Ghrelin in the regulation of feeding and energy balance

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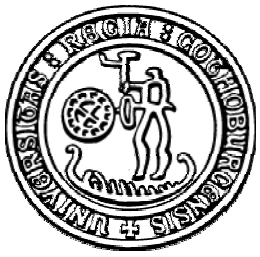

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”problems worthy of attack
prove their worth by hitting back”
Piet Hein

Abstract

Ghrelin, the first identified endogenous ligand for the growth hormone secretagogue receptor 1A, is a 28 amino acid peptide produced mainly by the stomach. Pharmacological studies indicate a role for ghrelin in the regulation of growth hormone secretion from the pituitary and also in the regulation of body weight, fat accumulation and food intake.

Using a classical endocrine deletion/replacement approach we found support for the notion that endogenous ghrelin is required for the maintenance of normal body weight and adiposity. Gastrectomy (Gx) surgery, that depleted animals of ~80% of circulating ghrelin, caused a reduction in body weight, fat mass and lean mass in adult mice. Ghrelin replacement (at a dose that restores circulating ghrelin levels in Gx mice and that is without effect on body weight in sham animals) fully or partially reversed the decrease in body weight, fat mass and lean mass following Gx. To further investigate the central mechanism behind these effects on body weight and fat mass following Gx-surgery and ghrelin treatment key hypothalamic genes involved in energy homeostasis were analysed by *in situ* hybridisation. Surprisingly the marked changes in body composition following Gx did not effect expression of the hypothalamic genes studied, to any large extent. By contrast ghrelin treatment increased mRNA expression of NPY and AgRP and decreased POMC mRNA expression in accordance with ghrelin's effects to increase fat mass and body weight.
Using growth hormone receptor (GHR) knockout animals we investigated the importance of a functional GHR signalling system for the acute effects of ghrelin on food intake. Ghrelin treatment increased food intake in wild type animals but not in GHR knockouts indicating that a functional GHR signalling system is needed for the acute effects of ghrelin on food intake.
In addition to impacting upon the hypothalamic circuits controlling energy balance, ghrelin was found to interact with the mesolimbic reward circuits (reflected by increased locomotor activity and dopamine release after ghrelin injection to the brain ventricles).
In conclusion, endogenous ghrelin from the stomach is important for maintaining normal body weight and body composition. Long term treatment with ghrelin increases body fat by a mechanism that appears to be independent of its acute affects on food intake. Long term ghrelin treatment still impacts upon hypothalamic genes regulating energy balance. Ghrelin's acute effect on food intake is dependant on a functional GHR signalling system. Moreover, this effect may be linked to dopamine release in areas of the brain intimately associated with reward-seeking activities.

List of publications

This thesis is based on the following publication, which are referred to by their Roman numerals in the text (I-V):

I Charlotta Dornonville de la Cour, Andreas Lindqvist, Emil Egecioglu, Loraine YC Tung, Vikas Surve, Claes Ohlsson, John-Olov Jansson, Charlotta Erlanson-Albertsson, Susanne L Dickson and Rolf Håkanson Ghrelin treatment reverses the reduction in weight gain and body fat in gastrectomised mice *Gut* 2005; 54:907-913; originally published online 21 April 2005

II Emil Egecioglu, Björn Stenström, Scarlett B. Pinnock, Loraine YC Tung, Charlotta Dornonville de la Cour, Andreas Lindqvist, Rolf Håkanson, Unni Syversen, Duan Chen, Suzanne L. Dickson. Effects of ghrelin and a ghrelin receptor agonist on the expression of hypothalamic genes involved in energy balance following gastrectomy of mice and rats. (Accepted by *Regulatory Peptides*, subject to revision)

III Elisabet Jerlhag, Emil Egecioglu, Suzanne L. Dickson, Malin Andersson, Lennart Svensson, Jörgen A. Engel. Ghrelin stimulates locomotor activity and accumbal dopamine-overflow via central cholinergic systems in mice: implications for its involvement in brain reward *Addiction Biology* May 2006; 11(1):45-54

IV Emil Egecioglu, Mikael Bjursell, Anna Ljungberg, Suzanne L. Dickson, John J Kopchick, Göran Bergström, Lennart Svensson, Jan Oscarsson, Jan Törnell and Mohammad Bohlooly-Y Growth hormone receptor deficiency results in blunted ghrelin feeding response, obesity, and hypolipidemia in mice *Am J Physiol Endocrinol Metab* 2006; 290:317-325; originally published online 20 September 2005

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Abbreviations

AgRP agouti-related peptide
CART cocaine and amphetamine regulated transcript
DEXA dual energy X-ray absorptiometry
GH growth hormone
GHR growth hormone receptor
GHSR-1A ”ghrelin receptor”
Gx gastrectomy
ICV intracerebrovetricular
MK-0677 synthetic ligand of the GHSR-1A (”ghrelin receptor”)
NPY neuropeptide Y
N. Acc nucleus accumbens
PCR polymerase chain reaction
POMC pro-opiomelanocortin
PYY peptide YY_{3-36}
RQ respiratory quotient
Wt wild-type; background strain of transgenic mouse strain
Note: some widely recognized abbreviations used in this thesis have deliberately been omitted from the list. Terms occurring only one or twice in the text are generally spelt out where they occur.

Introduction

The first law of thermodynamics states that energy can neither be created nor destroyed which in a biological setting could be expressed simply as:

Energy intake = Energy utilized + Energy stored

This fundamental concept of energy balance theoretically apply to all living organisms but is often much more complex when studied in relation to energy homeostasis *in vivo*. Food consumption, frequently referred to as energy intake, is comparatively easy to measure, but remains a fairly inexact measure of the actual energy put into the system. Instead, absorption of calories in the gastrointestinal tract, which requires a lot more effort to estimate, should be of consideration. More importantly, changes in energy expenditure or energy input are not inert within a biological system; rather they tend to affect one another in such a way that the achieved body weight or the "maximum energy status" of the system is defended in the long run (154). Consequently, increased physical activity ultimately leading to increased energy expenditure is often countered by increased appetite. Conversely, if food consumption is decreased deliberately by fasting (or involuntarily during famine), energy expenditure decreases making weight loss much harder than implied by the above equation. The set point theory of body weight implies that changes in body weight is counter acted by regulatory processes of energy homeostasis, whereby total energy intake closely match energy expenditure over long periods of time (Fig1).

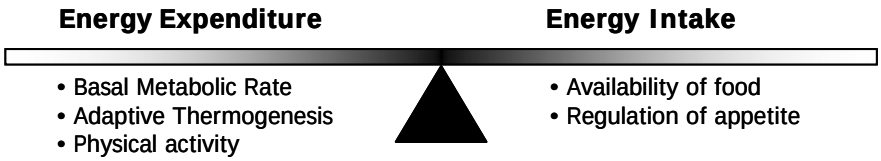


Figure 1. Energy balance and the components of energy balance in a biological setting
The first law of thermodynamics states that energy can neither be created nor destroyed. Thus, if energy intake exceeds the energy expenditure, the remaining energy must be stored. Both energy intake and energy expenditure are under tight physiological control in order to achieve long-term energy balance and a stable body weight. Caloric intake is entirely governed by food consumption and absorption over the intestine whereas energy expenditure is composed of several constituents, including basal metabolic rate, physical activity, and adaptive thermogenesis (a term used to describe diet-induced and cold-induced thermogenesis).

Underlying energy homeostasis are multiple intricate processes (neural, endocrine and metabolic) derived from the central nervous system (CNS) and periphery that act in concert to maintain a relatively constant body weight, despite great variations in daily energy expenditure and energy intake i.e. cumulative energy intake tends to match energy expenditure over time.

Throughout adult life body weight is also kept rather stable which reflects an active regulatory process promoting stability in body weight and in the amount of energy stored in adipose tissue. This requires input from endocrine and neuronal signals produced in proportion to body fat content informing the CNS of the current energy status of the body (153).
These long-term signals, exemplified by leptin, serve as major feedback regulators repressing neuronal hunger pathways, stimulating satiety pathways and increasing energy expenditure. Short-term signals arise mainly from the intestinal tract, hepatic portal vein and the liver and are transmitted via afferent fibres of the vagus nerve or via the blood stream to the CNS (125). Primarily these signals regulate acute indigestive behaviour by mediating satiety leading to meal termination. The gastrointestinal tract also produces at least one known peptide signal that stimulates appetite, namely ghrelin.

Ghrelin: its discovery and biological activity

Ghrelin is a 28 amino acid gut peptide that is mainly produced by the endocrine A-like cells in the acid producing part of the stomach (48, 55) , but ghrelin producing cells are also found throughout the whole GI tract, most abundantly in the stomach and decreasing aborally. Although the stomach is the major source of circulating ghrelin, exemplified by the 80-85% loss of circulating ghrelin following gastrectomy (Gx) (6, 55) other tissues including the hypothalamus, pancreas, kidney, testis and the placenta have been reported to produce ghrelin (43, 50, 74, 122, 170). Ghrelin was first isolated by Kojima and colleagues from rat stomach extracts and was given the name ghrelin for its ability to stimulate growth hormone (GH) secretion from the pituitary (101). Independently, another group isolated the same peptide in mouse and published the sequence under the name motilin-related peptide (172). Ghrelin is cleaved from a larger precursor, preproghrelin, and it is post-transcriptionally modified by covalently linking a medium-chain fatty acid with the serine-3 residue of the final peptide (101, 131). This modification occurs in ghrelin-secreting cells in the stomach and is required for the peptide to bind to and activate its established receptor, the growth hormone secretagogue receptor (GHSR) 1A (101). In the gastrointestinal tract and the circulation ghrelin exist in two major forms, acetylated and des-acylated ghrelin, the latter of which lacks the covalently linked fatty acid chain (87). Des-acylated ghrelin is the most common form in the circulation but does not activate the GHSR-1A. The physiological role, if any, of des-acylated ghrelin remains to be determined. Different research groups have reported that des-acylated ghrelin stimulate, inhibit or to have no effect on food consumption and GH secretion (7, 173). However, recent reports suggest that it has a role in gastric motility (38).

Ghrelin: a regulator of growth hormone secretion and energy balance.

