalized to 5 min of pre-stimulus base-line (see above). A regression analysis was made over all animals, comparing locomotor activity to average neuronal firing per session. Pearson's correlation coefficient was calculated and analyzed for significance.

Regression analysis

To assess if neuronal responses to events and to hormone/vehicle are functionally related neuronal responses, a regression analysis was performed. Correlation coefficients were analyzed for significance with ($p < 0.05$), responses identified as outliers by Cook's analysis ($D_i > 1$) were removed from the dataset.

*Individual neurons*

In addition to population responses to events and hormones, analyses were performed to assess if there are neurons that respond to specific stimuli.

Neuron classification

Following population analysis, individual neurons were analyzed for their response to events and hormone/vehicle application by comparing base-line firing frequency to averaged firing frequency during events and hormone application (t-test, $p < 0.05$). Based on these data cells were classified, per event/hormone, as non-responding, increasing- or decreasing to events and/or hormone/vehicle.

Event-related responses to hormone/vehicle

In case population analyses revealed in- or decreased activity during events, the possible relation of these changes to hormone/vehicle responses were analyzed. For that purpose average firing frequency in event-responsive neurons was compared with firing frequency following hormone/vehicle administration using paired-sample t-tests.

Regression analysis

Similarly to the population analyses, possible functional relations between responses to any event and hormone were analysed by means of regression analyses. Only cells that responded to both an event and hormone were included in this analysis (linear regression). Correlation coefficients were analyzed for significance with ($p < 0.05$), responses identified as outliers by Cook's analysis ($D_i > 1$) were removed from the dataset.

*Histology*

The final position of the tetrodes was marked by passing a 10 s, 25 µA current through one of the leads of each tetrode in order to induce a lesion and initiate gliosis. During this procedure the animals were anaesthetized. After 24 hours,

the animal was perfused transcardially using a 0.9% saline solution followed by 4% paraformaldahyde (PFA). After removal from the skull, the brain was stored overnight in PFA and subsequently transferred to a 30% sucrose solution with added sodium azide (0.05%). Brain sections (40 µm) were cut using a vibratome and Nissl-stained to identify the location of the probe and the final position of the tetrodes.

## RESULTS

*Anticipatory locomotor activity*

Locomotor activity, expressed as total distance moved, was measured during base-line, cue presentation, wait period and the first 5 minutes of food access. Locomotor activity was selectively increased during cue presentation, indicating that cue presentation was able to elicit FAA. In contrast, during a period of waiting, following cue presentation and preceding food delivery, locomotor activity was not different from base-line levels. (Figure 1).

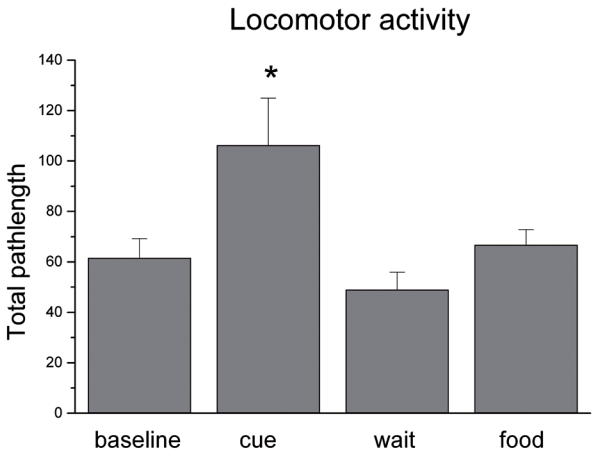


**Figure 1**

Locomotor activity in response to cue presentation, during the waiting period and food delivery. A repeated measures ANOVA indicated a significant effect of time (F=7.735, p=0.008, Greenhouse-Geisser corrected for sphericity). A one-way ANOVA (simple-first contrast) revealed that locomotor activity was exclusively increased during cue presentation (base-line vs. cue; F=11.27, p=0.005, base-line vs. wait; F=2.742, p=0.122, base-line vs. food; F=0.303, p=0.592).

*Electrophysiology*

During the 14 recording sessions, 93 individual neurons were recorded in the DMH/VMH in a total of 6 male Wistar rats. For each analysis, the number of cells that

were included based on our exclusion criteria (*see materials and methods* section) is mentioned. Figure S1 depicts example neurons recorded on a single tetrode.

Population analysis

Since previous Fos and lesion studies implicated the DMH/VMH nuclei in FAA, we first considered the role of the entire neuronal population in FAA and in response to leptin and ghrelin. Population responses to the cue and during waiting were analysed over the entire period. Responses to food, hormone and saline were analysed for two time-periods, the initial 5 minute- (acute) and 30-minute (sub-acute) time window.

Cue and wait

Population analyses of neuronal activity during cue-presentation and wait revealed a significant increase in firing frequency during cue presentation, whereas firing frequency during the wait period was not different from base-line activity (see Figure 2). A food-signalling cue thus activates the DMH/VMH neuronal population.

Acute effects (Food, hormone/vehicle, 5 min)

Population analysis showed that food presentation did not affect firing activity acutely (Figure 2). Similarly, saline and leptin injections did not affect firing activity in the first 5 minutes following administration. In contrast, ghrelin did significantly increase neuronal firing compared to base-line (see Figure 2).

Sub-acute effects (Food, hormone/vehicle, 30 min)

Regarding sub-acute effects of food presentation and hormone administration, only food availability induced a significant increase in firing frequency in the neuronal population, saline and ghrelin did not affect neuronal activity. Leptin showed a trend towards increased activity. Normalized firing activity for each of the events is illustrated in Figure 2.

Thus, data indicate that both a cue predictive of food as well as circulating ghrelin can acutely activate the DMH/VMH. Sub-acutely, the population increases firing frequency during food availability and shows a trend towards an increase in response to leptin.

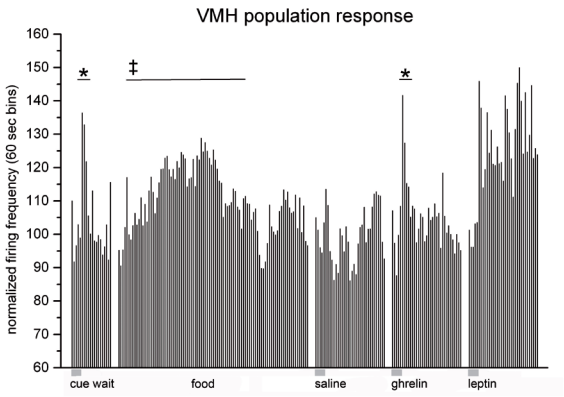


**Figure 2**

Neuronal population response to behavioural events and hormone administration. The grey horizontal bars indicate base-line measurements. An ANOVA was performed to assess effects of cue presentation, waiting period, food presentation and hormone administration. Firing frequency was analyzed over a period of 5 minutes (cue, acute effects of food availability and hormone administration), 10 minutes (waiting period), or 30 minutes (sub-acute effects of food presentation and hormone administration) and compared to base-line firing. Significant increases of neuronal firing activity were observed during cue presentation (N=75, F=8.960, p=0.004), food availability (30 min; N=75, F=7.394, p=0.007) and ghrelin administration (5 min; N=80, F=4.511, p=0.035). A trend towards an increase was shown for leptin (30 min; N=65, F=3.578, p=0.06). Saline injection did not affect overall neuronal activity (5 min; N=80, F=0.121, p=0.729, 30 min; N=81, F=0.936, p=0.335), nor did the waiting period (N=72 F=0.158, p=0.692), acute food availability (5 min; N=80, F=1.555, p=0.217), sub-acute ghrelin (30 min; N=80, F=0.831, p=0.363), or acute leptin (5 min; N=78, F=1.596, p=0.208) * significant increase (5 min) $p < 0.05$, ‡ significant increase (30 min) $p < 0.05$.

Regression analysis between locomotor activity and firing frequency

As one might argue that the increased firing frequency during cue presentation might be induced by pure locomotor activity, the relation between these two parameters was assessed during cue presentation and at a moment without cue, food or hormone influences. Regression analysis revealed that there is neither a significant correlation between neural activity in DMH/VMH and locomotor activity during cue presentation nor at a moment without other influences (resp.,$r^2$=0.003, F=0.169, p=0.682, $r^2$=0.000, F=0.001, p=0.972; data not shown). This indicates that increases in firing frequency during cue presentation are not due to the observed increase in locomotor activity in this period.

Regression analysis on a population level

To assess whether the neuronal response to leptin or ghrelin was predictive of the change in firing frequency during cue presentation, and thus functionally related , a regression analysis was performed on all neurons that were measured during both events (cue, wait, food) and subsequent hormone/vehicle administration. Due to exclusion of neurons based on Cook's analysis for outliers not all groups contained equal numbers of neurons. Population respon-

ses to cue and ghrelin (30 min) showed a significant positive relation ($r^2$=0.26). Wait and leptin (5 and 30 min), in contrast, showed a significant negative correlation (resp. $r^2$=-0.27 and -0.28). Responses to saline showed no relation to any event. A complete overview of all data is shown in the supplementary information S2.

Results so far revealed that a cue signalling food availability elicited both anticipatory locomotor and activation of neuronal activity on a population level. In addition, neuronal activity increased acutely in response to ghrelin, and sub-acutely following food presentation and leptin administration. On a population level the response to cue correlated only weakly with the sub-acute response to ghrelin (see figure S2).

Individual neurons

To assess the relation between event-related firing and hormone responsivity in individual neurons each cell was analyzed for its response to events and hormones and classified accordingly. Diversity in individual responses is expected given the low value of explained variance of correlated firing in the population and the heterogeneity of neuronal types such as in the VMH[40]. As such, absence of a population response, like for leptin, does not exclude responsivity to leptin of individual neurons.

Neuron classification

Firing activity during events and following hormone/vehicle administration were compared with normalized base-line activity using 2-tailed t-tests. Significant changes in firing activity are presented in table S3. For these analyses, 76 neurons were included for calculation of cue, wait and food presentation. Seven neurons were excluded from the analysis of hormone/vehicle due to missing data.

Event-related responses to hormone/vehicle

Neurons were grouped according to their response to cue, wait and/or food. Subsequently, we determined how these grouped neurons responded to ghrelin, leptin and saline. We could thus identify for instance those neurons that were activated during presentation of cue and assess whether this group also responded to ghrelin. Results showed that cells that decreased firing activity to cue, also selectively decreased firing in response to ghrelin (5 min and 30 minutes; see Figure 3), but not to leptin or saline. Although neurons responsive

to food (30 min) were also shown to respond to leptin and saline, regression analysis for these data showed that these responses were not related. In contrast, cue-related responsivity to ghrelin were found to be functionally related as shown by population regression analyses (see also below; *Regression analysis on a single neuron level*). No significant changes in response to any of the hormones could be detected in wait- or food availability (5 min) responding neurons. An overview of all responses is shown in table S4.

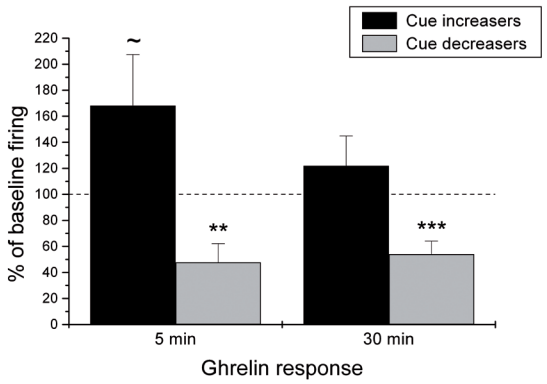


**Figure 3**
Response to ghrelin administration of neurons that significantly change their firing frequency following cue presentation. Paired Student's t-tests comparing base-line firing frequency to firing frequency in response to ghrelin administration revealed that neurons that reduce their firing frequency in response to cue, also decrease firing frequency in response to ghrelin (5 min and 30 min). Furthermore, cells that increased firing frequency during cue, showed a trend towards increased firing frequency following ghrelin administration (5 min). ** significant $p < 0.01$, *** significant $p < 0.001$, ~ trend, $p = 0.097$.

Regression analysis on a single neuron level

To investigate whether neuronal responsivity to events and hormone/vehicle were meaningfully related, a regression analysis was performed on neurons that responded significantly to both hormone/vehicle (saline, ghrelin, leptin) and a specific event (cue, wait, food access). These analyses were performed to assess the predictive value of the relation between events and hormone/vehicle. In these neurons, responses to cue and ghrelin (5 min and 30 min), and food (5 min) and ghrelin (5 min and 30 min) showed a significant, positive correlation, whereas no correlation could be detected in the responses to wait and ghrelin (5 min and 30 min) (see Figure 4). Leptin-responsive neurons (30 min) correlated positively with food presentation (5 min). No significant correlations were detected between responses to saline and cue, wait or food presentation. A complete overview of all data is shown in table S5.

Thus, analysis of individual neurons revealed a highly correlated response to ghrelin and cue presentation in a subset of neurons.

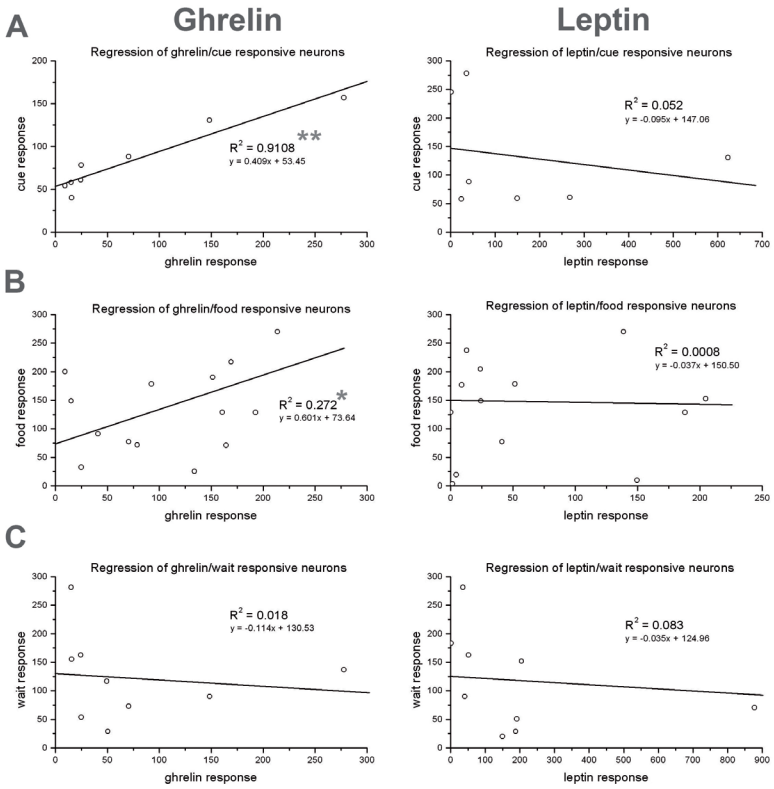


**Figure 4**

Regression analyses of ghrelin/leptin (5 min) and cue, wait and food presentation (5 min) in subsets of neurons that responded significantly to both cue/wait/food and ghrelin/leptin (5 min). Responses to ghrelin and cue ($r=0.95$, $p < 0.01$; panel A left), and ghrelin and food ($r=0.52$, $p < 0.05$; panel B left) showed a significant, positive correlation, whereas no correlation could be detected in the responses to ghrelin and wait ($r=-0.13$, $p=0.74$; panel C left). Acute responses to leptin and cue ($r=-0.23$, $p=0.62$; panel A right), leptin and food ($r=-0.03$, $p=0.93$; panel B right), and leptin and wait ($r=-0.29$, $p=0.42$; panel C right) did not display a significant correlation. * significant $p < 0.05$, ** significant $p < 0.01$.

*Histology*

Histological verification of the positions of the tetrodes confirmed that the recording sites were located in the DMH and the VMH. The average tetrode endpoints (± SD) and an example histological section are depicted in figure S6; figure adapted from Paxinos and Watson, 2005.

## DISCUSSION

The present study indicates that a neural population within VMH/DMH responds to food cues as well as ghrelin. Moreover, this population could drive FAA and feeding activity following stimulation by ghrelin. The need to understand what happens in the brain when food is expected is relevant to the understanding of obesity and eating disorders. Medial hypothalamic nuclei, as well as ghrelin have been implicated in FAA, but to date it has been poorly understood how. To the best of our knowledge, this is the first report that shows that DMH/VMH neurons respond to cues predictive of food availability, to food presentation and to ghrelin administration.

A cue that signals food availability induced FAA and increased firing frequency in the DMH/VMH. As outlined in the introduction, the DMH/VMH have been implicated in the network responsible for mediating FAA. Whereas FAA has been shown to induce Fos expression in these areas[6-10], lesions attenuated FAA[6,11-19]. An initial aim of these experiments was to examine how FAA is represented by neuronal activity in the DMH/VMH. in these experiments we aimed to FAA.w FAA in our behavioural setup modualtes

To assess FAA we used a pseudo-random feeding-schedule in which rats were given access to food at a variable time of the day. In order to induce FAA in these animals the presentation of food was preceded by a light cue. In line with previous studies[2-4], locomotor activity was specifically increased during cue presentation. Parallel to the increase in locomotor activity, the measured neuronal population responded with an increase in firing frequency to cue presentation. As non-specific locomotor activity and firing frequency did not correlate at a moment without cue, food or hormone/vehicle influences, the increased neu-

ronal population activity in the DMH/VMH during cue does not reflect general locomotor activity, but rather specifically FAA. Interestingly, this fits very well with previous data that implicated the DMH and VMH in FAA by revealing increased expression of immediate early genes *zif268* and *c-fos* in these areas during cue presentation.[2]

Together these data corroborate previous indirect evidence from both immediate-early-gene activity and lesion studies for the involvement of DMH/VMH in relation to FAA. Our data indicate that, in fact, the population response is one of increased activity.