Studies of ghrelin treatment have so far focused largely on the acetylated form of the peptide (referred to as ghrelin through out the text). Since the initial findings that ghrelin, apart from its effects on GH secretion (101, 166), robustly stimulates food intake and fat accumulation much attention has been drawn to its role in energy homeostasis (8, 127, 174, 188). Both central and peripheral injection of ghrelin potently stimulate food intake acutely in rodents as does

peripheral injections to humans, which is in line with earlier reports of the orexigenic effects of growth hormone secretagogue GHS (102). Furthermore, body weight and adiposity have been shown to increase following chronic administration of ghrelin as a result of anabolic effects on food intake, energy expenditure and nutrient partitioning (fuel utilisation) (8, 127, 174, 188).
The circulating levels of ghrelin have been shown to increase pre-prandially and decrease rapidly following meals (45). In subjects devoid of mealtime cues, circulating ghrelin levels match hunger scores suggesting that ghrelin may be the first identified circulating hunger hormone (44). This theory put forth by Cummings and colleagues is supported by several findings such as (a) the production of ghrelin by organs well-situated to sense ingested nutrients (6, 48), (b) the rapid and short lived orexigenic effects of ghrelin (8, 127, 174, 188) (c) the effects of ghrelin to decrease the latency to feed without increasing meal size i.e. increasing appetitive rather than consummatory feeding (65, 96) and (d) the effects of ghrelin to increase gastric motility, gastrin secretion and enzymatic activity of the intestine preparing the GI tract for effective transport and food processing (56, 114, 185). Furthermore, ghrelin targets the neurons in the arcuate nucleus of the hypothalamus co-expressing neuropeptide Y (NPY) and agouti related protein (AgRP) which are well known orexigens implicated in the central control of meal initiation (46). In line with this pharmacological blockade of these target neuropeptides attenuates the orexigenic effects of ghrelin and genetic deletion of NPY and AgRP completely abolishes ghrelin's effects on feeding (40, 127). Furthermore, almost all, of the NPY/AgRP containing cells in the arcuate express GHSR-1A (120, 180) and are activated by ghrelin and ghrelin mimetic, as demonstrated by increased firing rate and c-Fos expression in these neurons following ghrelin treatment or bath application (43, 54, 83).

Sites of ghrelin action within the CNS

Apart from the hypothalamic NPY/AgRP neurons, other areas of the brain such as the hindbrain and areas linked to mesocortico-limbic dopamine and serotonin have been implicated in ghrelin's effects on feeding (35, 36, 128). The expression of GHSR-1A in the brain is highly selective and restricted to areas known to be important for the regulation of food intake and energy balance including several hypothalamic nuclei, the dorsal vagal complex (area postrema, nucleus of the solitary tract and the dorsal motor nucleus of the vagus), the hippocampus, the ventral tegmental area (VTA) containing the mesocorticolimbic dopamine cell bodies and several areas that project into the VTA such as the laterdorsal tegmental area (LDTg), podunculopontine nucleus (PPTg) and the dorsal raphe (75, 88). Injections of ghrelin into several of these areas increase food intake indicating that ghrelin also exerts orexigenic effects by influencing circuits additional to the well documented orexigenic NPY/AgRP pathways (36, 65, 128, 187). However, it is not known how peripheral ghrelin is able to exert its effect on these different areas in the brain. There are studies showing that ghrelin can pass over the blood brain barrier from the periphery to the brain but also in the opposite direction (9, 139). Furthermore, the hypothalamic and hindbrain areas expressing GHSR-1A are close to circumventricular organs which are thought to be permeable to certain circulating factors and hormones indicating that ghrelin from the periphery

could reach these sites and maybe also deeper sites within the brain. However, ghrelin-containing neurons have been found in the hypothalamus suggesting that locally produced ghrelin could be acting as a neuropeptide influencing the circuitry important for energy homeostasis (43). It is noteworthy, however, that ghrelin lacZ reporter transgenic animals do not show any beta-galactosidase staining in the hypothalamus indicating that the hypothalamic ghrelin detected be of peripheral (ectopic) origin (186). Ghrelin in the circulation has also been suggested to exert effects on feeding through the vagal nerve since peripheral ghrelin injections fail to elicit feeding in vagotomized animals (49).

Growth hormone-independent effects of ghrelin

From the initial studies on ghrelin and food intake it was concluded that the increased food intake following central ghrelin treatment is independent of GH signalling since ghrelin is able to stimulate acute feeding in spontaneous dwarf rats (SDR), a growth hormone deficient (GHD) rat model that carries a disrupted GH gene (127). However, daily repeated injections of ghrelin peripherally does not affect food intake in female SDR but did increase cumulative body weight (174). Long term treatment with the ghrelin mimetics, GH-releasing peptide-6 (GHRP-6) and ipamorelin, have also been shown to increase body weight moderately in GH-deficient *lit/lit* mice (106).

Ghrelin status in relation to body mass

Ghrelin levels in circulation are found to be inversely proportionate to energy stores in the body and compensatory changes in ghrelin in response to alterations of these stores have been shown. In obese humans levels of ghrelin are low whereas patients with anorexia nervosa have increased circulating levels of ghrelin (6, 137, 155, 171). Levels of ghrelin are also altered in a compensatory fashion in response to weight loss or weight gain (47, 181). Moreover the expression of GHSR-1A in the hypothalamus is increased markedly in response to fasting or caloric restriction (98, 134) as well as the hypothalamic responsiveness to ghrelin mimetics (176). These observations are consistent with the criteria for an adiposity hormone (153) such as leptin, only ghrelin would be an orexigenic counterpart to for this classic feed back signal in the overall energy homeostasis.

In search of a physiological role of ghrelin

Pharmacological studies show effects of ghrelin on GH release, appetite and fat deposition but studies in gene deletion models aiming to elucidate the physiological role of ghrelin indicate that endogenous ghrelin may not be as important for these effects as first expected (163, 186). Two independent studies show that mice with deletion of the ghrelin gene appear perfectly normal with respect to body weight and body composition, appetitive behaviour, fat stores and body growth (163, 186). These authors report that the ghrelin deleted animals respond normally with respect to food intake and body weight when challenged with a high fat or high protein diet, indicating that ghrelin may not be a critical orexigenic factor (or that orexigenic factors are not critical for maintaining body weight) (163, 186). Ghrelin deficiency throughout

development may be compensated for by other systems, perhaps illustrating the need for many orexigenic signals for survival. It is noteworthy however, that ghrelin knockout mice described by Worley et al (186), were found to utilise fat rather that carbohydrates as energy source (ie decreased respiratory quotient, RQ) when challenged with a high fat diet which is in line with Matthias Tschöp's data showing that ghrelin treatment decreases RQ. Thus, endogenous ghrelin appears to be of importance for determining which type of metabolic substrate is used for maintenance of energy balance (174). While altered RQ does not necessarily reflect altered energy expenditure (that would be required for a change in energy balance), it has been shown to be a predictor for human obesity (195).

Deletion of key anorexigenic signals (eg leptin and melanocortins) render animals markedly obese which may reflect the essential role of these signals in the maintenance of body weight homeostasis (189, 192). By contrast, deletion of orexigenic signals as in the NPY, AgRP or the double NPY/AgRP knockout (ie key neuropeptides in target cells groups for ghrelin's central actions on appetite), like ghrelin knockouts, have little or no change in phenotype (63, 145). However, deletion of the GHSR-1A gives a modest bodyweight phenotype in mice on normal chow and protects from diet induced obesity (164, 194). These phenotype changes, following deletions of the GHSR-1A, in contrast to ghrelin deletion, is in line with reports of constitutive activity of the GHSR-1A (86) which also may explain why ghrelin knockouts lack a clear body weight phenotype.

From these studies in GHSR-1A knockouts it could be concluded that ghrelin´s acute effects on both feeding and GH secretion is dependent on GHSR-1A signalling (164, 194). Even though, no effect on bone parameters were found in the GHSR-1A deletion models nor following ghrelin deletion, indicating that the physiological role of ghrelin and GHSR-1A signalling is most likely energy balance rather than GH secretion, serum insulin like growth factor 1 (IGF-1) levels were decreased in one of these GHSR-1A knockout models (164). It has recently also been shown that familial short status in humans could be coupled to loss of constitutive signalling of GHSR-1A (140).

Thus, studies of ghrelin and GHSR-1A knockout mice are consistent with the role for ghrelin in promoting a positive energy balance, although it is clearly not the only system promoting a positive energy balance as the phenotype of the knockouts are rather modest. Although ghrelin stimulates GH secretion, it has yet to be fully determined whether ghrelin participates as a regulator of the normal GH secretory pattern. It is notable that GH secretion is not always coupled with increased plasma ghrelin levels, for example, GH increase in serum 2 h postprandially when ghrelin levels have been shown to be relatively low but during nutrient depravation ghrelin is thought to drive GH release (5, 124).

CNS control of body weight

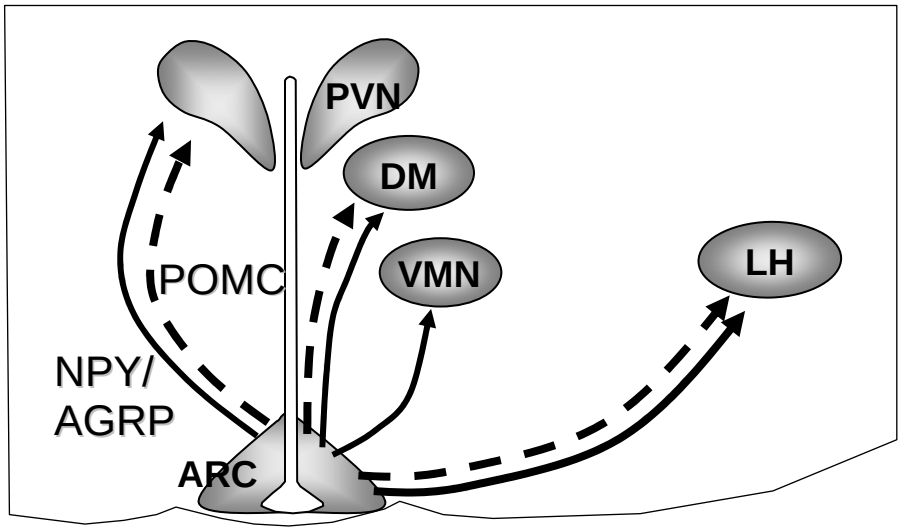
The complex process of regulating body weight involves several regions of the brain. Most attention has been focused on the hypothalamus and the brain stem and the neuronal populations within these brain regions that are important for the homeostatic regulation of feeding and energy balance. However, higher brain regions such as the prefrontal cortex, the mesocorticolimbic system and the amygdala (to mention a few) involved in regulating goal directed behaviour (motivation) and reward also seems to be involved in the central control of energy balance (18, 97). The CNS may influence energy balance and body weight through at least three mechanisms: 1) effects on feeding behaviour and physical activity 2) effects on the autonomic nervous system regulating basal metabolic rate and other aspects of metabolism 3) neuroendocrine effects on release of hormones such as thyroid hormone, GH, sex steroids, cortisol and insulin.