In addition to the response to cue, analyses of the population response revealed that neuronal activity increased acutely following ghrelin administration. Sub-acutely, food intake increased VMH neuronal activity, while leptin induced a trend towards increased activity. Leptin receptors[41-43] and GHS-R1a[21,44,45] are, amongst other brain areas, expressed in DMH and VMH. The observed increases in firing frequencies in response to ghrelin and leptin are in line with studies showing that leptin administration increased Fos immunoreactivity in DMH and VMH[46-49], while ghrelin induced Fos activation in DMH, but not in VMH.[34,50,51]

These data suggest that FAA may be regulated within the DMH/VMH network through ghrelin and possibly leptin signalling. However, to date, a direct modulation of DMH/VMH neurons that respond during FAA by these circulating hormones has not been reported. In order to address this issue, regression analyses were performed to assess the relation between neural responses during events and hormone administration. The sub-acute responses to ghrelin and the responses to cue were positively correlated (r=0.26) on a population level, whereas the sub-acute responses to leptin and to wait were negatively correlated (r=-0.28). Remarkably, ghrelin levels correlated with the amount of FAA in rats on a RFS[22] and ghrelin administration increased FAA[24]. Leptin, on the other hand, seems to have an inhibitory effect on FAA.[26,28,52-54]. Although the observed correlations are in line with current literature, the explained variance by these parameters is relatively low. Population cue responses explain only 7% of the variation in ghrelin responses. These data, thus, confirm earlier work, but

suggest that there is limited coherence in the DMH/VMH response to FAA and response to feeding hormones *on a population level*.

The most parsimonious explanation for this is that not all neurons in the DMH/VMH are functionally similar and that a proportion of the cells in the population does not respond to events and/or hormones or responds in an opposite manner. In a population analysis these neurons can, therefore, mask the effect of neurons that do respond. Previous work of Sabatier et al.[40], who measured VMH neurons, supports this explanation by showing the presence of several sub-types of neurons within the VMH. Such functional heterogeneity within the medial hypothalamic complex could explain the relatively limited impact of lesions on FAA and the lack of predictive power of cue responses to ghrelin-related activation of neuronal activity. Therefore, it is imperative to examine responses of individual neurons.

A subpopulation of neurons within the DMH/VMH exhibited highly correlated responses to cue and ghrelin. Following the population analyses, individual neurons were classified as increasing, decreasing or not responding to events/hormones. As expected, differential responses of individual neurons to these parameters were observed.

Population analyses revealed an overall increase in firing activity in response to cue. However, examination of individual responses showed that while a subset of neurons (15 %) responded with significant increases to cue, 10% of the population significantly reduced their firing frequency. Similar heterogeneity in response was observed for the events wait and food presentation. As indicated in the previous section, these responses are of particular interest when considered in relation to modulatory effects of ghrelin and leptin.

In response to ghrelin administration, approximately equal numbers of neurons increased and decreased firing frequency. A previous *in vivo* electrophysiology study showed that ghrelin's effects on firing frequency in VMH neurons depended on the cell type.[55] However, the effect of hormone administration in *in vivo* electrophysiology experiments can be direct, via receptors on the measured neuron, or indirect, via responses of projecting neurons. *In vitro* electrophysiology experiments, which measure direct responses of hormones on firing fre-

quency of individual neurons, increased firing frequency in the majority of VMH neurons.[56-58] Similarly, previous *in vivo* electrophysiological studies in anesthetized rats showed that the majority of VMH neurons increased firing frequency in response to leptin administration.[59], whereas others have shown with *in vitro* studies that the effects of leptin are depended on the specific cell-type.[60,61]
These previous studies indicate that neuronal activity in response to events or hormones depends on cell type and these responses can be observed within a single brain area, once again illustrating the need to couple the responses of individual neurons to events/hormone to behavioural activity.

To examine whether a relation exists between responses to events and hormones in individual neurons, regression analyses were performed on responses in neurons that exhibited significant effects of both a certain event and hormone. Sub-acute responses to leptin correlated significantly with acute responses to food availability (r=0.76). This is somewhat unexpected, as leptin is known as an anorexigenic hormone[62,63], moreover, neurons activated by food access (sub acutely) decreased firing frequency upon leptin administration. Furthermore, this correlation is not supported by regression analysis of leptin and food at other time-points (S5). In contrast to this unexpected finding, the effects of correlated responses to cue and ghrelin were observed at all examined levels, ranging from population to individual neurons.

Acute responses to food availability correlated significantly with acute (r=0.52) and sub-acute (r=0.57) responses to ghrelin. Ghrelin is known as an orexigenic hormone[33,64-66] and this neuronal population could potentially mediate this effect. Intriguingly, an even stronger correlation existed between responses to cue and the acute (r=0.95) and sub-acute (r=0.89) responses to ghrelin. Ghrelin levels are known to rise preprandially[67,68] and to correlate with FAA[22]. Although ghrelin knockout mice[69,70] still exhibited FAA, GHS-R1a knockout mice showed attenuated FAA[22-25]. In addition, ghrelin stimulated FAA[24], whereas GHS-R1a antagonism reduced FAA[22]. Taken together, the present study indicates that a subset of neurons within the DMH/VMH exhibits highly correlated responses to cue and ghrelin. As such, this population couples neuronal activity during FAA to ghrelin, and might be able to modulate FAA and food intake in response to high ghrelin levels.

The finding that a subpopulation of neurons responds to both FAA and ghrelin and that cue responsive neurons respond in the same manner (i.e. decrease or increase) fits very well with previous findings of distinct neural subpopulations within the VMH. Intriguingly, the relation between cue- and ghrelin- responsiveness is observed at the population level as well as in single cells. Whereas in the former case, cue responses are only modestly predictive of neuronal activity in response to ghrelin (7%), predictive value increases to as much as 91% in a sub population of DMH/VMH neurons that make up roughly 10% of the total number of neurons.

## CONCLUSIONS

This is the first study to directly measure neuronal activity in DMH/VMH during FAA in combination with ghrelin and leptin administration in awake, behaving animals. Cue-signalled food presentation, which occurred at random, elicited FAA and an increase in firing frequency of the neuronal population. Responses to ghrelin and to cue correlated in the measured neuronal population. Interestingly, on the level of individual neurons, a subset of neurons that responded significantly to both cue and ghrelin showed highly correlated responses. Altogether, this study reveals a role for ghrelin, but not leptin, signalling within DMH/VMH in FAA on both a population level as well as in single cells. These data thus support earlier Fos-data and lesion-studies that have implicated these areas in mediating FAA. Moreover, these data extent these previous findings by identifying a subset of neurons on which cue information and ghrelin signalling converge, possibly to drive hypothalamic FAA.

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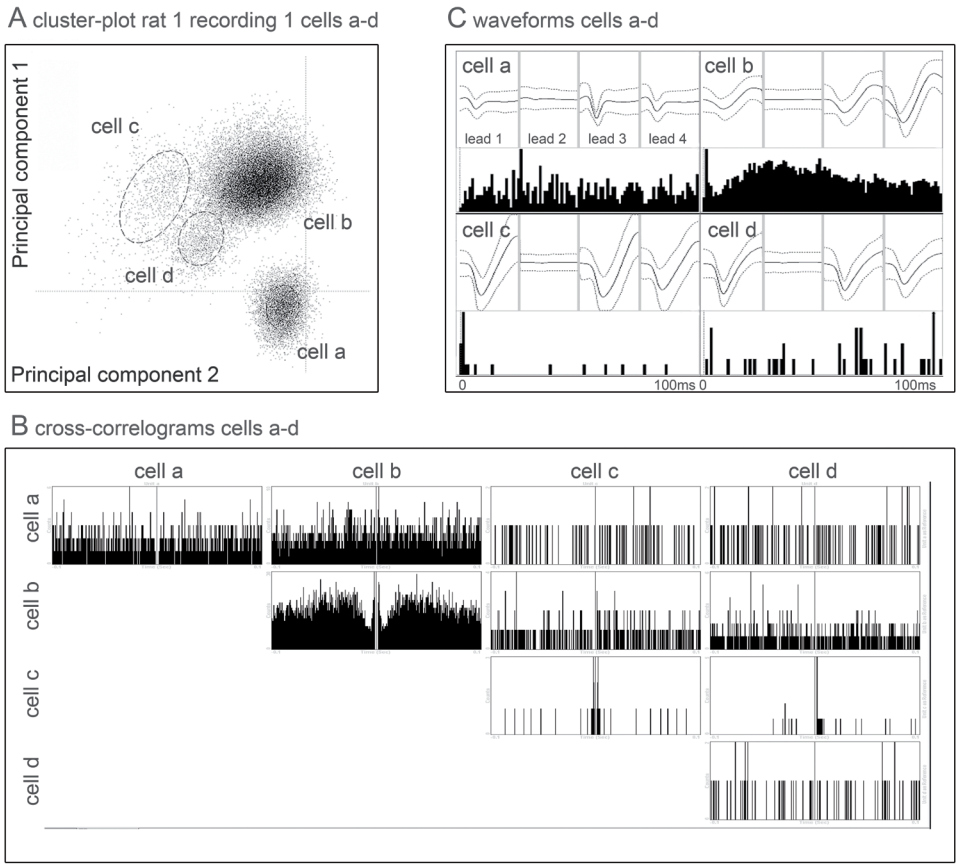
## SUPPLEMENTAL DATA




**Supplemental data 1**

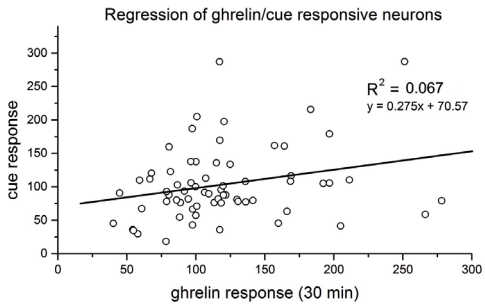
Cluster-plot, auto- and cross-correlograms and waveforms of 4 example neurons measured in a single session on a single tetrode.

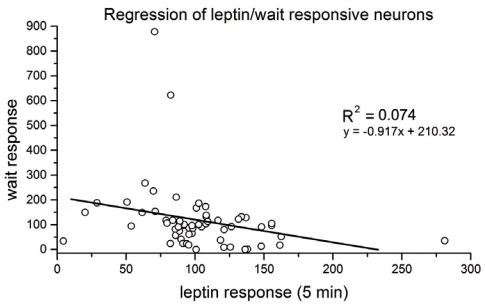
**A)** Plot of the principal component analysis showing 4 separate clusters, indicating 4 separate neurons. The circles in this plot delineate the center of each of the clusters

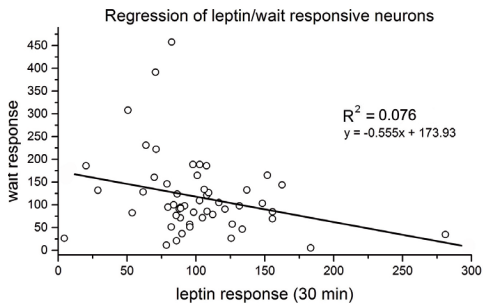
**B)** Auto – and cross-correlations between the four neurons depicted in (A). Auto-correlations (diagonal) were calculated to assess if individual spike-clusters (neurons) do not show spiking activity during their refractory period (indicative of contamination with spikes from a separate cluster). Cross-correlations were performed to analyze if separate neurons exhibit correlated firing activity. These data indicate related activity between neurons c and d (neuron d follows c), but otherwise unrelated firing activity.

**C)** This graph shows the waveforms of each recorded neuron and interspike-interval. The top graphs show the waveforms of neurons a-d as recorded on each of four tetrode leads (i.e. individual electrodes that are combined to make-up a tetrode). The bottom half of each graph shows the interval between successive action-potentials. The dashed vertical lines indicate the 1.5 ms period. These data were used to identify individual neurons.

| | | 5 min | | | | | 30 min | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *n* | *r* | r2 | p-value | | n | r | r2 | p-value | |
| saline | cue | 61 | 0.17 | 0.03 | 0.19 | | 68 | 0.05 | 0 | 0.71 | |
| | wait | 62 | 0.05 | 0 | 0.71 | | 69 | -0.03 | 0 | 0.83 | |
| | food 5 min | 62 | -0.15 | 0.02 | 0.24 | | 69 | -0.07 | 0 | 0.6 | |
| | food 30 min | 64 | -0.1 | 0.01 | 0.43 | | 71 | -0.11 | 0.01 | 0.36 | |
| ghrelin | cue | 60 | 0.19 | 0.04 | 0.15 | | 67 | 0.26 | 0.07 | 0.04 | * |
| | wait | 62 | 0.11 | 0.01 | 0.4 | | 68 | 0.05 | 0 | 0.71 | |
| | food 5 min | 62 | 0.2 | 0.04 | 0.12 | | 68 | 0.19 | 0.04 | 0.12 | |
| | food 30 min | 64 | 0.13 | 0.02 | 0.31 | | 70 | 0.08 | 0.01 | 0.51 | |
| leptin | cue | 61 | -0.13 | 0.02 | 0.33 | | 53 | -0.16 | 0.02 | 0.27 | |
| | wait | 61 | -0.27 | 0.07 | 0.03 | * | 53 | -0.28 | 0.08 | 0.04 | * |
| | food 5 min | 62 | -0.22 | 0.05 | 0.09 | | 54 | 0.03 | 0 | 0.86 | |
| | food 30 min | 64 | -0.14 | 0.02 | 0.27 | | 57 | 0.13 | 0.02 | 0.35 | |




**Supplemental data 2**

Population regression analyses for events (cue/wait) and food, hormone and saline. Regression plots of significant correlations (indicated by *) are shown below.

| | 5min | | 30min | |
|---|---|---|---|---|
| | increase | decrease | increase | decrease |
| cue | n=14, 188.78 ± 18.23 % | n=9, 59.82 ± 5.03 % | - | - |
| wait | n=11, 196.39 ± 26.92 % | n=10, 76.52 ± 8.64 % | - | - |
| food | n=21, 210.10 ± 17.74 % | n=18, 48.70 ± 26.92 % | n=20, 218.33 ± 15.62 % | n=16, 42.90 ± 7.46 % |
| | | | | |
| saline | n=11, 747.20 ± 1518.00 % | n=26, 42.60 ± 23.43 % | n=8, 1389.41 ± 950.32 % | n=24, 50.51 ± 3.91 % |
| ghrelin | n=14, 202.04 ± 21.13 % | n=16, 38.85 ± 6.29 % | n=12, 89.05 ± 11.93 % | n=15, 50.12 ± 5.00 % |
| leptin | n=14, 498.19 ± 160.72 % | n=19, 20.09 ± 4.98 % | n=11, 286.61 ± 84.21 % | n=18, 45.25 ± 4.93 % |

**Supplemental data 3**

Average normalized increases and decreases in firing frequency in response to events and hormones.

**Supplemental data 4**

Classification of all neurons according to their response to events and hormones.

| | | **Cue** | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **Increase** | | | **Decrease** | | | |
| | | *n* | *Average ± SEM* | *p-value* | *n* | *Average ± SEM* | *p-value* | |
| **Saline** | 5 min | 12 | 118.45 ± 23.29 | 0.437 | 9 | 64.37 ± 15.77 | 0.038 | |
| | 30 min | 12 | 116.10 ± 21.86 | 0.469 | 9 | 84.67 ± 19.30 | 0.439 | |
| **Ghrelin** | 5 min | 11 | 168.20 ± 39.12 | 0.097 | 9 | 47.71 ± 14.36 | 0.002 | ** |
| | 30 min | 11 | 121.92 ± 22.91 | 0.35 | 9 | 54.03 ± 10.01 | 0 | *** |
| **Leptin** | 5 min | 12 | 100.79 ± 14.87 | 0.958 | 8 | 98.66 ± 28.78 | 0.964 | |
| | 30 min | 13 | 119.15 ± 32.21 | 0.558 | 8 | 110.86 ± 30.36 | 0.726 | |

**Wait**

| | | Increase | | | | Decrease | | |
|---|---|---|---|---|---|---|---|---|
| | | *n* | *Average ± SEM* | *p-value* | | *n* | *Average ± SEM* | *p-value* |
| **Saline** | 5 min | 10 | 887.43 ± 761.81 | 0.315 | | 9 | 697.98 ± 575.86 | 0.315 |
| | 30 min | 10 | 908.00 ± 767.62 | 0.306 | | 9 | 313.38 ± 232.86 | 0.373 |
| **Ghrelin** | 5 min | 10 | 117.46 ± 21.15 | 0.42 | | 9 | 147.66 ± 42.68 | 0.281 |
| | 30 min | 10 | 91.62 ± 9.18 | 0.373 | | 9 | 108.48 ± 24.55 | 0.734 |
| **Leptin** | 5 min | 11 | 128.79 ± 38.11 | 0.459 | | 9 | 430.67 ± 231.30 | 0.172 |
| | 30 min | 11 | 113.01 ± 25.47 | 0.615 | | 9 | 262.87 ± 109.14 | 0.155 |

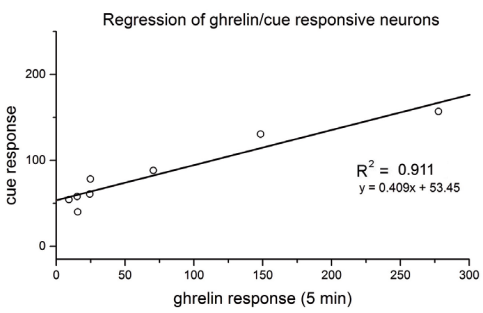
**Food 5 min**

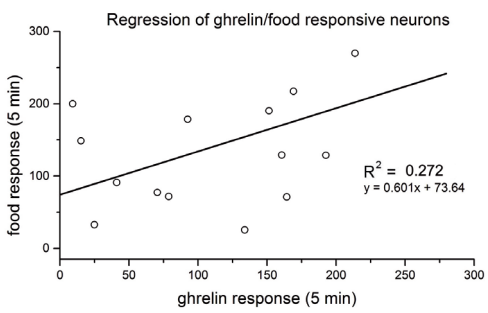
| | | Increase | | | | Decrease | | |
|---|---|---|---|---|---|---|---|---|
| | | *n* | *Average ± SEM* | *p-value* | | *n* | *Average ± SEM* | *p-value* |
| **Saline** | 5 min | 20 | 114.64 ± 34.30 | 0.672 | | 14 | 80.87 ± 10.11 | 0.069 |
| | 30 min | 20 | 98.79 ± 14.98 | 0.936 | | 14 | 88.26 ± 10.61 | 0.279 |
| **Ghrelin** | 5 min | 20 | 127.20 ± 15.47 | 0.087 | | 14 | 100.08 ± 11.97 | 0.995 |
| | 30 min | 20 | 114.51 ± 13.28 | 0.282 | | 14 | 92.86 ± 10.73 | 0.511 |
| **Leptin** | 5 min | 18 | 95.73 ± 26.29 | 0.872 | | 16 | 85.38 ± 11.25 | 0.204 |
| | 30 min | 10 | 132.27 ± 27.97 | 0.264 | | 16 | 89.04 ± 14.72 | 0.462 |