Hypothalamic regulation of energy intake and energy expenditure.

The hypothalamus has been seen as the prime central regulator of food intake and energy homeostasis for over half a century. Early studies in humans with hypothalamic injury (27) and in later studies using electrolytic lesions in animals identified the hypothalamus and even distinct areas within the hypothalamus as important nodes in the regulation of digestive behaviour and body weight. Hetherington and Ransom published a set of observations in the early forties on the effects of ventromedial hypothalamic lesions (that may in fact have included the ventromedial and the dorsomedial hypothalamic nuclei, the arcuate nucleus and the medial portion of the lateral hypothalamic area) on body weight and adiposity (1). Most of these lesions rendered animals hyperphagic which resulted in sever obesity within a few weeks following surgery. In the same studies it was also observed that lesions of more lateral areas had the opposite effect on body weight and adiposity. Later studies by Anand and Brobeck showed that bilateral lesion of the lateral hypothalamic area (LHA) induced pronounced aphagia (lack of food intake) even leading to death of the animal unless force fed and hydrated (4). While deletions of the medial parts of the hypothalamus induce hyperphagia, subsequent lesions of the LHA in the same animal cause hypophagia, implying that the LHA be of higher order then the ventromedial parts of the hypothalamus in the regulation of feeding. In contrast to lesions, electrical stimulation of the hypothalamus produced the opposite effects; lateral stimulation elicits feeding whereas ventromedial stimulation suppresses feeding. This functional neuroanatomical partitioning gave rise to the "dual-centre model" (161), with the lateral hypothalamus containing a feeding centre and the medial hypothalamus a satiety centre. Today the regulation of food intake and body weight is thought to be organised in neuronal networks, interconnected throughout the different structures of the hypothalamus (and the rest of the brain), rather then being governed by discrete centres regulating specific functions. Even though research since, has developed our understanding of the neurochemistry and the connections linking individual hypothalamic nuclei, as well as the links between the hypothalamus and the rest of the brain, the findings of Hetherington and Ransom stand remarkably true in that both the ventromedial and the lateral hypothalamus have been shown to contain neurons that express specific peptides and transmitters

that elicit or inhibit food intake. Figure 2a provides a schematic illustration of the hypothalamic areas important for the regulation of eating behaviour and the neurochemical signals therein.

A



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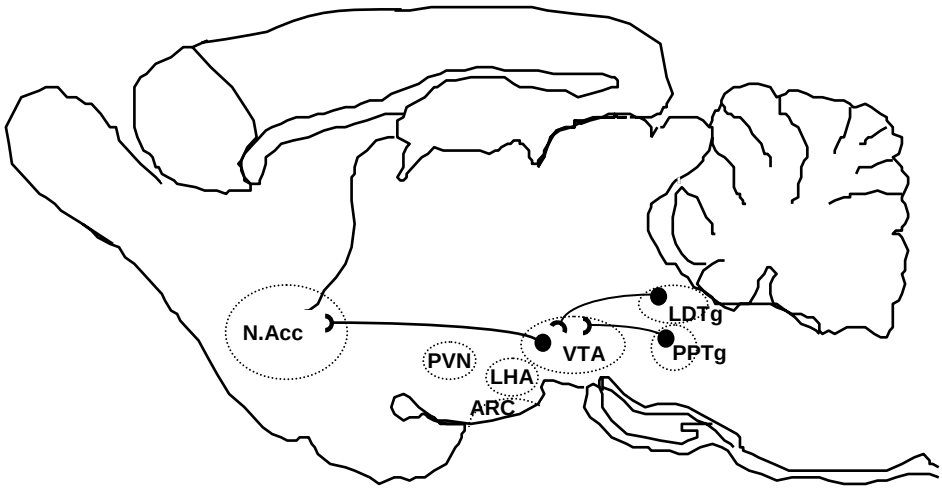

Figure 2a) Cross section of the hypothalamus, showing different areas important for food consumption and energy balance regulation. Neuropeptide Y (NPY) / Agouti-related peptide (AgRP) and pro-opiomelanocortin (POMC) containing cells in the arcuate nucleus (ARC) projecting to the paraventricular nucleus (PVN), lateral hypothalamic area (LHA) ventromedial nucleus (VMN) and the dorsomedial nucleus (DMN) **b)** Sagital section showing the acetylcholine dopamine reward link. Dopamine cells originating in the ventral tegmental area (VTA) project to the nucleus accumbens (N.Acc) and the acetylcholine producing cells from the laterodorsal tegmental area (LDTg) and podunculopontine nucleus (PPTg) innervating the VTA.

Lipostat theory and leptin

Unravelling of the hypothalamic mechanisms governing food intake and body weight regulation was given a tremendous push forwards by the discovery of the adiposity hormone leptin. Leptin is the product from the obese gene (*ob*) which was cloned in 1994 in the obese *ob*/*ob* mouse (76). Mutations in the *ob* gene as well as the receptor for leptin, discovered in 1995 in the diabetic (*db*) locus (169), result in severe obesity in both rodents and humans (39, 121). Leptin, produced predominantly in the white adipose tissue and circulating in proportion to adipose stores, is thought to be the main feedback regulator communicating body energy status to the brain (115, 153). Lack of leptin signalling elicits ingestive behaviour and increase the metabolic efficiency robustly as shown in the *ob*/*ob* mice.

Identifying sites of expression of the leptin receptor with in the CNS was instrumental for the unravelling of key hypothalamic circuits, but also other brain sites, important for control of food intake and energy balance. The receptor for leptin is found in high abundance in several nuclei of the hypothalamus but foremost in the arcuate nucleus (116). Apart from the hypothalamus, the receptors for leptin have been found in several structures of the brain such as the hippocampus, nucleus accumbens (N.Acc), VTA, thalamus, vagal afferent neurons and even taste receptor cells indicating leptin involvement in many, if not all, aspects of ingestive behaviour, such as taste perception, meal-related visceral feedback and motivational brain rewarding mechanisms (34, 59, 66, 69, 94, 116).

The arcuate nucleus: a key site for integration

Parts of the arcute nucleus, located at either side of the base of the third ventricle, is exposed to the circulation through the circumventricular medial eminence, which has a compromised blood brain barrier making it permeable to circulating hormones and nutrients. Consequently neurons in the arcuate, such as those co-expressing NPY/AgRP and pro-opiomelanocortin/ cocaine and amphetamine regulated transcript (POMC/CART), are perfectly situated to receive first hand information directly from the blood regarding the current energy status of the body (179). The sensory quality of these neurons is further emphasised by the fact that they express receptors for several peripheral signals known to influence food intake and energy expenditure such as leptin, peptide YY $(PYY)_{(3-36)}$, adiponectin, insulin and glucagons like peptide-1 (GLP-1) (12, 104, 116, 130, 180). Arcuate NPY/AgRP neurons also express the GHSR-1A and ghrelin has been found to regulate NYP/AgRP and POMC/CART neurons differentially (43, 180).

The actions of ghrelin, leptin and other peripheral signals on orexigenic NYP/AgRP and anorexigenic POMC/CART neurons (sometimes referred to as first order neurons in the literature) trigger a cascade of neuronal events, regulating the activity of other appetite controlling neurons (secondary order neurons) such as the melanin concentrating hormone (MCH) and orexin containing neurons in the LHA and the corticotrophin releasing hormone (CRF) neurons of the paraventricular nucleus (PVN). Immunoreactive NPY/AgRP and POMC/CART fibres innervate both the PVN and the LHA indicating that the orexigenic and the anorexigenic signals form the arcuate might be converging and integrated within these regions (58). NPY and α-MSH (the most potent

anorexigenic gene product of the POMC gene) injections into the PVN or the LHA have opposite effects on both feeding and regulation of gene expression. Furthermore, the GABA-evoked current of individual PVN neurons is differentially influenced by NPY and the melanocortin receptor agonist MTII (42). The PVN innervate regions important for autonomic and endocrine function (165) whereas the LHA is believed to integrate hypothalamic feeding signals with higher cortical brain functions (149). The neuronal populations of the LHA have also been shown to project into and affect limbic structures such as the VTA and the N.Acc (64).

Dopamine in reward and food intake

Body weight set point is often attributed to hypothalamic control mechanisms; however, it is reasonable to assume that factors/circuits other than purely metabolic ones might be of importance for eating behaviour. Sensory inputs from smell, sight and taste as well as texture of foods are undoubtedly of importance in regulating the day to day eating behaviour as are habitual and social behaviours. Increased or sometimes decreased appetite paired to external cues and conditioned stimuli such as these is known as a conditioned response and would most certainly involve dopamine signalling.

Since 1957 when Carlsson suggested it to be a transmitter in the brain (37), dopamine has been implicated in a variety of functions such as emotion, reward and reinforcement, cognition, locomotion and neuroendocrine regulation (3, 16, 24, 60, 68, 123, 182). The mesocorticolimbic dopamine projections, extending from distinct dopamine cell populations in the VTA, terminate in the ventral striatum and the prefrontal cortex (PFC) (fig 2b). These projections appear to be common denominators in the reward systems and participates in hedonic feelings of incentives, natural and artificial, as well as in reward conditioning and reinforcement, i.e. learning and motivation (52, 53, 61, 183). The activation of these dopaminergic projections, as quantified by electrophysiological, microdialysis, or voltammetric measures, is elicited by foods, sex, drugs of abuse and secondary reinforces of these incentives (16). However, the interpretation of the role of dopamine activation in reward or in association with rewarding stimuli is debated. The *anhedonia hypothesis* (or, in relation to normal dopamine function, the *hedonia hypothesis*) defined as the inability to experience pleasure in situations that normally would be pleasurable, proclaim that dopamine mediates the pleasure produced by natural, artificial and conditioned incentives and that blockade of dopamine results in anhedonia (184). Other interpretations of the role for dopamine in brain reward have developed from the understanding that dopamine appears to be linked to anticipatory, appetitive or approach phases of motivated behaviour as opposed to pure consummatory phases. The *motivational learning hypothesis* suggests that dopamine mediates some aspects of reward conditioning (learning), or the capacity to predict rewarding events from association correlations (53). The *incentive salience hypothesis* proposes that dopamine attributes attractive value to an otherwise neutral neuronal representation of a stimulus (mere information), transforming it into "wanting" and thus motivates the organism to work for such a stimulus (16).