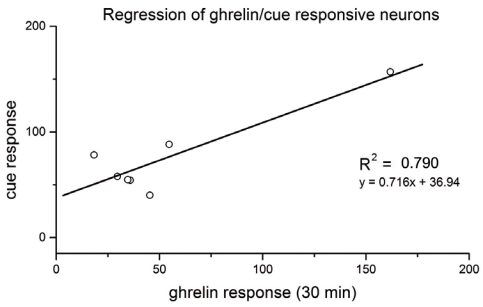
**Food 30 min**

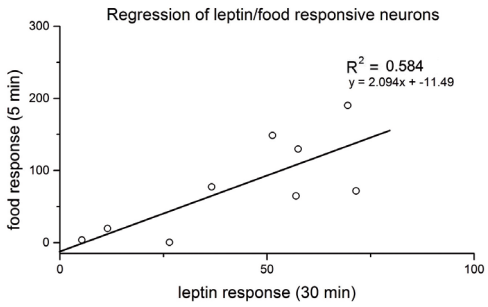
| | | Increase | | | | Decrease | | |
|---|---|---|---|---|---|---|---|---|
| | | *n* | *Average ± SEM* | *p-value* | | *n* | *Average ± SEM* | *p-value* |
| **Saline** | 5 min | 19 | 66.12 ± 7.94 | 0 | *** | 12 | 83.25 ± 9.81 | 0.102 |
| | 30 min | 20 | 81.77 ± 8.87 | 0.047 | * | 12 | 87.32 ± 10.96 | 0.26 |
| **Ghrelin** | 5 min | 20 | 114.52 ± 13.12 | 0.275 | | 12 | 103.54 ± 14.01 | 0.803 |
| | 30 min | 20 | 101.35 ± 11.48 | 0.907 | | 12 | 91.20 ± 12.26 | 0.48 |
| **Leptin** | 5 min | 18 | 67.42 ± 13.80 | 0.024 | * | 13 | 84.67 ± 13.82 | 0.278 |
| | 30 min | 10 | 123.12 ± 26.45 | 0.393 | | 14 | 117.50 ± 31.05 | 0.578 |

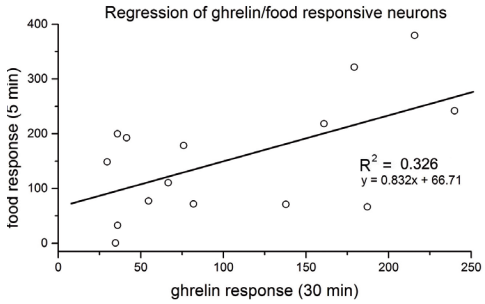
| | | 5 min | | | | | 30 min | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *n* | *r* | r2 | p-value | | n | r | r2 | p-value | |
| saline | cue | 10 | 0.15 | 0.02 | 0.68 | | 8 | -0.3 | 0.09 | 0.48 | |
| | wait | 6 | 0.11 | 0.01 | 0.83 | | 5 | 0.32 | 0.1 | 0.61 | |
| | food 5 min | 16 | -0.02 | 0 | 0.94 | | 13 | -0.01 | 0 | 0.97 | |
| | food 30 min | 13 | -0.45 | 0.2 | 0.12 | | 13 | -0.23 | 0.06 | 0.44 | |
| ghrelin | cue | 8 | 0.95 | 0.91 | 0 | ** | 7 | 0.89 | 0.79 | 0.01 | * |
| | wait | 9 | -0.13 | 0.02 | 0.74 | | 5 | -0.57 | 0.32 | 0.32 | |
| | food 5 min | 15 | 0.52 | 0.27 | 0.05 | * | 15 | 0.57 | 0.33 | 0.03 | * |
| | food 30 min | 13 | 0.19 | 0.04 | 0.54 | | 14 | 0.49 | 0.24 | 0.08 | |
| leptin | cue | 7 | -0.23 | 0.05 | 0.62 | | 12 | -0.17 | 0.03 | 0.59 | |
| | wait | 10 | -0.29 | 0.08 | 0.42 | * | 7 | -0.31 | 0.09 | 0.5 | |
| | food 5 min | 14 | -0.03 | 0 | 0.93 | | 9 | 0.76 | 0.58 | 0.02 | * |
| | food 30 min | 14 | 0 | 0 | 1 | | 10 | 0.22 | 0.05 | 0.55 | |



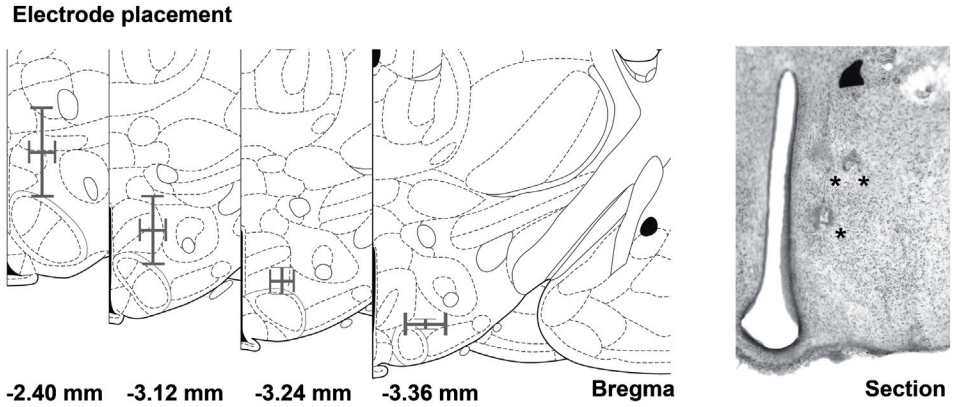






**Supplemental data 5**

Overview of all regression analyses between events and hormones for all neurons that significantly respond to and event and hormone administration. Regression plots of significant correlations (indicated by *) are shown below.




**Supplemental data 6**

Histological examination. Depicted are the average recording-endpoints (± SD) (left). A histological section with individual endpoints (indicated by an asterix) is shown on the right.

# Chapter 5

## GHS-R1A SIGNALLING IN THE DMH AND VMH CONTRIBUTES TO FOOD ANTICIPATORY ACTIVITY

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*Submitted*

## ABSTRACT

Rats that have restricted access to food at a fixed time point of the circadian phase display high levels of food anticipatory activity (FAA). The orexigenic hormone ghrelin has been implicated in the regulation of FAA. However, is not known via which brain area ghrelin exerts this effect. GHS-R1a is highly expressed in the hypothalamus, including the dorsomedial hypothalamus (DMH) and the ventromedial hypothalamus (VMH). These two hypothalamic areas are activated during FAA. To establish local knockdown of GHS-R1a in the DMH and VMH, we used AAV expressing a shRNA directed against GHS-R1a in rats. Under *ad libitum* conditions, knockdown of GHS-R1a in the VMH increased food intake and body weight gain. In addition, GHS-R1a knockdown in VMH and DMH reduced body temperature and running wheel activity. When rats were subjected to a restricted feeding schedule, the main effect of GHS-R1a knockdown in both DMH and VMH was a decrease in running wheel activity and an attenuation of body weight loss. Rats with knockdown of GHS-R1a in DMH and VMH showed a delay in onset of FAA. In addition, GHS-R1a knockdown in DMH resulted in a reduction of FAA amplitude. This is the first study to investigate the effect of local hypothalamic knockdown of GHS-R1a on FAA. Our results implicate hypothalamic GHS-R1a signalling in the regulation of FAA. Nevertheless, some FAA remained, suggesting that a distributed network of brain areas and signalling pathways is involved in the development of FAA.

## INTRODUCTION

The orexigenic peptide ghrelin is the endogenous ligand of growth hormone secretagogue receptor 1a (GHS-R1a).[1] It is produced in the stomach and released into the circulation.[1] Ghrelin stimulates growth hormone release[1], but also acts as a potent stimulator of appetite[2-7] and can cause an increase in fat mass[2]. Ghrelin plasma levels are increased in Anorexia Nervosa patients and after fasting[8]. In contrast, weight gain and obesity are associated with reduced plasma ghrelin levels[8,9]. Ghrelin's orexigenic effect is at least partly mediated via Agouti-related Peptide (AgRP) and Neuropeptide Y (NPY) in the arcuate nucleus.[5,10] In addition to the arcuate nucleus, GHS-R1a is also expressed in other hypothalamic regions, such as the ventromedial hypothalamus (VMH) and dorsomedial hypothalamus (DMH).[11-13]

Rats subjected to a restricted feeding schedule (RFS), with access to food for a limited period at a fixed time point of their circadian phase, show hyperactivity preceding their expected meal; a behaviour referred to as FAA.[14] Circadian rhythms are normally under control of the suprachiasmatic nucleus (SCN), which is entrained by the light-dark cycle. During a RFS, circadian rhythms in behaviour and in clock gene expression uncouple from the SCN and cycle in relation to food availability.[15] Plasma ghrelin levels show entrainment to habitual meal patterns in humans and rats[16-18] and central administration of ghrelin increases FAA[19]. In contrast, FAA is attenuated by a GHS-R1a antagonist[20] and in GHS-R1a -/- mice.[19-22] Ghrelin thus appears to play a role in food anticipatory activity (FAA), although the question remains via which brain nuclei ghrelin exerts its effect on FAA.

Lesioning studies and investigation of the expression of immediate early genes have implicated several brain areas in the network that regulates FAA.[23-28] These include several areas of the hypothalamus, a brain region that is intensively connected with the SCN and well known for its role in the regulation of energy balance and autonomic behaviours.

The VMH is the first brain area to become activated during anticipation after a shift in meal-time[29] and it has been proposed to amplify food entrainable rhythms[30]. The DMH also shows increased Fos expression during FAA[23,26,27,31,32]

and clock gene rhythms shift or change in DMH, Arc, and VMH in rodents on RFS.[33-37] However, the role of DMH in FAA is controversial, since one study has reported a decrease in FAA after lesioning DMH[32], whereas other studies still observe FAA in DMH-lesioned rodents[37-40]. Although lesions of VMH abolished FAA in rats on RFS[41,42], VMH-lesioned rats eventually regained the capacity to anticipate a restricted meal after a longer recovery period.[43,44]

Taken together, previous studies have implicated GHS-R1a, as well as DMH and VMH in FAA, but the present study is the first to examine the role of GHS-R1a in the VMH and DMH in FAA. An adeno-associated virus (AAV) vector containing a short hairpin RNA (shRNA) directed against rat GHS-R1a was injected in the VMH and DMH to determine the effects of local knockdown of GHS-R1a on locomotor activity, food intake, body weight gain and FAA in rats.

## MATERIAL AND METHODS

*Cell lines and plasmids*

Human embryonic kidney (HEK) 293 T cells were cultured at 37°C and 5% $CO_2$ in Dulbecco's Modified Eagles Medium (DMEM, Gibco, Paisley, Scotland) supplemented with 10% (v/v) fetal calf serum (FCS, Integro, Zaandam, the Netherlands), 2mM glutamine (Gibco, Paisley, Scotland), 100 units/ml penicillin, 100 units/ml streptomycin and non-essential amino acids (Gibco, Paisley, Scotland).
Bioinformatics analysis (via www.biopredsi.org and www.invitrogen.com) revealed three potential functional shRNA targets directed against the rat GHS-R1a gene (Table 1). As a control (pAAV-shCON), we designed oligonucleotides to target the Renilla gene (NW_001321314), which is not expressed in the rat. The annealed oligonucleotides (Sigma), which contained SapI and XbaI overhangs, were ligated into the pAAV-shbase plasmid (a kind gift from R.J. Dileone[45]), which was digested with SapI and XbaI. Expression of shRNA was under control of a mouse U6 promotor, and the sequence was followed by a terminator sequence. The plasmid was designed to co-express eGFP driven by a CMV promoter hybridized to a ß-actin intron, and a terminator sequence.
Rat GHS-R1a cDNA was amplified from hypothalamic rat cDNA by polymerase chain reaction (PCR). Primers were designed based on the published sequence

(NM_032075.3) and contained attB-sites to allow Gateway cloning, a Kozak sequence for expression in mammalian cells, and a Shine-Dalgano sequence for expression in E.Coli (Table 1). GHS-R1a cDNA was cloned into the Gateway entry vector pDONR201 (Invitrogen, Carlsbad, CA, USA) and subsequently cloned into a pBabe-puro vector (Invitrogen, Carlsbad, CA, USA) containing Renilla cDNA in order to get a rat GHS-R1a – Renilla fusion plasmid.

*Experiment 1: Luciferase assay – in vitro knockdown*

HEK293T cells were cultured in a 24-well plate and were transfected using polyethylenimine[46] (PEI) with 5 ng pcDNA4/TO-luc (a kind gift from M. van der Wetering), 500 ng pBabe GHS-R1a-Renilla plasmid and 2400 ng pAAV-shRNA (molar ratio pBabe GHS-R1a-Renilla : pAAV-shRNA is 1:4) per well. Transfections were performed *in duplo* for the three different pAAV-shRNA directed against rat GHS-R1a and pAAV-shbase. Three days after transfection, cells were lysed in passive lysis buffer and analyzed with a dual luciferase reporter assay according to manufacturer's protocol (Promega, Madison, Wi, USA). Firefly luciferase activities were measured using a Viktor 96-well plate reader (Perkin Elmer, Waltham, MA, USA). All values were normalized to luciferase values to account for differences in transfection efficiencies. These normalized values were expressed as percentage of measurements of pAAV-shbase, which does not contain a shRNA insert.

*Virus production and purification*

Virus production and purification were performed as described previously.[47,48] Fifteen 150 mm dishes of HEK293T cells were cultured to 80-90% confluency on the day of transfection. Two hours before transfection, 10% FCS DMEM was replaced with 2% FCS DMEM medium. Cells were transfected with AAV-shGHS-R1a #1, AAV-shGHS-R1a #3 or AAV-shCON and the helper plasmid pDp1 [49] (Plasmid factory, Germany) in a molar ratio of 1:1 using PEI. On the subsequent day, medium was refreshed with 2% FCS DMEM. Sixty hours post-transfection, cells were harvested, pelleted, washed with PBS containing 5mM ethylenediaminetetraacetic acid (EDTA), and resuspended in ice-cold lysis solution (150mM NaCl, 50mM Tris, pH 8.4). Cells were subjected to three freeze-thaw cycles between dry ice – ethanol and a 37 °C water bath and were incubated with Benzonase (Sigma, the Netherlands, 50 units/ml) for 30 minutes at 37 °C. Following centrifugations, the supernatant was loaded onto a Quick-seal tube (Beckman

Coulter, California, USA) containing an iodixanol gradient (60%, 40%, 25% and 15%, Optiprep, Lucron Bioproducts, Belgium). The gradient was centrifuged at 70,000 rpm for 1 hour at 20 °C in a Ti70 rotor (Beckman Coulter, California, USA), after which the 40% layer was extracted and subsequently applied to ion-exchange chromatograpy with 5 ml HiTrapQ columns (GE Healthcare, UK). A gradient with buffer A (20mM Tris, 15 mM NaCl, pH 8.5) and B (20mM Tris, 500 mM NaCl, pH 8.5) was applied to elute the column. Fractions of 2 ml were collected and screened by PCR using primers for GFP (Table 1) to determine which ones contained virus. AAV-positive fractions were pooled and transferred to a Centricon Plus-20 Biomax-100 concentrator column (Millipore, Massachusetts, USA) to concentrate the virus and exchange the buffer for PBS. The purified virus was then aliquotted and stored at -80 °C. The titer, in genomic copies per ml (gc/ml) was determined by real-time quantitative PCR in a LightCycler (Roche, Indianapolis, IN, USA) using primers for GFP (Table 1).

| | | |
|---|---|---|
| shGHS-R1a #1 | Top | 5' TTTGGACAAAGTCGAGCATCAACACTTCCTGTCATGTTGATGCTCGACTTTGTCCTTTTT 3' |
| | Bottom | 5' CTAGAAAAAGGACAAAGTCGAGCATCAACATGACAGGAAGTGTTGATGCTCGACTTTGTCC 3' |
| shGHS-R1a #2 | Top | 5' TTTGGCAGTGTTCAAACTGCTAGGCTTCCTGTCACCTAGCAGTTTGAACACTGCCTTTTT 3' |
| | Bottom | 5' CTAGAAAAAGGCAGTGTTCAAACTGCTAGGTGACAGGAAGCCTAGCAGTTTGAACACTGCC 3' |
| shGHS-R1a #3 | Top | 5' TTTGCGAGGAGCCGGAGCCTAACGCTTCCTGTCACGTTAGGCTCCGGCTCCTCGCTTTTT 3' |
| | Bottom | 5' CTAGAAAAAGCGAGGAGCCGGAGCCTAACGTGACAGGAAGCGTTAGGCTCCGGCTCCTCGC 3' |
| shCon | Top | 5' TTTGAATTATAATGCTTATCTACTTCCTGTCATAGATAAGCATTATAATTCTTTTT 3' |
| | Bottom | 5' CTAGAAAAAGAATTATAATGDTTATCTATGACAGGAAGTAGATAAGCATTATAATTC 3' |
| cDNA GHS-R1a | Forward | 5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATACCACCATGTGGAACGCGACCCCCAGC 3' |
| | Reverse | 5' GGGGACCACTTTGTACAAGAAAGCTGGGTCTGTGTTGATGCTCGACTTTG 3' |
| qPCR GHS-R1a | Forward | 5' CTCTGAAGGATGAGAGTTCCCGGGC 3' |
| | Reverse | 5' AAGTCCCGCTTGGCTACGGCT 3' |
| qPCR CycA | Forward | 5' AGCCTGGGGAGAAAGGATT 3' |
| | Reverse | 5' AGCCACTCGTCTTGGCAGT 3' |
| GFP | Forward | 5' CACAGACTTGTGGGAGAAGC 3' |
| | Reverse | 5' CCCCTGAACCTGAAACATAAA 3' |

**Table 1 Overview of oligonucleotides used in this study**

*Animals*

Male Wistar rats (Charles River, Germany) weighing 200 - 225 grams upon arrival were individually housed in transparent acrylic cages in an ambient temperature (21 ± 2°C) and humidity controlled room on a 12h/12h light-dark cycle with lights on at 7.00 PM (experiment 1) or 7.00 AM (i.e. zeitgeber time (ZT0, experiment 2). Animals had *ad libitum* access to water and food, unless mentioned otherwise. All described procedures were approved by the ethical committee on use and care of animals of the University of Utrecht, The Netherlands. For ethical reasons, the experiment had to be terminated when rats lost more than 20 % of their initial body weight.