Regardless of the different theories behind dopaminergic involvement in reward there is strong support for the hypothesis of a role of central dopamine in

appetitive and food consummatory behaviour. Already in the late 30s Lesses and Myerson suggested that amphetamine, later shown to be an indirect dopamine agonist, decreased food consumption (110). Later studies have shown that dopamine agonists such as apomorphine produce a dose-related decrease in food intake and that this anorexigenic effect is blocked by pimozide, a dopamine receptor antagonist (10). The site of action of these anorexic effects of dopamine has been proposed to be in the LHA or possibly the brain stem (80, 109). Feeding in itself has been associated with increased central turnover of dopamine in areas important for cognition and reinforcement such as the PFC (79, 81, 147). Electrical stimulation of the PFC induces food intake and disruption of dopamine input to the PFC interferes with food reinforced responses (20, 29, 156). Moreover, microdialysis studies have revealed that eating is strongly correlated to mesolimbic dopamine activity (11, 82, 113) .
In more recent years there has been an increasing interest in the overlap between peripheral signals of energy homeostasis mostly studied in relation to the regulation of feeding and energy balance and the reward systems such as leptin, insulin, cholecystokinin, ghrelin and GH.

Growth hormone, body composition and metabolism

The release of GH from the anterior pituitary is regulated by feedback signalling from circulating GH and IGF-I acting directly or indirect (through the hypothalamus) on the somatotrophic cells. The main regulators of GH secretion, growth hormone releasing hormone and somatostatin, are produced by hypothalamic neuroendocrine cells located foremost in the arcuate nucleus and the periventricular nucleus respectively and are released into the hypothalamo-hypophysial portal vessels that innervate the pituitary (67). Ghrelin also influences GH secretion from the pituitary but, as already discussed, the physiological importance of this effect is still unclear. NPY cells originating in the arcuate nucleus (that are established targets for ghrelin) have also been suggested to be a negative regulators of GH secretion from the pituitary (118, 144).
Growth hormone signal transduction occurs through binding and dimerization of the GH receptor (GHR), leading to intracellular activation of janus tyrosine kinas (JAK) and signal transducers and activators of transcription (STAT) pathways (193). In the circulation GH is bound by GH binding protein (GHBP) which is derived from the extracellular part of the GHR (51). The expression of GHR is found in the majority of peripheral tissues but also in central brain areas such as the hypothalamus and the thalamus (111, 126). Even though the somatotrophic cells are the main source of GH production small amounts of GH has also been reported to be produced with in the CNS (72, 84).
Growth hormone has important effects on postnatal growth while foetal and early postnatal growth is independent of GH. Growth hormone deficiency during stages of prepubertal development or loss of the GHR in humans leads to dwarfism while GH excess before puberty causes gigantism (77). Circulating GH regulates the production of IGF-1 from the liver as well as locally produced IGF-1 in target tissues. IGF-1 is thought to act in concert with GH on various tissues to stimulate growth (73). Although IGF-1 knockouts become growth retarded, liver specific IGF-1 knockouts have unaltered growth implying that locally produced IGF-1 is the major contributor to growth (157).

Apart from its growth-promoting effects, GH plays an important role in the regulation of body composition as well as protein, carbohydrate and lipid metabolism. It is well known that GH influences body composition by increasing lean mass and decreasing fat mass in a variety of species (17, 70, 119, 135). Human acromegalic patients, signified by overproduction of GH in adulthood, have reduced fat content where as GHD patients have increased fat content which is reduced by GH treatment (13, 141). Chronic, as opposed to acute effects of GH are diabetogenic, increasing circulating glucose by stimulating glucose out put from the liver and reducing peripheral uptake and utilisation of glucose. Postprandial glucose and insulin levels in circulation are increase by GH treatment and fasting glucose is decreased in GHD patients indicating a counter-regulatory effect of GH on hypoglycaemia (25, 33). In has been shown that long term GH infusion induce decreased insulin responsiveness/sensitivity by affecting intracellular signalling downstream of the insulin receptor (159). Adipocytes express GHR and GH has been shown to have a direct lipolytic effects on adipocytes through stimulation of triglyceride oxidation and inhibition lipid uptake from the circulation thereby decreasing lipid accumulation in adipocytes (71, 112, 152). However, the lipolytic effects of GH may also be mediated by decreased sensitivity/responsiveness to adipose tissue hormones.

Growth hormone is active in the CNS influencing feeding as well as the general sense of well being in humans (22, 160). Both central and peripheral administration of exogenous GH increase food intake in a variety of species (23, 26, 30, 89, 99, 168). Transgenic over-expression of bovine (b) GH in mice under the control of the glial acid fibrillary protein (GFAP) promoter, directing expression specifically to the CNS, results in hyperphagia induced obesity and increased hypothalamic AgRP and NPY mRNA expression (23) and general over-expression of bGH driven by the metallothionein promoter results in increased food consumption in mice on a HFD (136). Furthermore, expression of GH and its receptor have been found in central areas known to be important for feeding, indicating a role for central GH signalling in the regulation of feeding behaviour.

General objectives

The comprehensive aim of the present thesis was to investigate the role of ghrelin in food consumption and body weight regulation. Increased knowledge of the mechanisms behind ghrelin-induced feeding and adiposity is of general interest in a clinical setting for the treatment of obesity. Furthermore, our studies on the reward systems in relation to ghrelin could have implications in addictive biology.

Specific aims of the present thesis:

To investigate the effects of gastrectomy (a model of ghrelin depletion) and ghrelin replacement treatment on body weight, fat accumulation and bone density.

To study the effects of gastrectomy and long term ghrelin replacement/treatment on the regulation of hypothalamic peptides involved in regulation of food intake and body weight

To study the effects of ghrelin on acute locomotor activity and dopamine release in the nucleus accumbens, markers of the reward system.

To investigate the importance of GHR signalling for the acute effects of ghrelin-induced food consumption and to examine the effects of GHR deficiency on lipoprotein biology and body composition.

Methods

Animals

The experiments were approved by the Animal ethical committee (Centrala Försöksdjursnämden Göteborg, Göteborg, Sweden) (Papers III and IV), the Animal ethical committee (Centrala Försöksdjursnämden Malmö/Lund, Malmö, Sweden) (Papers I and II) and the Norwegian National Animal Research Authority (Forsøksdyrutvalget, FDU, Norway) (Paper II). The experiments in paper IV used mice on a Sv129Ola-Balb/c background with a deletion mutation in the GHR gene. The breeding and all experiments were preformed at Göteborg University, Sweden. Wild type (Wt) and knockout mice were derived from heterozygote breeding pairs. The mice experiments in Paper I and II were performed in Lund, Sweden, using female NMRI mice obtained from B&K (Sollentuna, Sweden), and for Paper III NMRI mice from B&K (Sollentuna, Sweden) were used at Göteborg Universitet.. The experiments done in rat in paper II were preformed in Trondheim, Norway and used animals obtained from Möllegaard/Taconic (Lille Skensved, Denmark) from the strain Sprague-Dawley.

The animals were housed in a temperature- and illumination-controlled environment in accordance with national and institutional guidelines: Makrolon Type-II, -III and IV cages, aspen wood chip bedding, 12:12-h light-dark cycle with a 1-h dawn/sunset function, relative humidity between 45 and 55%, unrestricted access to normal tap water and standard pellet chow (R-34; Lactamin, Vadstena, Sweden). For the experiments in paper IV using rats, chow was supplied by Special Diet Service (Witham, Essex, UK).

Considerations

Rodents are a good model for studying basic physiological functions of mammals and since humans have most genes and gene products in common with rodents there are also aspects of human disease that can be studied in depth using rodents. Practical advantages include small size making them easy to house and maintain, short generation time making them quick to breed into genetic homogeneity, the availability of inbreed strains, the abundance of data in the literature and that several in vivo equipment systems for rodents exist. In order to study the function and importance of gene products it has become more common to use genetically modified animals. Deletion mutations and transgenic over-expression models are most common in mouse and have turned out to be important for target identification and validation within industry but have also provided a tremendous tool for use in basic medical research.

When using genetically modified animals it is of importance to bear in mind the genetic drift that might occur during inbreeding. It is generally recommended to inbreed mice for 15-20 generations in order to reach a genetically uniform and standardised background but this also creates a possible genomic drift away from the original mouse strain. The GHR knockouts mice used in study IV are not backcrossed onto a new background strain but still remain inbreed for 20-25 generations on their original mixed Sv129Ola-Balb/c background. In order to avoid the kind of variability that might occur following heavy inbreeding (especially when the animals are backcrossed) it is always recommended, if possible, to use sibling controls. Other aspects important to consider when using genetically modified animals are the naturally occurring compensatory

effects following loss of a gene that might abolish the expected effects of deletion or even result in new additional phenotypes. Furthermore, deletion and replacement of parts of the genome may unintentionally delete or affect regulatory elements or important sites that yet have to be annotated and could change the expression of upstream or down stream genes.

Surgery

Gastrectomy surgery: In paper I and II both mice and rats were anaesthetised with an intraperitoneal injection of a mixture of fluanisone/fentanyl/midazolam before surgery. No antibiotics were used. Sham operation was performed by opening the abdomen with the midline incision, gentle manipulation of the stomach and immediate suture of the incision in 2 layers. Gx was carried out by resection of the stomach, followed by anastomosis of the oesophagus with the duodenum, end to end. The procedure of Gx includes bilateral subdiaphragmatic vagotomy. Mice and rats were allowed to recover from surgery for about a week before starting treatment with ghrelin, MK-0677 or vehicle.

ICV cannulation: In paper III and IV guide cannulas were implanted to the brain to facilitate injections into the dorsal third ventricle in mice. The animals were anesthetized with an initial 4% isoflurane (Baxter, Kista, Sweden) followed by a maintenance dose of 2% isoflurane and placed in a stereotaxic frame (Stoelting, Wood Dale, IL). A permanent 31-gauge stainless steel guide cannula (Eicom, Kyoto, Japan) was implanted into the brain (0.94 mm posterior to the bregma, 1.0 mm below the surface of the skull). The coordinates were chosen so that the protruding injection cannula reached the third dorsal ventricle upon injection. The guide cannulae were held in position by dental cement (Heraeus Kulzer, Hannau, Germany) and attached to two stainless steel screws driven into the skull. A stainless steel obtruder (Eicom) was inserted into the guide cannula to maintain cannula patency. The animals were allowed 4-7 days of postoperative recovery before injections.

Microdialysis: In paper III NMRI mice were implanted with both a microdialysis probe into the N.Acc. for measurement of extracellular DA levels, and an ICV guide cannula into the third ventricle for local drug administration. The microdialysis probe was alternated to both the left and right side of the brain. The coordinates used to isolate N.Acc. were 1.5 mm posterior to bregma, 0.8 mm bilateral to the midline and 4.7 mm ventral to the skull surface (Franklin & Paxinos 1996). The probe was slowly lowered into position and anchored to a screw in the skull bone with dental cement (DENTALON® plus). The animals were allowed to recover for 4 days before the microdialysis experiment. The exposed tip of the dialysis membrane (20 000 kDa cut-off with an o.d./i.d. of 310/220µm, HOSPAL, Gambro, Lund, Sweden) of the probe was 1 mm.

Treatment and doses

In papers I and II treatments were given by subcutaneous injection. Twelve Gx mice and twelve sham-operated mice received subcutaneous treatment with ghrelin (12 nmol) in 0.9% saline daily for eight weeks. Twelve Gx mice and 10 sham operated mice received daily injections of saline for 8 weeks. On the first 14 days, injections were given in the morning (08:00–09:00) and subsequent injections were made in the afternoon (17:00–18:00). The treatment dose of 12

nmol was chosen based on a preliminary study in which intact mice received either 12 nmol (n=6) or 24 nmol (n=6) of ghrelin by a subcutaneous bolus injection in the neck (corresponding to 400 and 800 nmol/kg, respectively). Blood samples (25µl) were drawn repeatedly by retro-orbital vein puncture at time 0, 15, 60, 120, 240, and 300 minutes post injection of ghrelin. Since both the 12 and 24 nmol dose of ghrelin generated blood levels in excess of physiological concentrations for more than 16 hours after injection, the lower dose of 12 nmol was selected. We aimed to give a replacement dose ghrelin to Gx mice using doses that generated near physiological plasma concentrations; there was no intent to induce sustained supraphysiological plasma concentrations of ghrelin. Fresh solutions of ghrelin were prepared before injections in all of the studies.

In paper II Gx and sham rats were treated orally with MK-0677, a GHSR-1A antagonist. The treatment was given daily by gavage at a dose of 4mg/kg per day. The oral treatment regime was chosen since MK-0677 has been demonstrated to have good oral bioavailability in mammals (158) and the dose chosen was 4-fold higher than what induced a GH response in dogs (142), as rats generally have a high metabolic rate. The rats were treated for 2 weeks since the fat-accumulating effects of ghrelin were apparent at this time point in mice (174).

ICV injections to GHR knockouts and Wt mice in paper IV were made with either ghrelin (0.4µg, n-acetylated-ghrelin; Bachem, Weil am Rhein, Germany) or an equal volume (1µl) of vehicle solution (Ringer). The duration of the injection was ~45 seconds. All injections were done 2 h after dawn (09:00h). Part of the study in paper IV used a crossover design where the mice were given the opposite treatment in a second ICV injection following a 4 day washout period. The dose used for the ICV injections were selected based on several reports showing robust effects on food consumption (127).

In paper III NMRI mice were injected with a dummy cannula that was carefully inserted and retracted into the guide cannula to remove clotted blood and to hamper spreading depression. The probe was then connected to a microperfusion pump (U-864 Syringe Pump, AgnThós AB) and perfused with Ringer solution at a rate of 1.5 µL/minute. After 1 hour of habituation to the microdialysis perfusion setup, perfusion samples were collected every 20 minutes. Mecamylamine, an unselective nicotinic acetylcholine receptor antagonist, was IP injected 10 min prior to ICV ghrelin injection (1µg, n-octanoylated-ghrelin; Bachem, Weil am Rheine, Germany) in a does previously shown to inhibit drug induced dopamine release in the N.Acc. After experiments the animals were give amphetamine to validate the location of ICV catheter and to verify the microdialysis and dopamine measuring setup. Animals that showed no amphetamine induce dopamine release were excluded. Animals were killed and brains cut to verify the correct position of the microdialysis probe.

Considerations

The choice of administration route of drugs or hormones used, intraperitoneal or subcutaneous injection, by ICV injections or orally by gavage was dependent on the aim of the study.

The ICV route is a very good way of giving bioactive drugs/hormones directly into the CNS and thereby bypassing biological barriers such as the blood brain barrier or enzymes metabolising the drug/hormone that may occur in the blood

or the gastrointestinal tract. Within the ventricles the injected substance disperses and can easily affect sites neighbouring the ventricle such as the PVN and arcuate nucleus of the hypothalamus. The cerebrospinal fluid flows from lateral ventricles to the 4th ventricle of brain stem and into the spine. When injecting substances ICV the proposed site of action should preferably be down stream of the injection point. It should be borne in mind that when the ICV route is chosen several sites will most probably be affected by the injected substance.
Intraperitoneal injection is an easy way of injecting a fairly large amount of fluid; such injections are easy to perform but should not be repeated on too many occasions. The substances injected generally have a fast uptake into the blood via absorption to the mesenteric vasculature in the abdomen.
The subcutaneous administration route is good for long term treatments since the injection place can be varied quite a lot but the volumes one can inject are very small.
Per oral injections via gavage resembles the natural rout for absorption of nutrients and drugs via the gastrointestinal tract. Variations in intestinal absorption as well as metabolic rate must be of consideration when administering substances orally.

Body weight and body composition
Body weight was monitored weekly in the long term studies using a conventional balance. In paper I and IV dual-energy X-ray absorptiometry (DEXA; PIXImus, Lunar Corporation, Madison, Michigan, USA) was used to determine total soft tissue lean body mass, body fat content and bone mineral density. In paper I and IV adipose depots (mesenteric, retroperitoneal, parametrial, inguinal and intra scapular brown adipose tissue), liver and brain were dissected and weighed. For paper I femurs were also collected and their lengths measured after which they were incinerated for 24 hours at 600˚C to determine ash weight.

Food intake
Food consumption was monitored every day in the long term studies by weighing and subtracting from the value from the day before (paper I). In papers IV acute food consumption following ICV injections of ghrelin was measured. In order to optimize the sensitivity of this measure cages (23 x 16 cm) were prepared with normal chow and incubated at 80 ºC for 1 h to correct for any differences in humidity. After 2 h at room temperature the cages were accurately weighted. Immediately after injection the animals were put in the preweighed cages with free access to food and water. The mice were left in the cage for 3 h and were then returned to their original home cages. All excrements were removed and the cages were reincubated at 80 ºC in order to dry out water spill and urine, and reweighed after 2 h. Animals were let to recover for at least 4 days between ICV injections in experiments where food intake was measured more than once.

Serum analysis

Papers IV used a commercial radioimmunoassay (RIA) kit (Linco Research, St Charles, Missouri, USA) with intra-assay variation of 3.3% to measure total ghrelin. In paper I total immunoreactive ghrelin was measured using a RIA kit (Phoenix Pharmaceuticals, Belmont, California, USA) with an antiserum raised against acylated human ghrelin; 125I labelled ghrelin-28 was used as tracer and rat ghrelin-28 as standard. The detection limit for total ghrelin was 12 fmol/l. Intra- and inter-assay variation was 3% and 8%, respectively. Furthermore, in paper III acetylated ghrelin was determined using an enzyme linked immunosorbent assay kit (Linco Research, St Charles, Missouri, USA). The antiserum does not recognise des-octanoylated ghrelin. The detection limit for active ghrelin is 1 pmol/l. Intra- and inter assay variation was 3% and 4%, respectively.

Insulin like growth factor I (IGF-I) was measured in papers I and IV by RIA kits from Diagnostic Systems (Webster, TX, USA, RIA DSL-2900) and Mediagnost (Reutlingen, Germany) respectively.

In papers I and IV insulin and leptin were measured using mouse-specific RIA kits (Linco Research, St. Charles, Missouri, USA; RI-13 K and ML-82 K, respectively). The intra-assay variation of insulin determinations was 1.4% (mean conc. 120 pM); the intra-assay variation of leptin measurements was 4.0% (mean conc. 2.2 ng/ml). Furthermore, corticosterone in serum was measured using an RIA kit (Amersham Life Science, Amersham International; RPA 548) and the intra-assay variation of these measurements were 5.2% (mean conc. 200 ng/ml). In paper I glucose levels were also measured in non-fasted mice using a photometric assay kit HK 125 (ABX Diagnostics-Parch Euromedecine, Montpellier, France). The intra-assay coefficient of variation (CV) of glucose measurements was 1.3% (mean conc. 5.2 mM, limit of detection 0.2 mM). The assays were performed using a Cobas Mira analyzer (Hoffman-La Roche, Basle, Switzerland).

Since the lipid profile of the GHR knockout model used in paper IV had not been studied in detail serum triglycerides and total cholesterol concentrations were measured using commercial reagent kits (Roche Diagnostics, Mannheim, Germany). The intra-assay CV of triglyceride measurements was 1.5% (mean conc. 1.21 mM, limit of detection 0.05 mM). The intra-assay CV of cholesterol measurements was 0.8% (mean conc. 6.0 mM, limit of detection 0.05 mM). Furthermore, in order to asses the cholesterol distribution in more detail The lipoproteins in a 10µl sample were separated over 60 min using a size exclusion high-performance liquid chromatography system, SMART, with column Superose 6 PC 3.2/30 (AmershamPharmacia Biotech, Uppsala, Sweden), and the area under the curve represents the cholesterol content. The peaks in the profiles were designated very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) for simplicity, even though it is clear that the separation is determined primarily by the size of the lipoproteins. Serum apolipoprotein (apo) B was measured by an electroimmunoassay.