*Surgical procedures*

Rats were allowed to acclimatize for at least one week after arrival. To obtain continuous measurements of core body temperature, rats received telemetric transmitter probes (TA10TA-F40, Data Sciences International, St. Paul, Minnesota) in the abdominal cavity (only experiment 2). Subsequently, rats were placed in a stereotactic apparatus and were injected bilaterally with AAV-virus in the VMH (coordinates: AP -2.6 mm from bregma, ML ± 1.2 mm from bregma, DV -9.7 mm below the skull, under an angle of 5°) or DMH (coordinates: AP -2.8 mm from bregma, ML ± 1.2 mm from bregma, DV -9.0 mm below the skull, under an angle of 5°). Per site, 1 µl of virus (1 * $10^{12}$ gc/ml) was delivered at a rate of 0.2 µl per minute, after which the needles remained in place for an additional 10 minutes. Surgery was performed under fentanyl /fluanisone (0.1 ml/100 g body weight, intramuscular; Hypnorm, Janssen Pharmacuetica, Beerse Belgium) and midazolam (0.05 ml/100g body weight, intraperitoneal; Dormicum, Hoffman-LaRoche, Mijdrecht, The Netherlands) anesthesia. In addition, rats received saline (6 ml, subcutaneous) post-operatively and carprofen (Rimadyl®, Pfizer Animal Health, Capelle a/d Ijssel, the Netherlands, 0.01 ml/100 g s.c.) as analgesic both pre-operatively and once a day post-operatively for 2 days.

*Experiment 2*

To determine *in vivo* knockdown, rats in experiment 1 received intra-VMH injections with AAV-shGHS-R1a #1 (n=3) or AAV-shGHS-R1a #5 (n=3) on one side, and an injection with AAV-shCON on the other side of the brain. In this way, each rat served as its own control for the establishment of *in vivo* knockdown. Rats were weighed weekly and were sacrificed five weeks after surgery.

*Experiment 3*

Baseline measurements of body weight, food intake, and water intake were taken in the week before surgery in order to divide rats (n=90) into three experimental groups (AAV-shGHS-R1a #1, AAV-shGHS-R1a #5, AAV-shCON). Two weeks after surgery, rats were transferred to cages with a running wheel. After a habituation period of two weeks, rats were subjected to a restricted feeding schedule (RFS), in which they had limited access to food for two hours in the middle of the light period from ZT6 – ZT8.

Food anticipatory activity (FAA) was defined as the locomotor activity in the three hour period before access to food. To control for differences in general locomotor activity, measures of FAA were normalized as a percentage of total daily locomotor activity. After 10-14 days on RFS, depending on the amount of weight loss, rats were sacrificed at ZT6 without receiving their expected meal.

*Collection of blood and tissues*

At the end of each experiment, animals were sacrificed by decapitation. Brains were quickly removed, immediately frozen and stored at -80 °C. Trunk blood was collected in heparinized tubes containing 83 _mol EDTA and 1 mg aprotinin, and placed on ice. Following centrifugation, plasma was stored at -20 °C until further analysis.

*qPCR – in vivo knockdown*

To establish *in vivo* knockdown, series of 16_m coronal sections of the hypothalamus were sliced on a cryostat (Leica, Rijswijk, the Netherlands), thaw-mounted onto RNAse-free 1.0 PEN membrane slides (Zeiss, Oberkochen, Germany) and stored at -80 °C until processing. Laser Capture Microdissection (LCM, PALM Robomover, Zeiss, Oberkcohen, Germany) was applied to dissect 8 VMH sections. For this the slides with the highest GFP density were chosen for each side of the brain. VMH sections were collected into RLT plus solution containing _-mercaptanol (Quiagen, Hilden, Germany) and kept at -80 °C. RNA was extracted from these sections with an RNeasy Plus Micro kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions. Expression of GHS-R1a was then detected with real-time quantitative PCR in a LightCycler (Roche, Mannheim, Germany) using primers for each short hairpin sequence (Table 1). GHS-R1a expression was first normalized to the expression of a household gene (CycA, Table 1). Next, the normalized GHS-R1a expression on the side with AAV-shGHS-

R1a was expressed as percentage of expression of GHS-R1a on the side with AAV-shCON.

*In situ hybridization*

To verify the injection sites, coronal sections (20 µm) were cut on a cryostat in series of 10 and collected on SuperFrost Plus slides (Menzel Gläser). One series was used for *in situ* hybridization (ISH) with a 720 basepair-long digoxigenin-labeled GFP riboprobe (NCBI gene DQ768212). Other series were used for Nissl or haemaluin-eosine (HE) staining. ISH was essentially conducted as described previously.[50] Briefly, sections were fixed in 4% paraformaldehyde (10') and acetylated in 0.25% acetic anhydride in 0.1M triethanolamine (10'). After prehybridization (120') in hybridization solution (containing 50% deionized formamide, 5XSSC, Denhardt's solution, 250 µg/ml tRNA Baker's yeast and 500 µg/ml sonificated salmon sperm DNA), 150 µl hybridization mixture with 400 ng/ml digoxigenin-labeled riboprobe was applied to each slide and slides were incubated overnight at 68 °C. Subsequently, the slides were quickly washed in 2XSSC, followed by a 2 hour-wash in 0.2XSSC, both at 68 °C. Digoxigenin was detected by an alkaline phosphatase labeled antibody (1:5000, Roche, Mannheim, Germany) using nitro-blue tetrazolium and bromochloroindolylphosphate as a substrate. Finally, sections were dehydrated in ethanol, leared in xylene and mounted with Entallan.

*Data analysis*

All data are expressed as mean ± standard error (SEM). Body weight, food intake and water intake were measured daily. Food intake data were also collected by Scales (Department Biomedical Engineering, UMC Utrecht, the Netherlands), which recorded the weight of food hoppers automatically every 12 seconds. A meal was defined as an episode with a minimal consumption of 0.3 g chow and a minimal intermeal interval of 5 min. [51] Measurements of core body temperature (°C) were sent by the transmitters to a receiver plate below the home cage via radio frequency signals. These data were automatically recorded every 10 minutes using DSI software (Data Science International, St. Paul, Minnesota, USA) and averaged per hour for statistical analysis. Running wheel activity (RWA) was continuously registered by a Cage Registration Program (Department Biomedical Engineering, UMC Utrecht, the Netherlands). SPSS 15.0 for Windows software was applied for statistical analysis. Outliers were defined using a boxplot analysis. Data points which exceeded three interquartile ranges

from the edge of the box were considered outliers and removed from the analysis. Differences between groups were calculated using (multivariate) ANOVA with a simple first contrast comparing sh1 and sh3 groups to shCON. Cumulative anticipatory activity was analyzed by a repeated measures ANOVA with time as a within subjects factor and a predefined simple contrast comparing sh1 and sh3 to shCON. In case of a significant group or time*group interaction, a multivariate ANOVA was run per time-point with a predefined simple contrast comparing sh1 and sh3 to shCON. Statistical significance was set at $p<0.05$.

## RESULTS

*Experiment 1: In vitro knockdown efficiency*

Once AAV-vectors containing the different shRNAs were constructed, the efficiency of these shRNAs to downregulate rat GHS-R1a was determined with

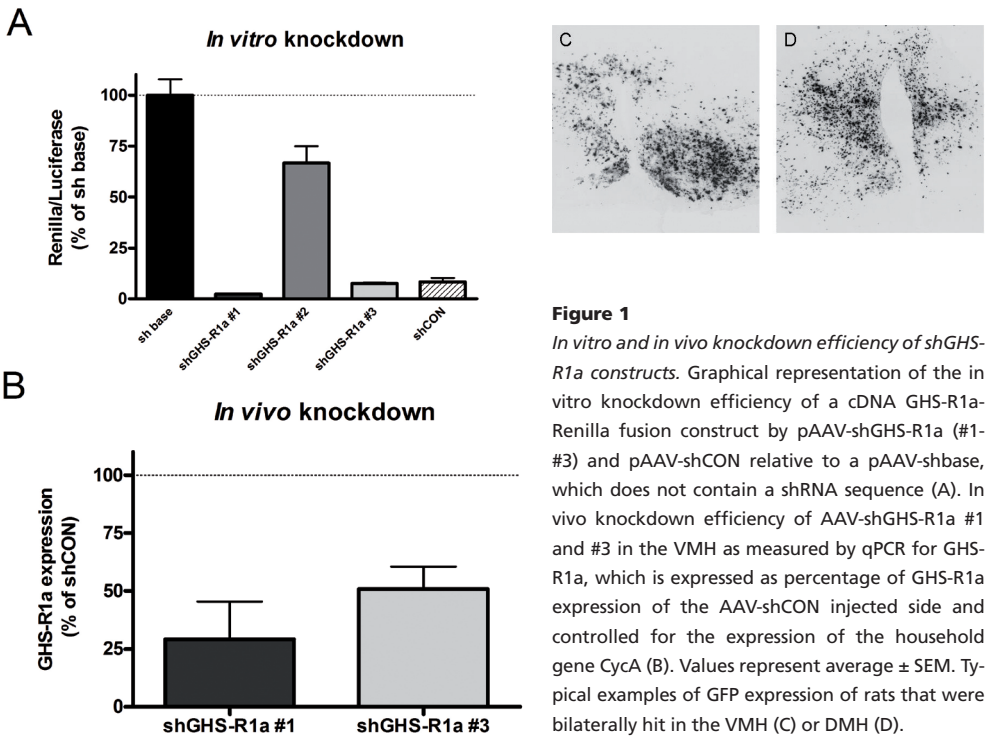


**Figure 1**

*In vitro and in vivo knockdown efficiency of shGHS-R1a constructs.* Graphical representation of the in vitro knockdown efficiency of a cDNA GHS-R1a-Renilla fusion construct by pAAV-shGHS-R1a (#1-#3) and pAAV-shCON relative to a pAAV-shbase, which does not contain a shRNA sequence (A). In vivo knockdown efficiency of AAV-shGHS-R1a #1 and #3 in the VMH as measured by qPCR for GHS-R1a, which is expressed as percentage of GHS-R1a expression of the AAV-shCON injected side and controlled for the expression of the household gene CycA (B). Values represent average ± SEM. Typical examples of GFP expression of rats that were bilaterally hit in the VMH (C) or DMH (D).

a renilla luciferase assay. HEK293T cells were co-transfected with a cDNA GHS-R1a-Renilla fusion construct and pAAV with shRNA against GHS-R1a (#1-#3) or pAAV-shbase. Furthermore, a plasmid containing luciferase was co-transfected to allow for correction of transfection efficiency. pAAV-shGHS-R1a #1 showed the highest percentage of knockdown, namely 98% compared to pAAV-shbase, followed by pAAV-shGHS-R1a #3, which exhibited 92% knockdown. pAAV-shGHS-R1a #2 only produced 33% knockdown of GHS-R1a (Figure 1A). Based on these results, AAV virus was produced of AAV-shGHS-R1a #1 and #3, which were used in the *in vivo* experiments. pAAV-shCON, which contains shRNA directed against Renilla, exhibited 92% knockdown of the GHS-R1a – Renilla fusion construct, and was, hence, a functional pAAV-shRNA.

*Experiment 2: In vivo knockdown efficiency*

To examine in vivo knockdown efficiency, rats were bilaterally injected in the VMH with AAV-shGHS-R1a #1 (n=3) or #3 (n=3) on one side and AAV-shCON on the other side. When rats were sacrificed after 5 weeks, downregulation of GHS-R1a mRNA was measured using qPCR on VMH-sections of each side of the brain (corrected for the amount of mRNA of a household gene, CycA). Figure 1B illustrates that AAV-shGHS-R1a #1 induced a 71% knockdown, whereas AAV-shGHS-R1a #3 downregulated GHS-R1a mRNA with 49%.

*Experiment 3: Hypothalamic injections of pAAV-shGHS-R1a*

Analysis of GFP expression in the rat brains revealed that 67 rats were bilaterally injected in the hypothalamus, of which 25 received pAAV-shCON (con), 21 received pAAV-shGHS-R1a #1 (sh1) and 21 received pAAV-shGHS-R1a #3 (sh3). Rats with DMH as the primary hypothalamic nucleus hit included 8 sh1 rats and 11 sh3 rats. The VMH was the main hypothalamic target for 13 sh1 rats and 10 sh3 rats. Of these rats, 11 (5 sh1, 6 sh3) showed GFP expression in the DMH as well.

Figures 1C and D represent typical examples of injections targeting the VMH and the DMH. In addition, the DMH and VMH were hit unilaterally in 5 (2 sh1, 3 sh3), respectively 6 rats (2 sh1, 4 sh3). Furthermore, in 12 rats (9 sh1, 3 sh3), no GFP expression was detectable in the VMH or DMH, these were considered missed injections. Rats with unilateral or missed injections were not included in the analysis due to small group sizes. Rats with missed injections lacked a clear phenotype and rats with unilateral injections of AAV-shGHS-R1a dis-

| **Ad libitum** | | | *DMH uni* | | *VMH uni* | | *missed* | | *VMH + DMH* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *shCON* | *sh1* | *sh3* | *sh1* | *sh3* | *sh1* | *sh3* | *sh1* | *sh3* |
| BW gain | | 105.90 ± 0.72 | 105.37 ± 0.25 | 108.96 ± 2.38 | 107.61 ± 0.21 | 112.60 ± 4.73 | 108.59 ± 1.21 | 111.99 ± 3.74 | 115.20 ± 4.58 | 114.06 ± 3.60 |
| Food intake | | 21.07 ± 0.39 | 19.31 ± 0.01 | 20.32 ± 1.79 | 21.65 ± 1.28 | 23.72 ± 1.23 | 21.88 ± 0.63 | 21.00 ± 0.52 | 28.78 ± 1.97 | 23.23 ± 0.97 |
| LMA | light | 204.10 ± 13.53 | 216.96 ± 72.56 | 109.33 ± 25.61 | 106.76 ± 9.68 | 143.05 ± 17.05 | 136.70 ± 8.12 | 180.28 ± 35.47 | 156.31 ± 43.37 | 161.28 ± 23.45 |
| | dark | 671.20 ± 34.99 | 716.18 ± 145.75 | 499.30 ± 47.98 | 480.79 ± 82.29 | 730.18 ± 246.26 | 477.71 ± 52.91 | 666.41 ± 67.46 | 334.14 ± 41.22 | 685.68 ± 123.21 |
| BT | light | 37.23 ± 0.03 | 37.22 ± 0.01 | 37.15 ± 0.08 | 37.18 ± 0.02 | 37.25 ± 0.03 | 37.18 ± 0.04 | 37.22 ± 0.02 | 37.23 ± 0.04 | 37.27 ± 0.05 |
| | dark | 37.64 ± 0.02 | 37.61 ± 0.05 | 37.65 ± 0.03 | 37.64 ± 0.06 | 37.67 ± 0.05 | 37.59 ± 0.03 | 37.71 ± 0.02 | 37.53 ± 0.05 | 37.77 ± 0.11 |
| RWA | light | 101.48 ± 18.92 | 66.43 ± 39.57 | 17.95 ± 1.41 | 24.71 ± 13.29 | 72.48 ± 27.07 | 107.08 ± 69.49 | 516.71 ± 194.84 | 63.80 ± 15.86 | 74.33 ± 37.67 |
| | dark | 544.74 ± 62.46 | 426.86 ± 185.43 | 477.24 ± 47.68 | 492.29 ± 159.86 | 353.43 ± 81.12 | 438.22 ± 54.74 | 450.24 ± 436.88 | 201.63 ± 58.90 | 327.21 ± 73.38 |

| **RFS** | | | *DMH uni* | | *VMH uni* | | *missed* | | *VMH + DMH* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *shCON* | *sh1* | *sh3* | *sh1* | *sh3* | *sh1* | *sh3* | *sh1* | *sh3* |
| BW gain | | 83.59 ± 0.64 | 86.38 ± 0.70 | 86.05 ± 0.52 | 86.34 ± 0.66 | 85.63 ± 1.75 | 86.15 ± 0.47 | 85.80 ± 1.07 | 88.07 ± 0.55 | 87.30 ± 1.32 |
| Food intake | | 8.58 ± 0.44 | 10.13 ± 0.30 | 9.17 ± 0.35 | 10.6 ± 2.57 | 10.57 ± 1.21 | 9.69 ± 0.39 | 9.28 ± 0.86 | 10.30 ± 1.15 | 8.49 ± 0.41 |
| LMA | light | 388.61 ± 38.90 | 419.09 ± 176.81 | 184.38 ± 37.70 | 180.58 ± 3.92 | 280.00 ± 123.47 | 205.79 ± 22.96 | 342.16 ± 37.67 | 128.70 ± 29.81 | 190.30 ± 40.49 |
| | dark | 762.77 ± 94.00 | 969.46 ± 350.36 | 525.45 ± 119.90 | 339.01 ± 105.31 | 730.18 ± 246.26 | 573.44 ± 121.69 | 803.81 ± 85.05 | 293.05 ± 52.89 | 489.05 ± 94.28 |
| BT | light | 37.03 ± 0.03 | 37.11 ± 0.10 | 36.95 ± 0.07 | 37.02 ± 0.06 | 37.11 ± 0.02 | 37.02 ± 0.06 | 37.15 ± 0.06 | 36.96 ± 0.04 | 37.16 ± 0.13 |
| | dark | 37.38 ± 0.05 | 37.46 ± 0.15 | 37.45 ± 0.03 | 37.36 ± 0.07 | 37.38 ± 0.11 | 37.38 ± 0.03 | 37.46 ± 0.10 | 37.10 ± 0.02 | 37.50 ± 0.15 |
| RWA | light | 458.35 ± 56.92 | 245.64 ± 175.36 | 247.43 ± 55.55 | 368.86 ± 46.86 | 280.21 ± 204.67 | 274.97 ± 40.41 | 243.38 ± 92.58 | 46.89 ± 25.40 | 109.67 ± 63.37 |
| | dark | 1198.54 ± 181.35 | 1067.09 ± 469.43 | 1065.37 ± 218.31 | 770.43 ± 183.43 | 782.54 ± 324.36 | 1165.87 ± 193.01 | 1191.36 ± 97.91 | 286.50 ± 70.18 | 541.09 ± 135.36 |
| | FAA abs | 335.95 ± 56.64 | 166.50 ± 95.83 | 170.44 ± 52.85 | 222.17 ± 30.17 | 260.33 ± 203.03 | 218.15 ± 38.03 | 219.22 ± 82.00 | 53.07 ± 27.44 | 104.61 ± 38.40 |
| | FAA norm | 17.04 ± 2.10 | 14.34 ± 10.44 | 13.30 ± 6.53 | 17.76 ± 5.63 | 17.38 ± 9.86 | 15.56 ± 3.02 | 9.46 ± 1.43 | 14.37 ± 6.34 | 0000 |

**Table 2 Overview of experimental parameters of rats with unilateral or missed injections**

played intermediate phenotypes. Average values of all parameters measured of these groups and of the VMH rats that also showed GFP in the DMH are depicted in Table 2.

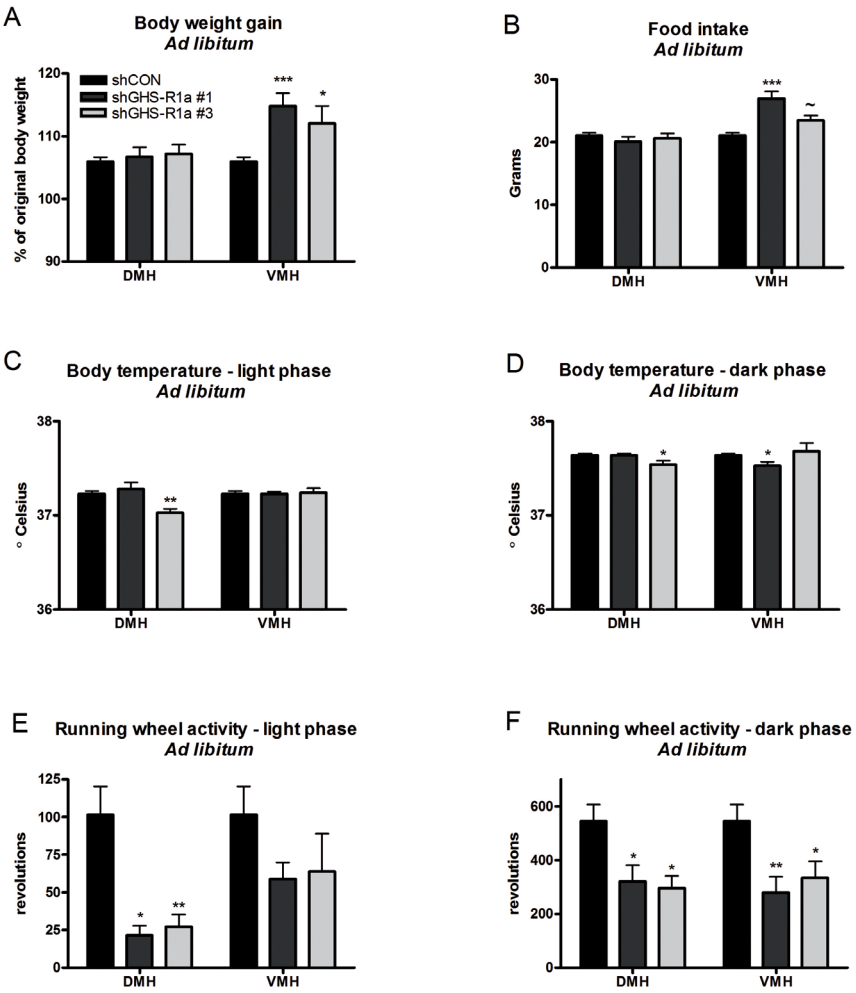


**Figure 2**

Effects of GHS-R1a knockdown in DMH and VMH during ad libitum feeding

Effects of knockdown of GHS-R1a in the DMH and VMH by pAAV-shGHS-R1a #1 and #3 compared to pAAV-shCON three weeks after surgery on body weight gain (A), food intake (B), locomotor activity (light phase (C); dark phase (D)), body temperature (light phase (E); dark phase (F)) and running wheel activity (light phase (G); dark phase (H)). Values represent absolute averages ± SEM. A multivariate ANOVA was conducted on with a predefined simple contrast comparing each pAAV-shGHS-R1a to the control group. ~ $p<0.10$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

*Effect of hypothalamic GHS-R1a knockdown during ad libitum conditions*
As AAV-mediated protein expression can usually be observed two weeks after injection[52], the effects of hypothalamic GHS-R1a knockdown under *ad libitum* conditions on body weigh gain, food intake, locomotor activity, body temperature and running wheel activity were examined in the third week following surgery.

Body weight gain
As depicted in Figure 2A, three weeks after surgery, body weight gain (expressed as % of base-line body weight) was significantly increased only in rats with knockdown of GHS-R1a in the VMH ($F=10.848$, $p<0.001$), shCON 105.90 ± 0.72, sh1 114.82 ± 2.02 ($p<0.001$), sh3 112.02 ± 2.81 % ($p<0.05$)). There was no significant effect of injection in the DMH group ($F=0.401$, $p=0.672$)

Food intake and meal patterns
In line with an increase in body weight gain, daily average food intake in the third week after surgery was increased in rats with hypothalamic GHS-R1a knockdown in the VMH ($F=20.173$, $p<0.001$, shCON 21.07 ± 0.39, sh1 26.94 ± 1.11 ($p<0.001$), sh3 23.47 ± 0.76 ($p=0.063$) grams), but this effect was lacking in the DMH group ($F=0.667$, $p=0.519$, shCON 21.07 ± 0.39, sh1 20.08 ± 0.81, sh3 20.62 ± 0.77 grams) (Figure 2B). The increase in food intake in the VMH group was due to an increase in meal size ($F=6.038$, $p<0.01$, shCON 1.39 ± 0.04, sh1 1.74 ± 0.10 ($p<0.01$), sh3 1.57 ± 0.14 ($p=0.150$) grams) without an effect on meal frequency ($F=0.481$, $p=0.621$, shCON 15.54 ± 0.53, sh1 14.54 ± 1.21, sh3 14.88 ± 0.57 meals) (Figures 4 A and B). In the DMH group, there was no effect on meal frequency ($F=2.390$, $p=0.104$, shCON 15.54 ± 0.53, sh1 13.13 ± 1.35, sh3 14.77 ± 0.56 meals) or meal size ($F=0.720$, $p=0.493$, shCON 1.39 ± 0.04, sh1 1.54 ± 0.18 ($p<0.01$), sh3 1.40 ± 0.09 grams) (Figures 4 A and B).

Body temperature
In the VMH group, average body temperature did not change in the light phase ($F=0.001$, $p=0.999$, shCON 37.23 ± 0.03, sh1 37.23 ± 0.02, sh3 37.24 ± 0.05 ° Celsius) and showed a trend towards reduction in the dark phase ($F=2.992$. $p=0.060$, shCON 37.64 ± 0.02, sh1 37.53 ± 0.04 ($p<0.05$), sh3 37.68 ± 0.09 ($p=0.531$) ° Celsius). In the DMH group, a significant decrease in body temperature could be observed in the light phase ($F=8.737$, $p<0.01$, shCON 37.23 ± 0.03, sh1 37.28 ± 0.07 ($p=0.474$), sh3 37.03 ± 0.04 ($p<0.01$) ° Celsius), but this effect remained a trend in the

dark phase (F=3.034, p=0.059, shCON 37.64 ± 0.02, sh1 37.64 ± 0.02 (p=0.997), sh3 37.54 ± 0.04 (p<0.05) ° Celsius). These effects are depicted in Figures 2C and D.

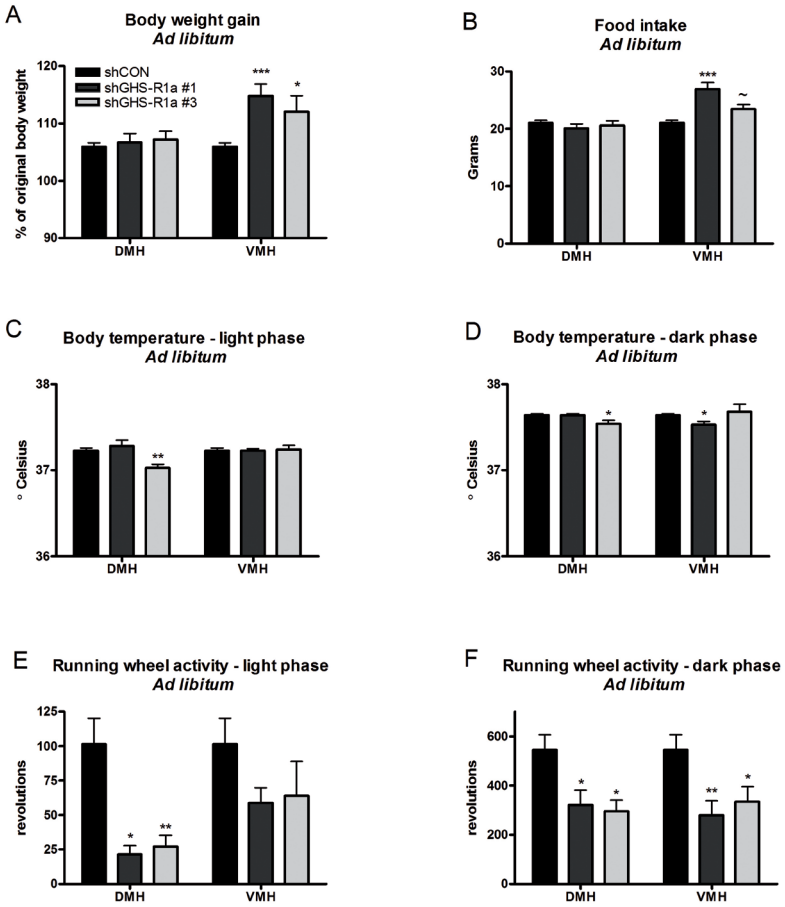


**Figure 3**

Effects of GHS-R1a knockdown in DMH and VMH during a restricted feeding schedule

Effects of knockdown of GHS-R1a in the DMH and VMH by pAAV-shGHS-R1a #1 and #3 compared to pAAV-shCON during the restricted feeding schedule on body weight gain (A), food intake (B), locomotor activity (light phase (C); dark phase (D)), body temperature (light phase (E); dark phase (F)) and running wheel activity (light phase (G); dark phase (H)). Values represent absolute averages ± SEM. A multivariate ANOVA was conducted on with a predefined simple contrast comparing each pAAV-shGHS-R1a to the control group. ~ p<0.10, * p<0.05, ** p<0.01, *** p<0.001

Running wheel activity

As can be observed in Figures 2E and F, in the third week following surgery, RWA was decreased in rats with knockdown of GHS-R1a in the VMH in the dark phase ($F=4.926$, $p<0.05$, shCON 544.74 ± 62.46, sh1 278.59 ± 59.75 ($p<0.01$), sh3 333.41 ± 62.10 ($p<0.05$) revolutions), but not in the light phase ($F=1.538$, $p=0.226$, shCON 101.48 ± 18.92, sh1 58.75 ± 10.91, sh3 63.85 ± 25.04 revolutions). In the DMH-group, there was a significant reduction in RWA both the light phase ($F=5.829$, $p<0.01$, shCON 101.48 ± 18.92, sh1 21.39 ± 6.36 ($p<0.05$), sh3 27.14 ± 8.09 ($p<0.01$) revolutions) and the dark phase ($F=4.567$, $p<0.05$, shCON 544.74 ± 62.46, sh1 321.11 ± 59.38 ($p<0.05$), sh3 295.55 ± 45.74 ($p<0.05$) revolutions).
Figures 5A and B depict the circadian rhythms of RWA per group, averaged per hour for the last 3 days of *ad libitum* feeding. Both the VMH and the DMH group are hypoactive compared to the control rats. However, when normalizing RWA per hour for total RWA, the circadian rhythms of RWA are identical for control rats and rats with knockdown of GHS-R1a in the DMH or VMH (Figures 5C and D).

*Effect of hypothalamic GHS-R1a knockdown during RFS*

Body weight gain

As illustrated in Figure 3A, in response to a RFS, rats with VMH knockdown of GHS-R1a lost less weight than control rats ($F= 13.402$, $p<0.001$, shCON 83.59 ± 0.64, sh1 88.04 ± 0.40 ($p<0.001$), sh3 87.19 ± 0.87 ($p<0.01$) % of pre-RFS body weight). This effect was also visible in rats with knockdown in DMH ($F= 11.417$, $p<0.001$, shCON 83.59 ± 0.64, sh1 88.07 ± 0.46 ($p<0.001$), sh3 86.89 ± 0.52 ($p<0.01$) % of pre-RFS body weight).

Food intake and meal patterns

During the last 3 days of the RFS, average daily food intake did not differ between control rats and rats with knockdown in VMH ($F= 0.099$, $p=0.906$, shCON 8.58 ± 0.44, sh1 8.47 ± 0.67, sh3 8.87 ± 0.39 grams) or DMH ($F=0.733$, $p=0.487$, shCON 8.58 ± 0.44, sh1 9.46 ± 0.73, sh3 8.44 ± 0.40 (Figure 3B). In addition, no changes were observed in meal frequency (VMH: $F=1.340$, $p=0.273$, shCON 2.76 ± 0.22, sh1 2.38 ± 0.27, sh3 2.22 ± 0.12 meals, DMH: $F=1.561$, $p=0.223$, shCON 2.76 ± 0.22, sh1 2.94 ± 0.31, sh3 2.23 ± 0.23 meals) or meal size (VMH: $F=0.357$, $p=0.702$, shCON 3.61 ± 0.41, sh1 4.13 ± 0.53, sh3 3.94 ± 0.27 grams, DMH: $F=1.038$, $p=0.364$, shCON 3.61 ± 0.41, sh1 3.28 ± 0.29, sh3 4.50 ± 0.77

grams) in the VMH or DMH group (Figures 4C and D). All groups spent on average an equal amount of time eating during the 120 minute-window of food availability (VMH: F=0.201, p=0.818, shCON 71.00 ± 4.18, sh1 74.18 ± 5.00, sh3 69.49 ± 3.48 minutes, DMH: F=0.374, p=0.690, shCON 71.00 ± 4.18, sh1 74.40 ± 7.41, sh3 76.64 ± 3.34 minutes).

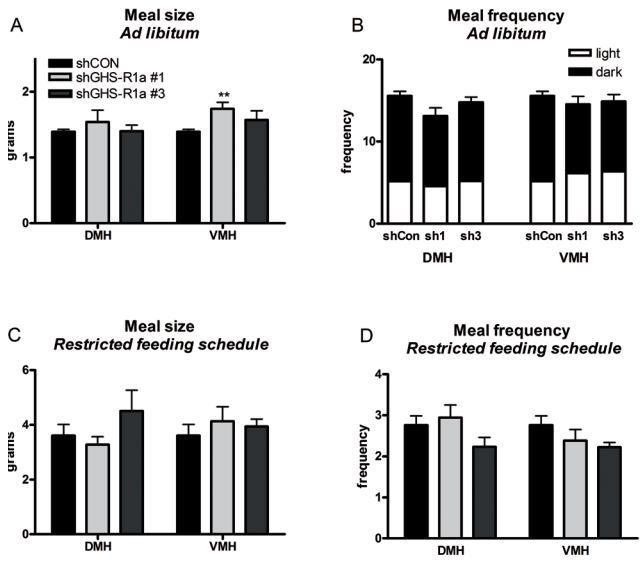


**Figure 4**
*Effects of GHS-R1a knockdown in DMH and VMH on meal patterns.* Effects of knockdown of GHS-R1a in the VMH or DMH on meal size (A and C) and meal frequency (B and D) during ad libitum feeding (A and B) and when subjected to a restricted feeding schedule (C and D). Values represent absolute averages ± SEM. A multivariate ANOVA was conducted on with a predefined simple contrast comparing each pAAV-shGHS-R1a to the control group. ~ p<0.10, * p<0.05, ** p<0.01, *** p<0.001

Body temperature

In contrast to the observed reductions in running wheel activity, average body temperature (Figures 3C and D) was not altered in rats with VMH knockdown of GHS-R1a in the light phase (F= 0.450, p=0.640, shCON 37.03 ± 0.03, sh1 37.01 ± 0.03, sh3 37.09 ± 0.10 (p=0.338) ° Celsius) or dark phase (F= 1.926, p=0.158, shCON 37.38 ± 0.05, sh1 37.22 ± 0.05 (p<0.05), sh3 37.93 ± 0.11 (p=0.982) ° Celsius). DMH knockdown of GHS-R1a had a dual effect on body temperature in the light phase (F= 5.973, p<0.01, shCON 37.03 ± 0.03, sh1 37.18 ± 0.08 (p=0.072), sh3 36.87 ± 0.07 (p<0.05) ° Celsius), but had no effect on body temperature in the dark phase (F= 1.893, p=0.164, shCON 37.38 ± 0.05, sh1 37.51 ± 0.05, sh3 37.31 ± 0.07 ° Celsius).