Analysis of mRNA

Real- time quantitative PCR analysis: In paper IV PCR was the method of choice to determine the expression levels of hypothalamic and pituitary mRNA

for the genes of interest. Total RNA preparation from dissected hypothalami and pituitary was performed using TRIzol Reagent kit (Invitrogen, Life Technologies, Carlsbad, CA). The RNA pellet was dissolved in RNAse-free water, and the concentration was measured using a spectrophotometer. Aliquots from all samples were loaded on a nuclease-free TAE agarose gel (1%) to confirm RNA quality. To eliminate DNA contamination in the samples, all cDNA synthesis was initiated with DNase treatment using a DNA-free kit (Ambion, Austin, TX). Both reverse transcriptase (RT) and negative RT controls were used. cDNA was synthesised using Superscript II RNAse H^- Reverse Transcriptase and random hexamer primers (Life Technologies, Frederck, MD). Quantification of mRNA levels was performed using Taqman real-time PCR on an ABI 7900 HT (Applied Biosystems). Both FAM/TAMRA-labelled fluorescence probe with TaqMan Universal PCR Master Mix (Roche Molecular Systems, Branchburg, NJ) and SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) was used. All samples were run in triplicates, and each triplicate was normalized using mouse acidic ribosomal phosphoprotein PO (M36B4) as an endogenous control. Primers were optimized and linear amplification was confirmed. Analysis of the data was done in SDS 2.1 (ABI Prism, Applied Biosystems).

In situ hybridisation: In brief, sections of brain from mouse and rat were cut at 10 μm thickness using a freezing cryostat (Bright Instruments, Leeds, UK) and transferred onto RNAse free poly-lycine coated glass slides and were air dried, paraformaldehyde fixed, rinsed in PBS and dehydrated in ethanol. Oligonucleotide probes complementary to the mRNA of the peptide under study were labelled with ^{35}S using a terminal deoxy transferase (Promega, Brighton, UK). The slides were incubated with the labelled probe overnight in a hybridisation buffer, washed and air dried. Sections were placed in X-ray cassettes and exposed to X-ray film (Kodak, Bio Max MR) for 5 days up to two months, depending on the peptide under investigation. The films were developed and the optical densities measured in six consecutive sections per animal, and presented as the average value.

Considerations

The two different ways of measuring mRNA used in this thesis work are very common but do have slightly divergent advantages. The real time PCR technique is very sensitive and can detect very small differences in the mRNA expression levels. Furthermore, several genes can be analysed quite rapidly. The *in situ* technique has one big advantage compared to the real time PCR in that one can pinpoint the exact location and at the same time get a fairly good quantification of the mRNA expression of discrete cell groups. This method is time consuming and only a few genes can be analysed due to limited parallel sections.

Summary of Results

paper I

This study was undertaken in order to investigate the effects of ghrelin depletion in adulthood following gastrectomy surgery (Gx) in mice on energy homeostasis and body composition. The effects of long term hyperghrelinemia in both sham and gastrectomised mice were also studied by giving daily subcutaneous injections of ghrelin for 8 weeks. Using this classical endocrine model we were able to study whether the effects of Gx (ie endocrine organ removal) could be reversed by ghrelin replacement and we could also determine the effects of long term hypo/hyperghrelinemia in mice.

To prevent supraphysiological accumulation of ghrelin over the 8 week treatment period we tested the elimination of two doses of ghrelin given by single subcutaneous bolus injection in the neck. The ghrelin levels 6h following injection using the lower dose of 12 nmol, were far lower than the peak value at 15-60 min but still elevated compared to ghrelin concentrations before injection. By the end of the 8 week treatment period ghrelin levels were somewhat elevated, but still within the physiological range, in ghrelin treated animals 16 h after the last injection.

Cumulative food intake did not differ between Gx and sham operated mice, and there was no difference in food intake between mice receiving a daily dose of ghrelin and those receiving saline. Gastrectomy surgery decreased the mean body weight by 15% compared to sham animals and daily injections of ghrelin to Gx mice partially reversed this body weight loss. The relative weight increase following 8 weeks ghrelin treatment to Gx mice was 8% whereas ghrelin treatment did not significantly alter the body weight in sham animals.

Dissection weights of four different fat pads showed that Gx reduced fat depots by approximately 30% compared with sham operation. Daily administration of ghrelin normalised the total amount of fat in Gx mice and increased it by 20 % in sham mice. The effect of ghrelin on individual fat pad weights was without significance.

Total BMD and bone ash weight of the femur was ~20% lower in Gx mice than in sham operated mice. Daily administration of ghrelin failed to affect BMD and femur ash weight in either Gx or sham operated mice and circulating IGF-1 levels were also unaffected by ghrelin treatment.

In conclusion, ghrelin replacement partially reverse the Gx-induced reduction in body weight, lean mass and fully reverse the body fat loss but does not effect bone mass. These results are in line with a physiological role of ghrelin in the regulation of body composition (notably fat), raising the possibility that ghrelin replacement may be used as a therapy to alleviate the weight loss associated with Gx in humans.

paper II

To further investigate the findings in article I – that ghrelin replacement to Gx animals restores adipose depose and body weight - we investigated the changes in hypothalamic gene expression following Gx and/or treatment with ghrelin for 8 weeks or the ghrelin mimetic MK-0677 for two weeks. The genes of interest in this study (NYP, AgRP, POMC and MCH) code for well known

hypothalamic neuropeptides involved in the regulation of food intake and energy expenditure. NPY and AgRP are also known to be regulated directly and POMC indirectly by acute treatment with ghrelin.
Since the stomach is the main producer of ghrelin removal of the stomach by Gx decrease circulating levels of ghrelin by ~80-85% in both rats and mice. Replacement treatment with ghrelin for 8 weeks increased the levels of both total and acetylated ghrelin about 1.5-2 fold at 16 h following the last injection of ghrelin compared to sham mice receiving vehicle treatment indicating that the ghrelin treated animals (both sham and Gx) would most likely be exposed to quite high levels of ghrelin for a majority of the treatment period. MK-0677 treatment for two weeks decreased endogenous ghrelin levels in sham operated control rats compared to vehicle treated sham rats.
In line with ghrelin's effects on adiposity and feeding, NPY and AgRP mRNA expression in the arcuate nucleus was increased by 8 weeks of ghrelin treatment in mice. Conversely POMC mRNA expression in the arcuate nucleus was reduced by ghrelin treatment. By contrast, MCH mRNA expression in the lateral hypothalamus was not affected by ghrelin. Surprisingly, no major alterations were found following Gx in mice in the genes studied. Only NPY was slightly increased by surgery. This may indicate that the Gx animals after 10 weeks have reached a new set point in the energy homeostasis.
In rats, NPY and AgRP mRNA in the arcuate nucleus was increased after 6 weeks of Gx and MK-0677 treatment for two weeks. Gx and MK-0677 had synergistic effects on both genes. POMC and MCH mRNA expression in the arcuate nucleus was, in contrast to ghrelin treatment in mice, increased by MK-0677 treatment for two weeks. Gx *per se* had no effect, but abolished the POMC response, and decreased expression of MCH in response to MK-0677 treatment.
In summary long term treatment with ghrelin or MK-0677 stimulates adipogenic pathways in the hypothalamus (including activation of the arcuate NPY/AgRP neurones) consistent with ghrelin's effects to increase body weight and fat accumulation in rodents. Furthermore, ghrelin also inhibited POMC gene expression providing another possible reason for the stimulation of fat accumulation by ghrelin. Unexpectedly, MK-0677 increased POMC expression in sham operated animals which might argue for divergent effects beyond dose and route of treatment

paper III
The homeostatic signals involved in long term body weight maintenance have been widely studied in relation to hypothalamic and brainstem regulation of energy expenditure and food consumption. It is, however, becoming increasingly apparent that there is a degree of neurochemical overlap between homeostatic signals such as leptin and ghrelin and the reward systems of the brain.
Injection of ghrelin into the third dorsal ventricle increased acute locomotor stimulation, an indirect measure of dopamine release. Furthermore, using microdialysis we found that extracellular dopamine was increased the N.Acc following central ghrelin injection indicating that ghrelin can activate the mesoaccumbal dopamine system originating in the VTA. Using the unspecific nicotine acetylcholine receptor antagonist, mecamylamine, we were also able to block both ghrelin-induced locomotor stimulation and extracellular dopamine

release in the N.Acc demonstrating that cholinergic projections into the VTA might be involved in modulating the effects of ghrelin on the mesolimbic reward systems.
The findings in this paper suggest that there is a connection between ghrelin and the dopaminergic reward pathways in the brain. The physiological relevance of this finding need further study but it may be that ghrelin could be involved in food related compulsive behaviours.

paper IV
In the initial studies on ghrelin, the endogenous GHS, it was central to clarify the involvement of GH in the effects on among other things food consumption since the GH secreting effects of GHS was known long before ghrelin was discovered. Using GH deficient rat models, several groups have shown that GH was not of importance for the acute effects of ghrelin on food intake. In this study we investigated the involvement of the receptor for GH in the acute effects of ghrelin on feeding using mice with a deleted GHR/GHBP gene. We also investigated the body composition and serum lipid distribution of these GHR knockout mice since GHD in humans is known to effect both body composition and lipid metabolism.
Central injection of ghrelin was found to stimulate feeding in both fasted and fed wt mice, but this effect was blunted in GHR knockout mice indicating that GHR is required for ghrelin's acute effects on food intake. Furthermore, it was also found that GHR knockout mice had elevated food consumption per body weight in comparison to wt animals. Injection of ghrelin increased the mRNA expression of the orexigenic neuropeptide AgRP in wt mice compared to GHR knockout mice treated with ghrelin. There was a similar tendency in the mRNA expression of hypothalamic NPY but this did not reach significance. The expression levels of several other neuropeptides involved in feeding such as POMC, CART, MCH and orexin were measured but no difference between treatment or GHR knockout and wt mice were found. The hypothalamic mRNA expression of GHSR-1A was also measured to see if ghrelin sensitivity might have been altered but no difference in expression was found between GHR knockout and Wt mice.
Male GHR knockout mice have retarded growth and become smaller than their Wt controls but their body fat percentage is increased 2.4 fold. In line with the increased percentage body fat, serum leptin levels were increased 4.8 fold. Conversely, relative lean mass is decreased by ~40% in male GHR knockout mice. Furthermore, bone content and area relative to crown rump length was also decreased in GHR knockouts. Weights of brain, retroperitoneal adipose tissue, and intrascapular brown adipose tissue were disproportionately larger, whereas liver weights were disproportionately smaller in the GHR knockout mice compared with Wt controls.
Serum levels of corticosterone are increased in GHR knockout mice compared to wt control mice. Despite obesity and increased corticosterone levels, known to increase glucose output from the liver as well as reducing insulin sensitivity, GHR knockouts were found to have low glucose and insulin indicating that the GHR knockouts are insulin sensitive. Growth hormone, as glucocorticoids, reduce peripheral insulin sensitivity and glucose utilisation in peripheral tissues indicating that the lack of GH signalling in the GHR knockout mice may

directly effect these functions. There are several other factors that could affect blood glucose such as glucagon and catecholamine but these were not measured.
Despite their obesity, GHR knockout mice have a marked decrease in triglycerides, total cholesterol and apoB containing lipoproteins. A cholesterol distribution profile revealed that both LDL and HDL fractions were decreased in GHR knockouts.
In summery, GHR deficiency in mice is associated with changes in food intake, body composition, leptin and corticosterone levels and lipoprotein metabolism. Moreover, our data suggests that ghrelin's acute central action to increase food intake requires functionally intact GHR signalling.

General discussion

Paper I and II

Studies in gene deletion animal models aiming to elucidate the physiological role of ghrelin indicate that endogenous ghrelin may not be as important for food intake and body weight maintenance as first expected. Even though pharmacological studies show effects of ghrelin on GH release, appetite and fat deposition, ghrelin knockout mice do not have an altered body weight phenotype when fed normal chow and GHSR-1A deletion gives a very modest lean phenotype. (163, 164, 186, 194). In an evolutionary ("thrifty gene") perspective this might not be so surprising since the ability to put on body weight (and maintain that weight) is vital for survival and loss of one orexigenic signal should therefore be backed up by several others. On the other hand ghrelin is the only orexigenic signal found in the periphery to date whereas several anorexigenic signals have been identified. Hence, the default situation might be to have a continuous orexigenic drive which is inhibited by feedback homeostatic adiposity signals rather than a "neutral" system fuelled by orexigenic/adipogenic "feed forward" signals. Future studies using selective ghrelin blockers will give us the definite answer as to what extent endogenous ghrelin influence energy homeostasis and whether this blockade will be of practical use in a clinical setting.

Using a classical endocrine deletion/replacement approach we found support for the notion that ghrelin is required for the maintenance of normal body weight and adiposity. By complete Gx we were able to deplete animals of ~80% of both total and acetylated circulating ghrelin causing a reduction in body weight, fat mass and lean mass in adult mice. Ghrelin replacement treatment at a dose that restored circulating ghrelin levels but without effecting body weight in sham animals, fully or partially reversed the decrease in body weight, fat mass and lean mass following Gx. This finding suggests that loss of ghrelin following Gx, and maybe also following other bariatric surgery procedures that decrease circulating ghrelin levels, contributes to the weight loss associated with these procedures raising the possibility that ghrelin replacement may be used as a therapy to alleviate involuntary weight loss following gastrectomy in humans (gastric cancer patients that have to undergo surgery). In a study presented by Kaplan et al Gx rats were treated with a ghrelin antagonist to investigate the importance of the remaining ghrelin in the circulation (92). Interestingly it was found that treatment with ghrelin antagonist could increase body weight loss drastically in Gx animals (92). This, in combination with our own study indicates, contrary to what has been found in ghrelin knockout studies, that endogenous ghrelin is of great importance in normal physiology. In light of the fact that many obese people fail to lose a clinically satisfactory amount of weight following bariatric surgery, which currently is the only effective obesity treatment, combining or extending surgical procedures for weight loss with ghrelin antagonists could improve the end result of surgery/treatment tremendously.

Although we saw an increase in hypothalamic NPY and AgRP mRNA expression and a decrease in POMC mRNA expression following long term ghrelin treatment, which is in line with the appetite promoting effects of ghrelin as well as previous studies indicating that NPY and AgRP mediate ghrelin's

effects on feeding, we were unable to observe the orexigenic effect of ghrelin (ie no detectable change in cumulative food consumption). Several studies have shown that ghrelin is a strong orexigen when administered both centrally and peripherally (8, 90, 91, 127, 188). Tang-Christensen et al showed that a single ICV injection of ghrelin made rats over eat, increasing cumulative food intake for more than three days compared to vehicle treated animals (167). On the other hand Wren et al reported that the orexigenic effects of peripherally administered ghrelin is very short lived which possibly could explain why we were not able to detect any changes in day to day food intake or in cumulative intake during the whole treatment period (188). Furthermore, Tschöp et al failed to induce an increase in cumulative food intake in mice following peripheral treatment for 12 days with ghrelin using a 6 times higher dose than we used (174). It is, however, also possible that our detection system was not sensitive enough to observe modest changes in food intake.

Explaining the adipogenic effects of ghrelin in our model is complicated by the fact that the acute effects of ghrelin on food intake are not always seen in long term studies. However, there might be explanations for the fat accumulating effect other than increased food consumption. It has been shown that a single central injection of ghrelin decrease spontaneous locomotor activity for up to three days. Likewise, a single central injection of $AgRP_{(83-132)}$ decreases spontaneous locomotor activity long term (167). Even though we did not measure activity in our study the increased hypothalamic AgRP mRNA expression may indicate that ghrelin treated Gx and sham animals move less in order to saving energy, hence increase their fat mass.

Furthermore, the decrease in hypothalamic POMC mRNA expression following 8 weeks of ghrelin treatment may also account for the adipogenic effects found as blockade of melanocortin receptors or deletion of POMC leads to decreased energy expenditure and increased body weight (32, 167).

Another explanation to our findings, as has been suggested by others, might be that ghrelin affect nutrient partitioning, modulating the metabolic substrate preferentially used for maintaining energy balance. Consistent with this, ghrelin treatment decreases fat utilisation and ghrelin knockouts put on a HFD have decreased RQ (174, 186). Furthermore, the influence on nutrient partitioning might be of importance in a physiological setting as ghrelin secretion is reduced in situations of positive energy balance, such as obesity, possibly reflecting an adaptive counter regulatory process pushing the metabolic fuel preference towards fat utilisation (62, 175). A shift to increased glucose utilisation, rather than fat, in the ghrelin treated Gx and sham animals might possibly explain the increase in fat mass although without a concomitant decrease in energy expenditure, it would be hard to accept as the underlying mechanism.

We cannot exclude the possibility that ghrelin's adipogenic actions reflect, at least in part, a peripheral action, even at the level of the adipocyte where GHSR-1A receptors have been demonstrated (41).

The finding that ghrelin treatment enhanced body weight and body fat mass in Gx mice (that were also vagotomised) is interesting in view of the fact that subdiaphragmatic vagotomy *per se* has been reported to prevent the meal initiating effect of ghrelin. Conceivably, vagotomy impairs ghrelin-stimulated meal initiation but not the long term effects of ghrelin on body fat (49). In paper I we did not investigate the effects of ghrelin in animals that were vagotomised only; this would have perhaps made an unnecessary control as we

saw ghrelin-induced adipogenic effects in Gx rats (that also have subdiaphragmatic vagotomy).
Further studies in Gx and sham animals with low dose ghrelin replacement treatment will reveal whether activity, basal metabolic rate or RQ or maybe a combination of these is causative of the adiposity.
In paper I, we hoped to discover that ghrelin is the long sought after hormone whose deficiency might explain why Gx leads to bone loss (osteopenia). We found, in line with several previous publications, that Gx decreased bone mineral density and bone ash weight of the femur compared with sham operated mice indicating that the acid producing part of the stomach is required for maintaining normal bone density. However, ghrelin failed to prevent Gx-induced bone loss and did not affect bone in sham operated mice. Furthermore, ghrelin treatment or loss of ghrelin following Gx surgery did not affect IGF-I levels in mice.

In paper II we did not directly compare the effects of ghrelin versus MK-0677 on hypothalamic gene expression however, in separate studies, we found that their effects were largely similar but with a few notable differences. The stronger induction of mRNAs for NPY and AgRP by MK-0677 could simply be an effect of dose or efficacy provided that MK-0677 and ghrelin stimulate the same receptor. The opposite effect of ghrelin and MK-0677 treatment on the expression of POMC however was quite surprising. An explanation to this might be differences in the levels of des-acylated ghrelin or differences in the ratio between acetylated and des-acylated ghrelin (or MK-0677) signalling in the two studies. Treatment with acetylated ghrelin gave an increase in des-acylated ghrelin in both sham and Gx mice whereas MK-0677 treatment in rats decreased endogenous production of total ghrelin in sham animals and did not affect the low levels of total ghrelin in Gx rats. Apart for the difference in des-acylated ghrelin the ratio between des-acylated ghrelin and acetylated ghrelin (or MK-0677) signalling could possibly also explain the differences in mRNA expression. Acetylated ghrelin was normal in ghrelin treated sham mice and decreased in Gx mice regardless of treatment indicating a low acetyl/des-acetyl ghrelin ratio since des-acylated ghrelin was increased in both sham and Gx mice. Even though des-acylated ghrelin was not measured in the MK-0677 study the total ghrelin levels were decreased in both sham and Gx rats following MK-0677 treatment implying that the ratio between GHSR-1A signalling, stimulated by MK-0677, and des-acylated ghrelin signalling would be increased. In any case, our studies suggest that the term “ghrelin mimetic” should be applied with some caution when referring to GHS. While MK-0677 has effects that are rather similar to ghrelin, there are clear differences when we consider the effects on the expression of key hypothalamic genes involved in body weight regulation.

Paper III
To eat is rewarding and it is also pleasurable. In recent years, this fact has been overlooked by the obesity research community that have embraced the “set point hypothesis” that places the hypothalamus as a homeostatic regulator of body weight, receiving feedback regulation from the periphery via endocrine and nutrient messengers. In this article, we provide the first demonstration that

ghrelin activates an important brain circuits involved in reward-seeking behaviour, a circuit that is a known target for alcohol and other addictive substances. We found that ICV ghrelin increases locomotor activity and extracellular dopamine release in the N.Acc in mice suggesting that ghrelin can activate the mesoaccumbal dopamine pathway which is implicated in brain reward and motivational behaviour. Given the dynamic changes in circulating ghrelin in relation to meal times (45) together with its ability to induce hunger feelings in humans (187) and to increase food intake in rodents (127) it is conceivable that ghrelin may have incentive value for goal directed behaviour such as meal initiation and food searching. Indeed, administration of ghrelin has been shown to increase primarily appetitive, rather than consummatory, feeding behaviour in animals and in addition to increased meal initiation events, these behaviours include increases in hoarding and foraging for foods (95, 96) and, in accordance with the incentive salience hypothesis of reward related dopamine put forth by Berridge and Robinson, the work animals will perform to acquire food (15).