Running wheel activity

In both the VMH and DMH group, RWA during RFS was diminished in the light phase (Figure 3E)(VMH: F=16.085, p<0.001, shCON 458.35 ± 56.92, sh1 58.75

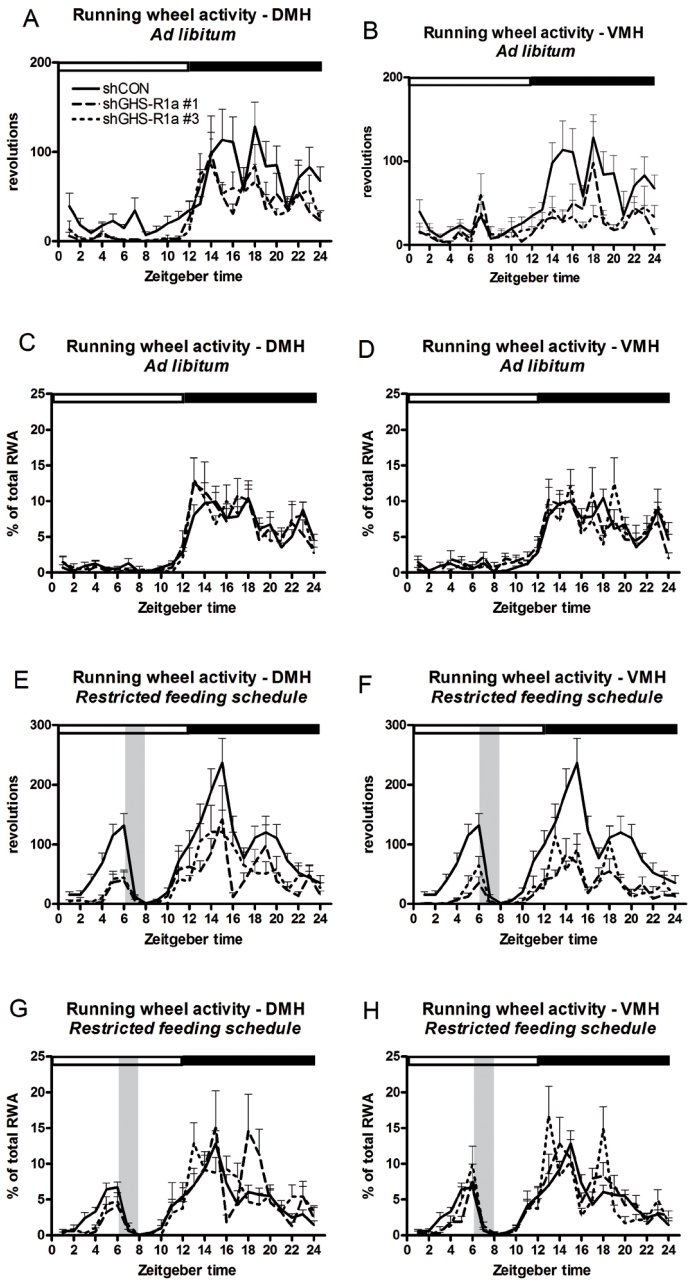


**Figure 5**

*Effects of GHS-R1a knockdown in DMH and VMH on running wheel activity*. Running wheel activity (RWA) was averaged per hour per group for the last 3 days of ad libitum feeding. Hypoactivity is present in rats with GHS-R1a knockdown in DMH (A) and VMH (B). When RWA per hour was normalized to total RWA, no differences in the circadian rhythms of RWA could be detected in the DMH (C) or VMH (D) group. In addition, average RWA per hour during the last 3 days of the restricted feeding schedule was decreased in the dark phase and in anticipation to the meal (from ZT6-ZT8, indicated by the grey vertical bar) and to the dark phase in rats with knockdown of GHS-R1a in the DMH (E) or VMH (F). When examining normalized RWA, it becomes evident that the DMH group (G) and the VMH group (H) show slightly reduced food anticipatory activity. Values represent absolute averages ± SEM. A multivariate ANOVA was conducted with a predefined simple contrast comparing each pAAV-shGHS-R1a to the control group. ~ $p<0.10$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

± 10.91 ($p<0.001$), sh3 108.44 ± 41.97 ($p<0.001$) revolutions, DMH: $F= 7.480$, $p<0.01$, shCON 458.35 ± 56.92, sh1 155.79 ± 65.13 ($p<0.01$), sh3 182.65 ± 49.06 ($p<0.01$) revolutions). Dark phase RWA during RFS (Figure 3F) was reduced in the VMH group ($F= 6.556$, $p<0.01$, shCON 1198.54 ± 181.35, sh1 278.59 ± 59.75 ($p<0.01$), sh3 505.31 ± 91.96 ($p<0.05$) revolutions) and showed a trend towards reduction in the DMH group ($F= 2.443$, $p=0.099$, shCON 1198.54 ± 181.35, sh1 683.55 ± 88.74 ($p=0.089$), sh3 730.22 ± 115.94 ($p=0.082$) revolutions).

Anticipatory running wheel activity

Figures 5E and F depict the circadian rhythms of RWA per group, averaged per hour for the last 3 days of the RFS. Both VMH and DMH groups were hypoactive compared to control rats and show reduced levels of anticipatory RWA preceding food access, but also preceding the dark phase. To control for the reduced levels of RWA in general, RWA is also expressed as a percentage of total RWA per hour (Figures 5G and H).

To examine whether there was also a difference in relative anticipatory RWA (anticipation to food compared to anticipation of the dark phase), the cumulative percentages of total RWA during the first 6 hours of the light phase were calculated, to investigate anticipation to food. Next, cumulative percentages of total RWA during the last 6 hours of the dark phase were calculated, to examine anticipation to the dark phase. In the DMH group, a repeated measured ANOVA revealed a significant time*group interaction for the first half of the light phase ($F=4.526$, $p<0.01$) (Figure 6A), but not for the second half of the light phase ($F=0.488$, $p=0.656$) (Figure 6C). In this group, the time of onset of FAA was attenuated (indicated by the reduced cumulative percentage of total RWA at ZT3, 4 and 5), as well as the amplitude of FAA (indicated by the decreased value at ZT6). Similarly, in the VMH group, there was a significant affect on FAA ($F=2.247$, $p<0.05$) (Figure 6B), while anticipation to the dark phase remained unaffected ($F=0.186$, $p=0.883$) (Figure 6D). The time of onset of FAA was delayed, as the cumulative percentage of RWA at ZT 2, 3, 4 and 5 was attenuated in the GHS-R1a knockdown groups. However, in contrast to the DMH group, the total amplitude of FAA was similar to the control group, as indicated by the datapoints at ZT6.

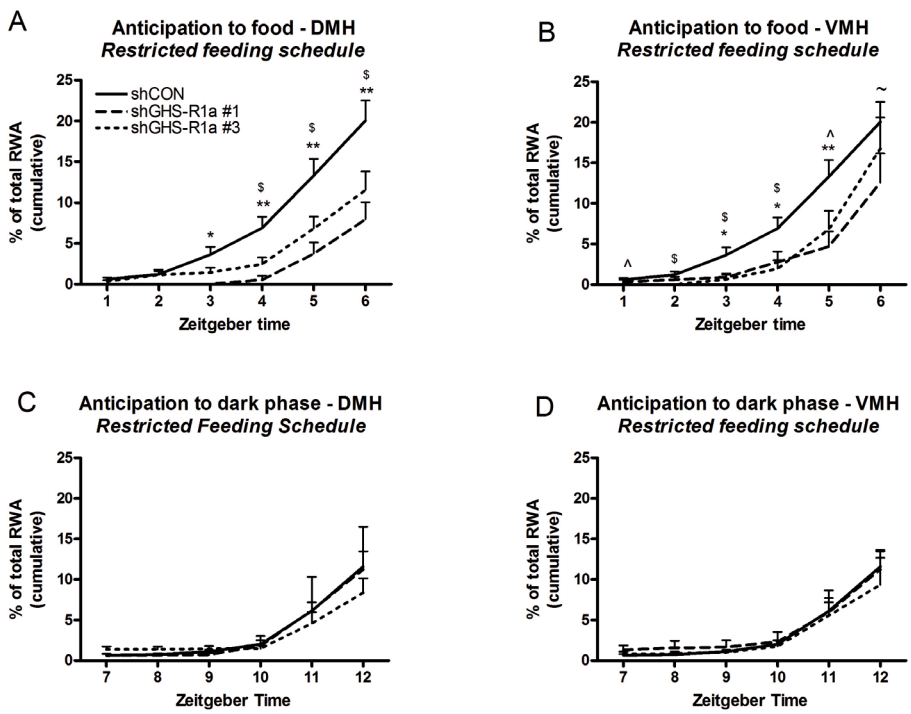


**Figure 6**

*Effects of GHS-R1a knockdown in DMH and VMH on food anticipatory activity*

Cumulative running wheel activity (RWA), expressed as percentage of total RWA, in anticipation to food (ZT1-6, A and B) and to the dark phase (ZT7-12, C and D). Rats with knockdown of GHS-R1a in the DMH exhibit a delayed onset and attenuated amplitude of anticipation to food (A), but anticipation to the dark phase (C) remains unaffected. Anticipation to the dark phase (D) is similar to control rats in the VMH group as well, while time of onset of FAA is delayed without affecting amplitude of FAA (D). Values represent absolute averages ± SEM. A repeated measures ANOVA was conducted. In case of a significant time*group interaction, a multivariate ANOVA was conducted with a predefined simple contrast comparing each pAAV-shGHS-R1a to the control group. ~ $p<0.10$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for shGHS-R1a #1 compared to shControl. ^ $p<0.10$, $ $p<0.05$, $$ $p<0.01$, $$$ $p<0.001$ for shGHS-R1a #3 compared to shControl.

## DISCUSSION

In this study we determined the effect of hypothalamic knockdown of GHS-R1a on FAA. Two of the three constructed pAAV-shGHS-R1a were shown to be able to efficiently knockdown GHS-R1a both *in vitro* and *in vivo*, sh1 being more efficient than sh3. These two viruses and a control virus were injected in the medial hypothalamus targeting the DMH or the VMH. Under *ad libitum* conditions, body weight gain was increased in rats with knockdown of GHS-R1a in the VMH, presumably due to an increase in food intake. In addition, DMH and VMH knockdown of GHS-R1a led to reductions in BT and RWA. In line with its increased *in vitro* and *in vivo* knockdown efficiency, physiological effects of sh1 were larger than those of sh3. Furthermore, rats with unilateral injections of shGHS-R1a showed intermediate phenotypes, while rats with missed injections lacked a phenotype (see Table 2). Although some rats in the VMH group also showed GFP expression in the DMH (which could be caused by the injection needle that passed the DMH in order to reach the VMH) no additive phenotype could be observed in these rats (Table 2), indicating that the VMH was the primary target.

When subjected to a RFS schedule, rats with knockdown of GHS-R1a in DHM or VMH lost less body weight than control rats, which was most likely due to decreased energy expen-diture by reduced RWA, since food intake was similar amongst groups. However, we did not measure other components of energy expenditure, such as resting metabolic rate. Hence, we cannot exclude that these components contributed to the attenuates body weight loss. Anticipatory RWA was strongly decreased as well, although this could have been due to the general decrease in

| | | **Ad libitum** | | **RFS** | |
|---|---|---|---|---|---|
| | | *DMH* | *VMH* | *DMH* | *VMH* |
| BW gain | | = | ↑ | ↑ | ↑ |
| Food intake | | = | ↑ | = | = |
| BT | light | ↓ | = | ↓ / ↑ | = |
| | dark | = / ↓ | = / ↓ | = | = |
| RWA | light | ↓ | = | ↓ | ↓ |
| | dark | ↓ | ↓ | = / ↓ | ↓ |
| | FAA onset | | | ↓ | ↓ |
| | FAA ampl. | | | ↓ | = |

**Table 3 Overview of the effects of GHS-R1a knockdown in VMH or DMH**

locomotor activity. Therefore, RWA was normalized for total daily RWA. After normalization, both onset and amplitude of FAA were reduced in the DMH group, whereas the VMH group exhibited only a delay in time of onset of FAA. In contrast, anticipation to the dark phase remained unaffected in both groups. An overview of the observed effects is depicted in Table 3. Although FAA was attenuated in rats with GHS-R1a knockdown in DMH as well as VMH, some anticipatory RWA remained. This could have several reasons. First, in contrast to knockout models, AAV-mediated knockdown of GHS-R1a did not result in complete ablation of a gene; therefore GHS-R1a signalling was reduced, but not abolished. Second, GHS-R1a signalling in the DMH and VMH is presumably part of a distributed network of brain areas that control FAA. Thus, interfering with one node of this network is likely to attenuate, but not completely prevent the development of FAA.

In the present study, the strongest effect of hypothalamic GHS-R1a knockdown was the observed hypoactivity. This is in line with the finding that GHS-R1a antagonism reduced drug-induced locomotor activity[53-55], although central administration of ghrelin has been reported both to decrease[6] as well as increase[56] locomotor activity In addition to the reduction in total RWA, rats with knockdown of GHS-R1a in DMH also exhibited a decrease in normalized FAA. Similarly, GHS-R1a -/- mice were shown to exhibit a reduction in FAA.[19-22]. However, a decrease in general locomotor activity was not reported in GHS-R1a -/- mice[19,21,57]. This suggests opposite effects of ghrelin on locomotor activity in different nuclei that counteract the effect in the observed in the present study. Taken together, these findings suggest that GHS-R1a signalling in the DMH and VMH plays a role in FAA.

Remarkably, ghrelin -/- mice showed normal levels of FAA[58,59]. This might indicate that another, to date unknown, substrate of GHS-R1a is involved in FAA, or can replace ghrelin's role in FAA. Further research is required to answer this question. Moreover, in contrast to knockout models, AAV-mediated shRNA injection provided local knockdown without affecting development. Hence, brain area-specific physiological effects might not be visible in knockout models.

Ghrelin is known as an orexigenic hormone[1-3,5] and GHS-R1a signalling in the VMH has been shown to be involved in ghrelin's orexigenic effect.[60] Therefore,

the appetite-stimulating effect of GHS-R1a knockdown in VMH is remarkable. GHS-R1a -/- mice did not exhibit increases in food intake or body weight gain, they rather show reductions in body weight gain.[21,22,57,61,62]
The VMH is considered as a satiety area and stimulation of this brain region resulted in a reduction in food intake.[63] The direct effect of ghrelin on VMH neurons is predominantly stimulatory.[64] However, ghrelin could mediate VMH activity via other brain areas as well. NPY/AgRP neurons within the Arc are known to be activated by ghrelin[5,65]. These orexigenic neurons were shown to project to the shell of the VMH.[66] Furthermore, both AgRP and NPY predominantly inhibited excitatory VMH neurons.[66,67] These data suggest that the VMH is stimulated by ghrelin directly and inhibited indirectly via activation of NPY/AgRP neurons. By reducing the stimulatory tone of ghrelin by reducing GHS-R1a expression in the VMH, the indirect inhibitory effect of ghrelin might dominate. This could result in the observed increase in food intake.

In previous studies, there have been reports of sequence-specific cellular toxicity following injection of AAV containing shRNA into the liver[68], red nucleus[69], VTA / substantia nigra[70] and the striatum[71,72]. Such damage might provide a phenotype itself, that could be mistaken for effects of the shRNA. In this study, we were unable to detect any cellular damage in brain slices when stained for Nissl or HE. In addition, the dose of AAV used in this study has been injected in the hypothalamus by others without reported side effects.[47,73] Moreover, any toxic effects of expression of shRNA in hypothalamic neurons would have been induced in control rats as well as the knockdown groups. Therefore, fact that we observed differences between AAV-shCON and AAV-shGHS-R1a groups supports an effect of the shRNA that is independent of tissue damage, However, as the induction of cellular toxicity has been reported to be sequence specific[68], we cannot fully exclude the possibility that the observed phenotypes by injecting AAV-shGHS-R1a in VMH and DMH are in part due to cellular damage.

Previous studies showed that lesioning of the DMH leads to lower levels of locomotor activity, decreased body temperature, reduced nocturnality ratio and hypophagia under *ad libitum* conditions.[32,38,74,75] Furthermore, when rats were subjected to a RFS, ablation of the DMH induced a decrease in FAA, in addition to diminished overall locomotor activity[32]. However, others reported no effect of DMH ablation on FAA, but only an attenuation of nocturnality ratio.[39,75]

Although, in the present study, we observed hypoactivity and reduced FAA in rats with knockdown of GHS-R1a in the DMH, the nocturnality ratio was unaltered, as depicted by the identical distributions of normalized RWA in shCON and shGHS-R1a groups. In addition, we did not observe a decrease in food intake in the DMH group.

Under *ad libitum* conditions, ablation of VMH was previously shown to result in hypoactivity, reduced body temperature, attenuated nocturnality ratio, hyperphagia, and obesity.[30,44,76] In contrast, under RFS conditions, rats with a VMH lesion exhibited decreased food intake. Furthermore, although it was initially attenuated, FAA eventually recovered.[41-44] The reduced activity, diminished FAA and hyperphagia, observed in the present study, therefore correspond to the phenotype of rats with a VMH lesion. In contrast, nocturnality ratio remained identical to shCON rats.

Taken together, the phenotypes induced by injection of AAV-shGHS-r1a in the DMH and VMH are not identical to phenotypes due to lesioning of these brain areas. Nevertheless, this apparent discrepancy further supports the idea that effects in this study were mediated by knockdown of GHS-R1a rather than cellular damage.

In conclusion, although studies in GHS-R1a -/- mice had already implicated GHS-R1a signalling in the regulation of FAA in a RFS model[19-22], it was still unknown via which brain area this effect could be mediated. In the present study, the predominant effect of GHS-R1a knockdown in the medial hypothalamus during RFS was a decrease in RWA, which attenuated body weight loss. In addition, the amplitude of FAA was reduced in rats with DMH knockdown of GHS-R1a, whereas the onset of FAA was delayed in rats with knockdown of GHS-R1a in VMH as well as DMH. Together, this implicates GHS-R1a signalling in the DMH and VMH as an important part of the network that regulates FAA.

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# Chapter 6

## GENERAL DISCUSSION

## CONTENTS

In light of the still increasing prevalence of obesity and other eating disorders it is crucial to examine what happens in our brain when we are triggered to think about food. Food anticipatory activity (FAA) consists of hyperactivity and arousal preceding a time-restricted meal and reflects the underlying appetitive mechanisms when a rodent gets ready for its meal. In this thesis, two models of FAA are described. Rats display FAA under food-restricted conditions, anticipating their daily meal (restricted feeding schedule (RFS)), but also under ad libitum conditions, anticipating a daily palatable meal (palatable feeding schedule (PFS)). Despite many years of research, the mechanisms underlying FAA are still not completely understood. The studies described in this thesis aimed to examine the neuronal and molecular substrates of FAA, and in particular the role of ghrelin in FAA. In this chapter, the main findings will be summarized and discussed, followed by suggestions for future directions.

## Summary of main findings

### Physiology of animal models of FAA

As described in **Chapter 2**, in *ad libitum* fed rats (AL), circadian rhythms of locomotor activity (LMA) and body temperature (BT) were coupled to the light-dark cycle, with high levels during the dark phase and low levels during the light phase. These circadian rhythms disengaged from the light-dark cycle in rats subjected to a RFS with food provided for 2 hours in the middle of the light phase. Due to the RFS, rhythms of LMA and BT cycled in relation to the period of food availability. In addition, RFS rats showed a reduction in nadir value of BT, probably reflecting decreased basal metabolic rate to compensate food restriction. Important features of the RFS model were the development of FAA and the anticipatory rise in BT. The limited availability of food during this model induced body weight loss due to decreased food intake. In the course of the experiment, RFS rats learned to consume more calories during the 2 hour-period of food availability and dramatically increased their average meal size, indicating reduced satiation, which allowed increased food intake during these 2 hours.
Remarkably, rats subjected to a random RFS schedule (R-RFS) who also had access to food for 2 hours during the light phase, but then at random time-points, exhibited many of the physiological changes that occurred in RFS rats, including uncoupling of circadian rhythms of LMA and BT from the light-dark cycle, lower

nadir of BT, body weight loss, reduced food intake and increased average meal size. R-RFS rats were more active during the light phase in general, but lacked a specific anticipatory peak in LMA preceding ZT6, the time of onset of food access in RFS rats. Furthermore, R-RFS rats lacked an anticipatory peak in BT. Although not statistically significant, R-RFS rats lost more weight and consumed less during the 2-hour period of food access, due to a decreased meal size compared to RFS rats, which likely reflected that they were unable to anticipate their daily meal and were less prepared to eat.
In contrast to RFS rats, rats subjected to a PFS model did not uncouple their circadian rhythms of BT and LMA from the light-dark phase. Apart from a small anticipatory increase in LMA, but not in BT, these circadian rhythms were indistinguishable from AL rats. FAA could not be detected until week 3 of the experiment. Although PFS rats compensated chocolate intake by decreasing their chow intake already in the first week of PFS, they exhibited increased caloric intake in weeks 1 and 2. A decrease in meal frequency accounted for this reduction in chow intake. Body weight gain and the amount of epididymal fat, however, were identical to AL rats.

Altogether, food restriction to the light phase, but not necessarily timed restriction, led to disengagement of circadian rhythms to the light-dark cycle. An anticipatory increase in BT was detected in RFS rats exclusively. FAA, on the other hand, was observed in RFS and PFS rats, although to a much lesser extent in the PFS group.

## Activation of brain areas during FAA

To identify brain areas involved in FAA, we examined expression of the neuronal activation marker Fos in AL, RFS, R-RFS and PFS rats sacrificed during FAA (**Chapter 2**) in the hypothalamus and the nucleus accumbens (NAc). The hypothalamus is considered as the homeostatic regulator of food intake and receives peripheral input about the metabolic state of the body[1], and could hence mediate the "hunger"-component of FAA. The NAc, in contrast, has been implicated in reward-related behaviours, and could be involved in the motivational aspects of FAA.[2]
As already described in literature[3-5], the hypothalamus was activated in RFS rats, more specifically the dorsal lateral hypothalamus (dLH) and dorsomedial hypothalamus (DMH). Notably, hypothalamic Fos expression in R-RFS rats did not

differ from that in RFS rats in most areas. R-RFS rats had received their last meal at the same moment as RFS rats. Only in the paraventricular nucleus (PVN) R-RFS rats showed higher levels of Fos activation than RFS rats, which could reflect increased stress due to unpredictable meal timing. Interestingly, Fos activation in the ventromedial hypothalamus (VMH), DMH and arcuate nucleus (Arc) correlated significantly with anticipatory LMA in the RFS group exclusively. In agreement with previous reports[6], activation of the hypothalamus lacked in PFS rats. To gain more insight into specific neuronal populations activate during FAA, we investigated co-localization of Fos and a-MSH, which represents the anorexigenic neuronal population in the Arc. All three experimental groups, RFS, R-RFS and PFS, exhibited decreased activation of the anorexigenic population, suggesting an increased orexigenic drive.
Using an *in vivo* electrophysiology approach, we measured neuronal responses in the VMH/DMH area during cue-induced anticipation to restricted food access, feeding and following peripheral injections of leptin and ghrelin (**Chapter 4**). In line with increased Fos expression in the DMH, and correlations of anticipatory LMA with Fos activation in DMH and VMH, the neuronal population in the VMH/DMH increased firing frequency during cue presentation.

NAc was activated in both RFS and R-RFS rats, but not in PFS rats (**Chapter 2**), in contrast to previous findings. The latter effect could be due to the fact that PFS rats did not show FAA until the last week of the experiment.

In summary, hypothalamic and accumbal activation occurred in RFS as well as R-RFS rats, revealing that this reflects a negative energy balance rather than anticipation, as has been shown for the Arc and DMH before[7]. Cue-induced anticipation induced activation of the VMH/DMH neuronal population as well. In addition, Fos expression correlated specifically in the RFS group with anticipatory LMA. While we could not detect any activation of the hypothalamus or NAc in the PFS group, these rats showed a decreased anorexigenic drive, just as RFS and R-RFS rats.

## Ghrelin and FAA

During anticipation to a RFS or PFS, plasma levels of total ghrelin were increased (**Chapters 2&3**). RFS rats showed a trend towards higher levels of ghrelin com-

pared to PFS rats, in agreement with the decreased amplitude of FAA observed in the PFS group. R-RFS rats exhibited increased ghrelin levels as well, indicating an increase in orexigenic drive in these rats, in line with the decreased activation of the anorexigenic population.

Ghrelin levels are known to rise in anticipation of an expected meal in humans as well as rodents.[8-10] Furthermore, ghrelin increased FAA in a RFS model[11], and GHS-R1a antagonism as well as general knockout of GHS-R1a decreased FAA[11-14]. As ghrelin has also been implicated in the rewarding aspects of food intake[15-17], we aimed to examine whether ghrelin is involved in the regulation of FAA in rats on a PFS in **Chapter 3**. Central administration of ghrelin increased FAA in PFS rats, whereas GHS-R1a antagonism reduced anticipatory LMA in rats that exhibited a minimum amount of FAA under control conditions. This was the first study to suggest that ghrelin signalling does not only contribute to FAA under restricted conditions, but also in a non-restricted paradigm with limited access to palatable food.

## Hypothalamic ghrelin signalling and FAA

In **chapters 2 and 4**, we showed that the DMH and VMH could be involved in the regulation of FAA in a restricted paradigm. Furthermore, plasma ghrelin levels were increased during FAA, and literature has implicated ghrelin signalling in the control of FAA[11-13]. Therefore, we sought to determine whether hypothalamic ghrelin signalling could drive FAA. Using *in vivo* electrophysiology, we revealed that the DMH/VMH neuronal population is activated during presentation of a cue that signals access to food and also following peripheral administration of ghrelin, as described in **Chapter 4**. Intriguingly, a subset of neurons that responded significantly to both cue and ghrelin, exhibited a strong positive correlation (r=0.95) between these two parameters. Potentially, this specific neuronal population could contribute to the regulation of FAA by ghrelin. Therefore, in **chapter 5**, we examined the effect of reduced ghrelin signalling in the VMH and DMH on FAA by injecting AAV-vectors containing shRNA against GHS-R1a in either VMH or DMH. Under *ad libitum* conditions, the main effect of decreased GHS-R1a in both DMH and VMH was a reduction in running wheel activity. VMH knockdown of GHS-R1a led, in addition, to augmented body weight gain by increasing food intake. When rats were subjected to a RFS, the effect on running wheel activity remained. Absolute values of anticipatory running wheel

activity were attenuated in both the VMH and the DMH group. However, when corrected for the reduction in running wheel activity in general, only rats with reduced GHS-R1a signalling in the DMH exhibited a decrease in amplitude of FAA. The onset of FAA was delayed in rats with knockdown of GHS-R1a in the DMH as well as in rats with GHS-R1a knockdown in the VMH. Hence, GHS-R1a signalling in the DMH and VMH contributes to FAA. Since FAA was still apparent, its regulation does not exclusively depend on GHS-R1a signalling in the DMH or VMH. However, GHS-R1a signalling in these brain areas is presumably part of the distributed network that is suggested to regulate FAA.

## Limitations

### FAA in a palatable feeding schedule; not solely motivation?

FAA is delayed in onset and decreased in amplitude in rats on a PFS compared to rats on a RFS (**Chapter 2**).[18,19] Whereas a RFS activated both hypothalamic systems, as well as reward-related areas[6,20], a PFS has been suggested to exclusively activate reward-related areas[6]. Others have suggested that a palatable meal size has to exceed a certain caloric threshold to evoke FAA.[21,22] When using RWA as a read-out parameter, only 37% of the rats anticipating a palatable treat exhibited FAA.[23] However, 5 grams of chocolate, the same amount as used in our studies, induced FAA when assessed with general locomotor activity measurements.[6,18,19] Indeed, we observed higher levels of FAA when PFS rats had 15 minutes access to chocolate as opposed to 5 grams (**Chapter 3**). In addition, FAA correlated strongly with the amount of chocolate intake in these rats. Collectively, this could indicate, that the PFS model does not solely induce a motivational component of FAA, but that a certain hunger-component is required as well to provoke FAA. Although we did not observe hypothalamic Fos activation in PFS rats (**Chapter 2**), the anorexigenic neuronal population of the Arc was less activated in PFS rats, indicating an increased orexigenic drive, hence hunger. Unfortunately, we could not investigate the activation of the orexigenic neuronal population, due to lack of cytoplasmatic staining of the NPY antibody, a problem encountered before[3].

## Local knockdown with AAV-shRNA

In order to establish local knockdown of GHS-R1a in adult rats, we injected AAV containing shRNA sequences directed against GHS-R1a in specific brain areas. AAV is a widely used tool to locally over-express or knockdown genes in rats.[24] However, in recent years, reports have arisen that AAV containing shRNA might induce cellular toxicity.[25-29] This toxicity is suggested to be sequence-specific[25] and might provide a lesion-like phenotype itself. Oversaturation of the endogenous microRNA / shRNA processing pathway, more specific exportin-5, has been proposed as the underlying cause of this cellular damage.[25] Interestingly, sequences that elicited cellular toxicity in a shRNA-context, lacked or exhibited attenuated toxicity when cloned into a microRNA context.[28]

AAV-shRNA induced cellular toxicity might lead to a similar phenotype as in rats with lesions of the targeted brain area. Lesioning of the DMH led to reduced locomotor activity, decreased body temperature, attenuated nocturnality ratio, hypophagia and hypodipsia under *ad libitum* conditions.[30-33] Under RFS conditions, DMH lesions elicited hypoactivity and, in addition, decreased FAA.[30] However, others reported no effect of DMH ablation on FAA, only nocturnality ratio was attenuated.[33,34] Lesions of the VMH resulted in hypoactivity, decreased body temperature, hyperphagia, increased water intake and obesity under *ad libitum* conditions.[35,36] When subjected to a RFS, rats with a VMH lesion exhibited decreased food intake and, initially, attenuated FAA, which eventually recovered.[36-39] The hypoactivity (in DMH and VMH groups), reduction of FAA (in DMH and VMH groups) and hyperphagia (in VMH group) correspond to the phenotypes in lesioned rats. However, nocturnality ratios were unaltered in both groups, as observed by the identical distributions of normalized running wheel activity in shCON and shGHS-R1a group (**Chapter 5**). In addition, no effect on food intake was observed in the DMH groups. Moreover, the observed differences are between AAV-shCON and AAV-shGHS-R1a groups. Hence, any side-effect of expression of shRNA in hypothalamic neurons would be induced in control as well as GHS-R1a knockdown rats and could not explain the differences observed between these groups. However, as the induction of cellular toxicity has been reported to be sequence specific[25], we cannot exclude the possibility that the observed phenotypes by injecting AAV-shGHS-R1a in VMH and DMH were due to cellular damage. Therefore, for future experiments it is recommended to apply AAV containing microRNA to establish reliable *in vivo* knockdown.

## Ghrelin effects versus GHS-R1a effects

As mentioned before, GHS-R1a -/- mice exhibited a reduction in FAA.[11-14] Remarkably, ghrelin -/- mice showed normal levels of FAA.[40,41] The phenotype of GHS-R1a -/- mice is in line with the main findings of this thesis, namely that antagonism of GHS-R1a attenuated FAA in PFS rats (**Chapter 3**) and that knockdown of GHS-R1a in DMH and VMH diminished FAA in RFS rats (**Chapter 5**). This might indicate that another, to date unknown, substrate of GHS-R1a is involved in FAA, or can replace ghrelin's role in FAA. Furthermore, GHS-R1a is known to display constitutive activity[42,43], which might be implicated in the regulation of FAA. However, we also observed effects of ghrelin on FAA. Ghrelin increased FAA to a palatable meal (**Chapter 3**) and a specific subset of neurons within the DMH/VMH showed a highly correlated response to ghrelin and a food-signalling cue (**Chapter 4**). Ghrelin -/- mice do not only lack ghrelin, but also obestatin, which originates from the same precursor gene. The biological function of obestatin is controversial, as initial reports that it antagonized ghrelin's effects on food intake and body weight gain[44] could not be replicated[45-47]. We hypothesize that compensatory changes in ghrelin -/- mice prevent impairment of FAA, and that ghrelin and GHS-R1a signalling are able to modulate the behavioural response during FAA. Further research is required to answer this question.

## The ability of Fos to detect neuronal activation

Fos immunohistochemistry is widely applied as a marker of neuronal activity. It has several advantages. First, its basal levels are low, providing a good signal-to-noise ratio, and its expression is rapidly induced by various stimulants. Second, it is widely expressed in the brain. Third, it has a short half-life, thus providing a good temporal resolution.[48] Hence, we (**Chapter 2**) and many others[3-6,20] used Fos activation to examine which brain areas are activated during FAA. However, Fos immunohistochemistry might not detect all neuronal activation, and does not differentiate between activation of inhibitory and excitatory neurons. And since basal Fos levels are in general low, Fos studies are only able to detect an increase in activity. Furthermore, only one time-point per rat can be measured using Fos. Therefore, in order to further examine the role of the DMH/VMH in FAA, we used *in vivo* electrophysiology to measure neuronal activity during FAA in awake, behaving rats (**Chapter 4**). *In vivo* electrophysiology offers benefits

compared to Fos studies, since it enables measurements of neuronal activity on the level of a single neuron as well as of a population, which can be combined with behavioural and pharmacological experiments. This technique enabled us to confirm previous Fos studies (**Chapter 2**, [3,4,30,49]) showing activation of the DMH/VMH region during FAA, by demonstrating that firing frequency of this neuronal population was increased during FAA, and that within a subset of neurons, responses during FAA and ghrelin were highly correlated (**Chapter 4**). Although *in vivo* electrophysiology is more expensive, invasive and time-consuming than Fos immunohistochemistry, it provides additional information about the longitudinal responses of neurons and responses to drugs. Hence, Fos studies can screen the potential involvement of brain areas in FAA, whereas *in vivo* electrophysiology allows a more detailed analysis of the involvement of individual neurons within one specific region of interest in FAA.

## Implications and future directions

This thesis has implicated GHS-R1a signalling in the regulation of food anticipatory activity in rats subjected to a RFS as well as a PFS. It would be interesting to examine whether ghrelin plasma levels continue to rise at the time of the expected meal after discontinuation of the feeding schedule. FAA is known to remain for a few days when RFS rats are food-deprived, or when PFS rats are restraint from chocolate.[18] However, how ghrelin signalling fits in the network of brain areas regulating FAA requires further investigation. GHS-R1a can be found in the hypothalamus, including in the Arc, VMH and DMH, but also in the VTA. How could ghrelin signalling in these areas contribute to FAA?

### Reward-related ghrelin signalling

Dopamine is known for its involvement in the regulation of reward and motivation via the mesolimbic dopamine system.[2] Dopaminergic neurons in the VTA project to the NAc, which in turn projects to many other limbic areas. Initially, ingestion of cue-signalled palatable food intake increased dopamine release in the NAc.[50,51] However, following habituation, augmented dopamine release transferred to the cue[52-55], hence in anticipation of the predicted reward. Additionally, stimulation of dopamine release in NAc induced locomotor activity.[56,57] Hence, increased release of dopamine in the NAc could be involved in FAA.

Around 50% of the dopaminergic neurons in the VTA co-express GHS-R1a[58], hence ghrelin might augment afferent reward signals via increased dopaminergic transmission from the VTA to the NAc[59]. Indeed ghrelin administration (central, peripheral and intra-VTA) resulted in increased dopaminergic levels in the NAc.[58,60,61] Furthermore, VTA ghrelin signalling is essential for ghrelin's orexigenic effects on palatable food intake, whereas ghrelin-induced increase in non-palatable food intake does not require VTA GHS-R1a.[15] In addition, VTA GHS-R1a are involved in ghrelin's effect on the augmented rewarding value of palatable food.[15,16,59] Interestingly, ghrelin can also amplify the effects of other reinforcing behaviours, such as drug- and alcohol-induced behaviours.[62-70]

In summary, via GHS-R1a in the VTA, ghrelin increases dopaminergic release in the NAc, which could lead to hyperactivity and increased reward value of a (palatable) meal. In light of these findings, it would be interesting to examine whether attenuating ghrelin signalling at the level of the VTA would reduce FAA to a restricted or palatable meal, and whether this involves ghrelin's well documented effects on the mesolimbic dopamine system[58,61].

## Hypothalamic ghrelin signalling

Orexin neurons are predominantly located in the LH, and orexin is known for its orexigenic and arousal-stimulating properties. Orexin acts downstream of ghrelin's effect on food intake. Ghrelin activates specifically orexin neurons in the LH[71-73] and blocking orexin signalling attenuated ghrelin-induced feeding[72] and motivation for a palatable reward[16]. We observed increased Fos activation in LH neurons during FAA in RFS rats (**Chapter 2**). Others have shown that orexin neurons were activated during FAA in rats expecting a chow meal and in those that anticipated a palatable meal.[74-78] Orexin knock out mice still exhibited FAA, although reduced or with a delayed acquisition.[40,76,78-80] However, specific ablation of orexin neurons in LH did not impair FAA.[81] Orexin neurons project to and receive projections from many other brain areas, including VTA and NAc.[82] Intra-VTA administration of orexin enhanced dopamine release in the NAc.[83] However, application of an orexin A receptor antagonist in the VTA did not affect ghrelin-induced hyperactivity.[84] The paraventricular nucleus of the thalamus (PVT) receives dense orexinergic projections[85] as well and was activated during FAA[5,20,86,87] and by a cue signalling palatable food intake[75]. Interestingly, the electrical stimulation of the PVT enhanced dopamine release in the NAc

independent of VTA [88]. Lesions of the PVT have been reported to attenuate anticipatory locomotor activity[86], although others still observed robust anticipatory activity[89]. Hence, recruitment of orexin neurons by ghrelin signalling might increase dopamine release in the NAc, either via VTA or PVT, and contribute to FAA. To shed more light on the components of the network that regulates FAA, it would be interesting to examine, whether the effects of ghrelin on FAA to a RFS[11] and a PFS (**Chapter 3**) are mediated via orexin signalling, if so, whether VTA-induced dopamine release is required for this.

In this thesis, we showed that the DMH exhibited Fos activation (**Chapter 2**) and that the DMH/VMH increased firing frequency (**Chapter 4**) in anticipation of a meal. Furthermore, reduced GHS-R1a signalling in the DMH delayed the onset and attenuated the amplitude of FAA (**Chapter 5**) and a subpopulation of the DMH/VMH shows a highly correlated response to ghrelin and a food-signalling cue (**Chapter 4**). As described in the introduction, the role of the DMH in FAA is under debate. Lesions have been reported to attenuate FAA in some studies[30,33], but did not affect FAA at all in other studies[32,34,90] Recently, it was demonstrated that a DMH lesion reduced FAA, but this effect disappeared when in addition the SCN was ablated, indicating a role for the DMH in silencing output from the SCN to permit FAA.[49] This potential role of the DMH fits with the finding that DMH lesions reduced FAA to a daytime meal, but not to a nighttime meal.[91] Future research could determine whether ghrelin's effect on the DMH during FAA is involved in silencing SCN output.