Electrophysiological and voltammetric measurements have shown that dopamine systems become active before a meal, prior to the taste of food, to the same or even greater extent than during food consumption (148, 151). Some of these studies based on the use of conditioned stimuli paired with food, show complete separation in the response to the conditioned stimuli and presentation of foods making them difficult to interpret in a physiological setting (21, 150). The studies of Kiyatkin et al and Kosobud et al, however, have measured dopamine activation in the N. Acc and the VTA respectively in rats trained to perform a bar press response and found that dopamine-related signalling increased in anticipation of a food reward (100, 105). In this context it is conceivable that under normal physiological circumstances the anticipatory dopamine activation is influenced by the preprandial increase in ghrelin and that this increase in ghrelin influences goal directed behaviour.

The locomotor stimulatory and dopamine enhancing effects of ghrelin could be of both direct and indirect origin. The receptor for ghrelin has been found in the VTA on tyrosine hydroxylase positive cells (marker of monoaminergic neurons) indicating that there might be a direct stimulatory component on to dopaminergic cells in the VTA (2, 75). However the receptors have also been identified presynaptically in the VTA (2). Electrophysiological studies have revealed that the effect of ghrelin to increase firing frequency in dopaminergic VTA neurons is absent in the presence of glutamatergic antagonists but not GABA receptor antagonists, indicating that presynaptic glutamate is needed for the stimulatory effects of ghrelin on the mesolimbic dopamine system (2). Others, however, have failed to induce a stimulatory response with ghrelin in the VTA using whole cell patch clamp techniques (103). In situ hybridization analysis of the distribution of the GHSR-1A in the brain has identified the receptor in other neurochemical systems associated with reward such as the LDTg and the PPTg nucleus known to project cholinergic input to the VTA (75). There is accumulating evidence suggesting that cholinergic excitatory input to the dopaminergic cells in the VTA may be important for this circuitry mediating natural as well as drug-rewarding behaviour (107, 108, 146). Our finding that the dopamine enhancing and locomotor stimulatory effect of ghrelin is abolished by the unselective nicotinic antagonist mecamylamine indicates that central nicotine acetylcholine receptors might be involved in mediating these

effects of ghrelin. Interestingly, studies by Dulloo et al have shown that long term mecamylamine treatment of genetically obese rodents reduces their obesity by decreasing their food intake rather than their energy expenditure (57). In future studies it will be interesting to investigate the potential use of more specific nicotinic antagonists in the treatment of not only smoking but also in general obesity and in eating disorders where ghrelin levels are elevated such as Prader-Willi patients, that are compulsive overeaters, and in patients with anorexia nervosa. Furthermore, our findings indicate that use of ghrelin antagonists may be of clinical interest more generally in the field of addiction.

Paper IV

Since the main bulk of knowledge preceding the discovery of ghrelin, based on the GH releasing peptides and GHS, was focused on GH secretion it was important to the discoverers of ghrelin and in the initial papers that followed to distinguish between primary effects of ghrelin and secondary GH-dependent effects. Using the spontaneous dwarf (SD) rat, a GHD rat model that carries a disrupted GH gene, Nakasato et al showed that acute ghrelin injection induced food intake, following ICV administration, was independent of GH (127). It is noteworthy, however, that the vehicle-injected SD rats in this study did not consume any food during the experiment and no control group on a Wt background, which could have revealed any impairment or improvement of ghrelin's effect on food intake, was included. In contrast to these findings using SD rats, we could not to induce an increase in food intake in either fasted or fed GHR knockout mice following ICV injection of ghrelin. It is not clear why deletion of the receptor for GH blunt the effect of ICV ghrelin but three possible explanations might be considered; 1) that GH signalling within the CNS, notably the hypothalamus, might be down stream of ghrelin, mediating food intake, 2) that GH is important for the foundation and organisation of neuronal populations targeted by ghrelin, 3) the increased food intake seen in the GHR knockouts at baseline might mask the effects of ghrelin treatment.

It has been shown that exogenous administration of GH both centrally and peripherally increases food intake in mammalian, avian, fish, and reptiles whereas inhibition of GH secretion inhibits food intake (23, 26, 30, 89, 99, 168). Furthermore, Bohlooly-Y et al showed that transgenic over-expression of bGH in mice under control of the GFAP promoter, directing expression specifically to the CNS, results in hyperphagia induced obesity and increased hypothalamic AgRP and NPY mRNA expression (23). These effects in the GFAP-bGH mice are thought to be causative of GH signalling in the brain and not due to peripheral GH deficiency. The circulating levels of peripheral bGH were slightly elevated whereas IGF-I levels were unchanged in the GFAP-bGH mice. Immunoreactivity for GH has been found in the VMN, one of the key hypothalamic area regulating food consumption, indicating direct GH involvement in the regulation of feeding (84, 85). Other neuronal sites of GH action indicated by the expression of GHR mRNA and/or GHR/GHRBP immunoreactivity are the hippocampus, cerebral cortex, amygdala and, with in the hypothalamus, the arcuate nucleus, periventricular nucleus, ventro lateral region, VMH, PVN, supraoptic nucleus, DMN and the medial tubal nucleus (31, 117). This expression of GHR has been shown not to coincide with somatostatin or GHRH in several of the extra hypothalamic areas as well as in

the arcuate nucleus and the DMH suggesting actions of GH within the brain unrelated to GH feedback regulation (31).
A possible integrative site of GH and ghrelin action on food intake might be the NPY/AgRP cell-population in the arcuate nucleus. A majority of these cells express both GHR and GHSR-1A and have been shown to be activated by both GH and ghrelin. Furthermore, the acute effect of central administration of ghrelin on food intake is abolished by co-administration of AgRP and NPY IgG and peripheral ghrelin administration fails to stimulate food intake in AgRP and NPY double knockout mice indicating that these neuropeptides are important for mediating the orexigenic effects of ghrelin. Indeed, ghrelin injections have been shown to increase NPY and AgRP in the hypothalamus in several studies but in our hands ICV injections of ghrelin to GHR knockout mice does not increase hypothalamic expression of AgRP or NPY.
Interestingly, GHSR-1A expression in the arcuate nucleus has also been suggested to be regulated by GH. GHSR-1A expression in the arcuate nucleus is known to be regulated positively by ghrelin and in response to fasting but ICV injections of ghrelin fail to up-regulate mRNA expression of GHSR-1A in the arcuate nucleus in GH deficient Lewis rats (DR) (134). Although the latter study did not see any changes in the expression of GHSR-1A between vehicle treated DR and Wt rats others have shown that SD rats have increased GHSR-1A expression in both arcuate and the VMN (14, 93). These finding suggest that GH deficiency in some strains of rats increase GHSR-1A expression and that ghrelin's feedback regulation of its own receptor is dependent on GH.
Long term treatment with GH has also been shown to effect GHSR-1A expression in the brain and has been thought to be a direct negative feedback effect (14). Circulating ghrelin and mRNA expression of ghrelin in the stomach is, however, not effected by chronic changes in GH levels as analysed in two different GH transgenic mice and in GHR knockout mice (129). In our study we could not find any genotype specific changes at baseline or following ghrelin treatment in neither hypothalamic mRNA expression of GHSR-1A nor in circulating ghrelin levels.
Long term peripheral treatment with ghrelin has been shown to affect adiposity without increasing food consumption (see paper I). In SD rats peripheral administration of ghrelin for 7 days did not increase cumulative food intake significantly in but did induce a strong cumulative weight gain compared to vehicle treatment in SD rats (174). Using ipamorelin, a GHS, Lall *et al* showed that GHS also could induce body weight gain and that this effect was GH independent (106). Eight weeks of treatment with ipamorelin increased body weight and adiposity in both heterozygous and homozygous *lit* mice, another GH-deficient rodent model. These findings indicating that the effects of ghrelin and GHS on body weight gain following long term treatment are GH independent. We have not studied the effect of long term ghrelin or GHS treatment on body weight and adiposity in the GHR knockouts but this is something that would be interesting to do. To verify whether GH acts as a signal down stream of ghrelin it would be interesting to pre-treat animals with GH antagonists prior to ghrelin injections. Furthermore, to test if there is an overlap between GH and ghrelin signalling in the regulation of feeding it would be interesting to investigate whether long term GH treatment, alternatively using GH transgenic mice, would change central ghrelin sensitivity.

Even though it is unknown whether GH has mitogenic effects in the adult brain it is possible that GH might effect the early neonatal organisation of the circuitry important for ghrelin's effects of feeding. No studies to elucidate the wiring of hypothalamic neurons in GH-deficient or GHR knockout animals have been done but given that GH stimulates neuronal and glial proliferation and increases cranial size in young animals (138, 190, 191) and that GH deficiency impairs proliferation and myelination of neurons and glial cells (143), an impairment reversed by GH treatment (132, 133), it is plausible that GH also could affect specific neuronal populations. In GH deficient Snell dwarf mice and GH deficient cretinoid rats exogenous GH corrects the altered neural glucose metabolism, nucleic acid and protein synthesis, lipid content and enzymatic activity only when given prior to critical periods of neonatal development (19, 132, 178). Furthermore, levels of GH is transiently elevated in the brain during late fetal - early neonatal stages, when GHR expression in the brain is at a maximum and interconnections of neural circuitry and formation of glial cells are maximal, indicating that GH might be a possible agent promoting the maturation of the CNS (84, 111). These effects have been suggested to be mediated by of both IGF-I and thyroid hormone (111, 162). Future studies using different genetically modified animals models and in progeny of GH treated mothers as well as GH treatment in neonatal animals might reveal if hypothalamic NPY/AgRP neuronal and possibly also the POMC/CART neuronal projections are differentially effected by GH as have been shown for ghrelin and leptin (43).

The increased food consumption seen in vehicle treated GHR knockouts could be a result of hypoglycaemia and/or the very low circulating insulin levels observed in our study and by others (78). This effect could possibly also mask the orexigenic effects of ghrelin treatment in the GHR knockouts. Ghrelin is, on the other hand, the most potent orexigen, next to NPY, known to man and injections of ghrelin increase food intake in other models with increased appetite (28). The increased corticosterone levels might also be causative of the increased food intake as well as the obesity observed in GHR knockout mice. Conversely, loss of corticosterione following adrenalectomy has been shown to abolish the adipogenic effects of ghrelin mimetic (177).

Conclusion

Endogenous ghrelin from the stomach is important for maintaining normal body weight and body composition.

Long term treatment with ghrelin increases body fat by a mechanism that appears to be independent of its acute affects on food intake.

Long term ghrelin treatment impacts upon hypothalamic genes regulating energy balance.

Ghrelin's acute effect on food intake is dependant on a functional GHR signalling system.

Ghrelin induce dopamine activation in areas of the brain intimately associated with reward-seeking activities.

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