In order to examine whether GHS-R1a within a specific brain area is involved in FAA, we would, ideally, want to reversibly silence these neurons during FAA. Optogenetics involves the use of light-sensitive channel proteins, 'opsins', to manipulate neuronal function (see [92] for review). When activated by light, channelorhodopsins cause depolarization of the neuron, whereas halorhodopsin activation results in hyperpolarization. The use of AAV vectors that encode for cre-dependent halorhodopsin in combination with rodents expressing cre in GHS-R1a positive neurons, could answer this research question in future studies.

## Clinical relevance

The study of FAA and its underlying mechanisms is clinically relevant to disorders with a disturbed food intake, such as anorexia nervosa and obesity. Firstly, hyperactivity is observed in many anorexia nervosa patients and worsens outcome of the disease. The hyperactivity observed in rats on a RFS could reflect this hyperactivity observed in people suffering from anorexia nervosa, and might share common regulatory mechanisms. Secondly, metabolism is tightly coupled to circadian rhythms of locomotor activity and the sleep-wake cycle. In nature, it is pivotal to link the period of activity with food accessibility. As observed in **chapter 2**, when rats are subjected to a RFS, circadian rhythms uncouple from the light-dark cycle, and start cycling in relation to the period of food availability. This ability to shift circadian rhythms to food availability might be hampered in rodents with deficiencies in FAA.

Lastly, cues that are associated with food can induce food intake in satiated rats[93] and humans[94]. Therefore, these conditioned cues are able to evoke vulnerability to overconsume food in a specific time period or in a typical context. In the PFS model, rats associate a specific time of the day with a palatable meal. Insight into the regulation of FAA in this paradigm could shed light on the mechanisms of cue-induced overconsumption.

This study has implicated ghrelin signalling in the modulation of FAA in response to a RFS as well as a PFS. Interfering with ghrelin signalling could decrease hyperactivity, specifically anticipatory hyperactivity, and decrease the rewarding value of palatable food. Unfortunately, to date, administration of GHS-R1a antagonists has not been proven successful in obese patients.[95] Inverse agonists or GOAT inhibitors have not been tested yet in obese patients.

## Conclusion

In this thesis, we started out addressing the underlying mechanisms of FAA in rats subjected to a RFS or a PFS, and more specific to investigate the role of ghrelin signalling in FAA. Ghrelin had already been implicated in the regulation of FAA in rodents on a RFS, as ghrelin levels were known to rise preprandially[8-10] and decreased GHS-R1a signalling attenuated FAA[11-14]. However, whether ghre-

lin and/or GHS-R1a signalling would be involved in FAA in rats on a PFS was still unknown. Furthermore, it remained to be examined in which brain areas GHS-R1a signalling would contribute to FAA.

We have shown that ghrelin levels were increased during FAA in rats subjected to either RFS or PFS. As already had been shown for FAA in response to a RFS[11,12], ghrelin increased and GHS-R1a antagonism reduced FAA to a palatable meal. A subpopulation of neurons within the DMH/VMH responded in a highly correlated manner to a food signalling cue and to ghrelin, thereby providing a potential anatomical substrate that could drive FAA following stimulation by ghrelin. Although local hypothalamic knockdown of GHS-R1a delayed the onset of FAA (DMH, VMH) and attenuated the amplitude of FAA (DMH), FAA could still be detected. Hence, ghrelin signalling within the DMH/VMH is not essential for the development of FAA. Nevertheless, these findings underline a modulatory role of ghrelin in the regulation of FAA in RFS and PFS models, and suggest ghrelin signalling as a potential target in the treatment of disorders such as anorexia nervosa and obesity.

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# Addendum

# CURRICULUM VITAE

Myrte Merkestein was born on January 12th 1983 in Assen, the Netherlands. In 2001, she graduated from secondary school (Gymnasium, Sint-Odulphuslyseum, Tilburg, *cum laude*) and started studying Biomedical Sciences at the University of Utrecht. After obtaining her bachelor-degree in 2005, she continued with the master Neuroscience & Cognition also at the University of Utrecht. During her master she performed scientific internships in the research groups of Prof. dr. Roger Adan at the Rudolf Magnus Institute of Neuroscience, Utrecht and of Dr. Perry Barrett at the Rowett Research Institute, Aberdeen. After obtaining her master degree (*cum laude*), she was awarded a NWO Toptalent grant to fund her PhD-project entitled "What happens in our brain when we get ready for dinner?" on which she worked in the research group of Prof.dr. Roger Adan from September 2007 to October 2011. The results of this research project are presented in this thesis. In November 2011, Myrte started working as a postdoctoral research assistant in the research group of Prof.dr. Frances Ashcroft at the University of Oxford.

Myrte Merkestein werd geboren op 12 januari 1983 te Assen. In 2001 behaalde zij het gymnasium diploma (*cum laude*) aan het Sint-Odulphuslyceum te Tilburg. In datzelfde jaar begon zij met de studie Biomedische Wetenschappen aan de Universiteit Utrecht. Na het behalen van haar bachelor-diploma in 2005, vervolgde zij haar opleiding met de master Neuroscience & Cognition. Gedurende deze master heeft Myrte onderzoek gedaan bij de groep van Prof.dr. Roger Adan (Rudolf Magnus Instituut voor Neurowetenschappen, Universiteit Utrecht) en bij de onderzoeksgroep van Dr. Perry Barrett (Rowett Research Institute, Aberdeen). Na het behalen van haar master-diploma (*cum laude*), kreeg zij een NWO Toptalent beurs voor het AIO-project "What happens in our brain when we get ready for dinner?". In september 2007 begon zij met dit project in de onderzoeksgroep van Prof. Dr. Roger Adan. De resultaten van haar onderzoek zijn beschreven in dit proefschrift. In November 2011 is Myrte gestart als postdoctoral research assistant in de onderzoeksgroep van Prof.dr. Frances Ashcroft aan de Universiteit van Oxford.

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[a,b] Authors contributed equally to this work

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## BOOK CHAPTERS

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# SAMENVATTING IN HET NEDERLANDS

Obesitas wordt wereldwijd een steeds groter probleem. Men eet te veel en beweegt te weinig. Onze hersenen spelen een belangrijke rol bij de regulatie van onze energiebalans. Ze bepalen wanneer we gaan eten, en wanneer juist niet. Om meer inzicht te krijgen in de processen die betrokken zijn bij verstoord eetgedrag, zoals bij obesitas en anorexia nervosa, is het van groot belang te achterhalen wat er gebeurt in onze hersenen als we ons voorbereiden om te gaan eten. Dit proefschrift gaat over hyperactiviteit voorafgaand aan een maaltijd ("food anticipatory activity" (FAA)) in ratten.

Er zijn twee verschillende diermodellen die FAA opwekken. De eerste is het restrictieve maaltijdschema ("restricted feeding schedule" (RFS)). Ratten worden gehuisvest in kamers waar het afwisselend 12 uur licht en 12 uur donker is. Omdat ratten nachtdieren zijn, is de donkerperiode hun actieve periode. Ratten op een RFS hebben maar twee uur per dag tijdens de lichtfase, normaalgesproken de rustperiode, toegang tot hun normale voedsel, genaamd chow. Gaandeweg leren de ratten wanneer ze hun eten kunnen verwachten en worden ze hyperactief en extra alert in de periode voorafgaand aan het eten, dit noemen we FAA. Omdat de ratten in twee uur niet hun normale dagelijkse hoeveelheid eten consumeren, hebben deze ratten honger. In dit model vertegenwoordigt FAA waarschijnlijk dan ook honger en motivatie om te eten.

Ratten op een lekker maaltijdschema ("palatable feeding schedule" PFS) kunnen 24 uur per dag chow eten, maar krijgen als extraatje in het midden van de lichtfase 5 gram chocolade, wat ze erg lekker vinden. Ook ratten op een PFS leren wanneer ze hun chocolade kunnen verwachten en worden actiever en alerter in de periode voorafgaand aan de chocolade, hoewel dit effect minder groot is dan bij ratten op een RFS. Aangezien PFS-ratten de hele dag door chow kunnen eten, denkt men dat FAA in dit model vooral de motivatie om te eten representeert.

In dit proefschrift hebben we naar verschillende aspecten van FAA gekeken. Allereerst hebben we de fysiologische kenmerken van beide modellen beschreven en hebben we onderzocht welke hersengebieden actief worden tijdens FAA. Vervolgens hebben we gekeken naar de rol van het hormoon ghrelin, dat de eetlust stimuleert, en de receptor voor ghrelin (GHS-R1a) in de regulatie van FAA.

## FYSIOLOGIE VAN FAA-DIERMODELLEN

In *hoofdstuk 2* worden ratten op een RFS en PFS vergeleken met twee controlegroepen. Ad libitum (AL) ratten hebben 24 uur per dag toegang tot chow en zouden dus geen honger moeten hebben danwel FAA moeten vertonen. Random RFS (R-RFS) ratten hebben net als RFS-ratten twee uur per dag toegang tot chow, maar elke dag op een ander tijdstip in de lichtperiode. Deze ratten hebben dus net zoveel honger als RFS-ratten, maar zouden geen FAA moeten vertonen.
Omdat RFS en R-RFS-ratten maar twee uur per dag kunnen eten, verliezen ze lichaamsgewicht. Gedurende de drie weken van het experiment, gaan ze in die twee uur steeds meer eten door een grotere maaltijdgrootte. De maaltijdgrootte van RFS-ratten is in de laatste week groter dan van R-RFS-ratten, waarschijnlijk omdat de RFS-ratten weten wanneer hun eten komt.
Zoals eerder gezegd zijn ratten nachtdieren; dagelijkse ritmes van lichaamstemperatuur en activiteit zijn gekoppeld aan het licht-donkerritme en AL-ratten zijn dan ook actiever in de donkerperiode dan in de lichtperiode. Echter, wanneer eten alleen in de lichtperiode wordt aangeboden, zoals bij RFS- en R-RFS-ratten, verschuift de activiteit van de donker- naar de lichtperiode. R-RFS-ratten zijn gedurende de hele lichtperiode meer actief, maar RFS-ratten vertonen FAA: er is een piek van hyperactiviteit voorafgaand aan het moment dat ze eten krijgen. PFS-ratten laten ook hyperactiviteit zien net voordat ze chocolade krijgen. Deze piek is veel kleiner dan bij RFS-ratten. PFS-ratten vertonen geen verschuiving van hun activiteit, ze zijn nog steeds meer actief tijdens de donkerperiode dan tijdens de lichtperiode. Doordat RFS- en R-RFS-ratten minder te eten hebben, daalt hun lichaamstemperatuur, vooral in de lichtperiode. RFS-ratten vertonen echter wel een piek in lichaamstemperatuur voorafgaand aan hun maaltijd.
In de eerste twee weken van het experiment eten PFS-ratten meer calorieën per dag dan AL-ratten. Ze verminderen wel het aantal chowmaaltijden dat ze consumeren, maar pas in de derde week compenseren ze volledig voor de extra calorieën die ze innemen met de chocolade. Aan het eind van het experiment zijn PFS-ratten niet zwaarder dan AL-ratten. In tegenstelling tot RFS- en R-RFS-ratten, blijft het dagelijkse ritme in activiteit en lichaamstemperatuur in PFS-ratten gekoppeld aan het licht-donkerritme. PFS-ratten vertonen pas in de derde week van het experiment FAA, een toename in activiteit vlak voordat ze chocolade krijgen. Een piek in lichaamstemperatuur laten ze echter niet zien voorafgaand aan het eten van chocolade.

Samengevat leidt voedselrestrictie tot de lichtperiode ertoe, dat ritmes in activiteit en lichaamstemperatuur niet meer gekoppeld zijn aan het licht-donkerritme. RFS-ratten laten FAA zien in de vorm van hyperactiviteit en een verhoging van de lichaamstemperatuur, terwijl PFS-ratten alleen hyperactiviteit laten zien.

## ACTIVATIE VAN HERSENGEBIEDEN TIJDENS FAA

Om te onderzoeken welke hersengebieden actief worden tijdens FAA, hebben we gebruik gemaakt van een kleuring voor een eiwit dat voorkomt in neuronen die actief zijn. We hebben ons gericht op twee hersengebieden; de hypothalamus en de nucleus accumbens. De hypothalamus ontvangt signalen vanuit het lichaam over de energiebalans en reguleert dat energie-inname en –verbruik in balans zijn. Dit hersengebied zou dus de hongercomponent van FAA kunnen reguleren. De nucleus accumbens is meer betrokken bij beloning en motivatie van gedrag en zou dus de motivationele component van FAA kunnen reguleren.
In *hoofdstuk 2* wordt beschreven dat de hypothalamus in RFS en R-RFS-ratten inderdaad is geactiveerd in vergelijking met AL ratten, maar in PFS-ratten was dit niet het geval. Het lijkt er dus op dat honger, en niet zozeer anticipatie, activatie van de hypothalamus veroorzaakt. Interessant genoeg vonden we alleen in de RFS-groep correlaties tussen het aantal geactiveerde neuronen en de mate van FAA in een aantal regio's van de hypothalamus. Foskleuringen zeggen alleen iets over activatie van neuronen, maar niets over welk type neuronen geactiveerd zijn. Om te onderzoeken of een specifieke populatie van neuronen meer betrokken is bij anticipatie hebben we een dubbelkleuring gedaan van Fos met a-MSH, een marker voor neuronen die geactiveerd worden als de energiebalans positief is. Zowel RFS, R-RFS als PFS-ratten vertoonden minder activatie van deze populatie, wat inderdaad suggereert dat deze ratten meer honger hebben op het moment net voordat eten komt.
In tegenstelling tot wat we verwacht hadden, was de nucleus accumbens wel geactiveerd in RFS en R-RFS-ratten, maar niet in PFS-ratten (*hoofdstuk 2*). Dit zou kunnen komen doordat de PFS-ratten pas in de laatste week van het experiment FAA vertoonden.
In *hoofdstuk 4* beschrijven we een *in vivo* electrofysiologie methode, waarbij we electrodes hebben geïmplanteerd in de mediale hypothalamus en zo in vrij-

bewegende ratten hersenactiviteit in dit gebied konden meten. Een lichtsignaal dat de naderende aanwezigheid van voedsel signaleerde, veroorzaakte FAA en verhoogde hersenactiviteit in de mediale hypothalamus, wat overeenkomt met onze bevindingen van de Fos-kleuringen in *hoofdstuk 2*.

## GHRELIN EN FAA

Levels van het hormoon ghrelin in het bloedplasma zijn verhoogd tijdens FAA in RFS- en PFS-ratten (*hoofdstukken 2 & 3*). In overeenstemming met het verschil in FAA tussen beide groepen, waren ghrelin-levels ook iets verhoogd in RFS-ratten ten opzichte van PFS-ratten. R-RFS-ratten vertoonden ook verhoogde ghrelin-levels, wat suggereert dat deze ratten hongerig zijn. Dit komt overeen met de verminderde activatie van de a-MSH-populatie.
Het is bekend uit de literatuur dat zowel in mensen als knaagdieren ghrelin-levels stijgen wanneer een maaltijd verwacht wordt.[6-8] Bovendien versterkt toediening van ghrelin FAA in een RFS-model[9], en verminderde signalering via de ghrelin receptor GHS-R1a vermindert FAA[9-12]. Ghrelin staat niet alleen bekend om zijn eetluststimulerende functie, maar kan ook motivationele aspecten van eten beïnvloeden.[13-15] In *hoofdstuk 3* hebben we gekeken of ghrelin ook betrokken is bij FAA in PFS-ratten. Toediening van ghrelin stimuleerde FAA in PFS-ratten, en een antagonist van GHS-R1a remde FAA in PFS-ratten die een minimale hoeveelheid FAA vertoonden onder normale omstandigheden.

## GHRELIN SIGNALERING IN DE HYPOTHALAMUS EN FAA

In de *hoofdstukken 2 en 4* hebben we laten zien dat de mediale hypothalamus betrokken zou kunnen zijn bij FAA in RFS-ratten. Ook hebben wij laten zien dat ghrelin-levels stijgen gedurende FAA en in de literatuur is beschreven dat GHS-R1a-signalering een rol zou kunnen spelen in FAA. [9-11] Daarom wilden we onderzoeken of GHS-R1a-signalering in de mediale hypothalamus belangrijk is voor FAA.
Met *in vivo* electrofysiologie hebben we aangetoond dat de dorsomediale en ventromediale hypothalamus (DMH/VMH) geactiveerd wordt door zowel een

lichtsignaal dat aankondigt dat er eten aankomt als door toediening van ghrelin *(hoofdstuk 4)*. Er is een subpopulatie van neuronen die significant reageren op het lichtsignaal en op ghrelin, en die twee responsen zijn sterk aan elkaar gecorreleerd (r=0.95). Deze specifieke populatie zou dus bij kunnen dragen aan de regulatie van FAA door ghrelin.

Daarom hebben we in *hoofdstuk 5* gekeken naar het effect van verminderde GHS-R1a in de VMH en DMH op FAA. We hebben AAV-vectors geïnjecteerd in de DMH en VMH die een shRNA-sequentie bevatten tegen de GHS-R1a. Het resultaat hiervan is verminderde expressie van GHS-R1a in specifiek de DMH of de VMH. Onder normale omstandigheden resulteerde dit in een reductie in renwielactiviteit in beide groepen en in de VMH groep ook tot een hoger lichaamsgewicht door een toename van voedselinname.

Ook wanneer de ratten onderworpen werden aan een RFS bleef renwielactiviteit verminderd, waardoor absolute waarden van FAA ook lager waren dan die van de controlegroep. Wanneer FAA gecorrigeerd werd voor de afname in renwielactiviteit in het algemeen, vertoonden alleen ratten met verminderde expressie van GHS-R1a in de DMH een reductie in de hoeveelheid FAA. Hoewel VMH-knockdown van GHS-R1a niet leidde tot een vermindering in de hoeveelheid FAA, begonnen ratten wel later met FAA, net als de DMH-groep. GHS-R1a-signalering in de DMH en VMH draagt dus bij tot FAA. Echter, FAA is niet alleen afhankelijk van GHS-R1a-signalering in de mediale hypothalamus, omdat FAA niet helemaal verdween. Dit betekent dat GHS-R1a-signalering in de VMH en DMH waarschijnlijk onderdeel is van het netwerk in de hersenen dat FAA reguleert.

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