Growth Hormone and Melanin-Concentrating Hormone receptor in the regulation of energy balance and metabolism

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Göteborg, Sweden, 2007
ISBN: 978-91-628-7075-1
Printed by Intellecta Docusys, Västra Frölunda, Sweden

Abstract
Energy homeostasis – the balance of energy intake, expenditure, and storage – is controlled by autonomic regulation originating in the hypothalamus and the brain stem, which receive input from the periphery. Upon receiving signals from the periphery, centres in the central nervous system (CNS) react through endocrine or neuronal responses to maintain a steady balance. Growth hormone (GH) and melanin-concentrating hormone (MCH) act in the CNS to influence the energy balance and may be connected to the peripheral signals ghrelin and leptin. The overall aim of this thesis was to investigate how these different hormonal systems interact.

To investigate the metabolic role of GH in the CNS, transgenic mice that overexpress bovine GH in the CNS (GFAP-bGH) were studied. GFAP-bGH mice have higher food intake and body weight and are obese compared with wild-type (WT) mice. Moreover, GFAP-bGH mice had hyperinsulinemia, pancreas islet hyperplasia, and dyslipidemia, but no changes in energy expenditure were observed. Thus, GH is an orexigenic signal in the CNS that leads to obesity and alters insulin and blood lipid profiles.

Mice deficient in the gene encoding GHr (GHr KO) were injected in the CNS with ghrelin to study whether the orexigenic signal from ghrelin is dependent on functional GH signalling. The stimulatory effect of ghrelin on food intake was blunted in GHr KO mice, which suggests that the effects of ghrelin on food intake involve the central GH/GHr system. Furthermore, GHr KO mice were growth retarded and obese with higher leptin and corticosterone levels, low insulin and glucose levels and altered circulating lipids. Functional GH signalling is thus required for normal carbohydrate metabolism and lipid biology.

The orexigenic neuropeptide MCH may also be involved in ghrelin-induced food intake and GH secretion. Food intake of mice that were deficient in the gene encoding MCHr (MCHr KO) and were injected in the CNS with ghrelin was similar to that of ghrelin injected WT mice, which suggests that MCHr is not required for the stimulating effect of ghrelin on food intake. But ghrelin had no effect on pituitary GH expression in MCHr KO mice, which suggests that MCHr is involved in ghrelin-mediated GH expression. Furthermore, MCHr is important for the acute effect of intracerebroventricular ghrelin on serum insulin but not on corticosterone levels. Thus, functional MCHr is required for the effects of ghrelin on GH expression and insulin secretion.

Since leptin and MCH act in common pathways in the hypothalamus to regulate energy balance, leptin-deficient MCHr KO (MCHr KO ob/ob) mice were studied to investigate the importance of MCHr on the phenotype of ob/ob mice. MCHr KO ob/ob mice were similar to ob/ob mice concerning body weight, food intake, hepatic steatosis, blood lipid profile, and energy expenditure. But normal glucose tolerance and markedly reduced insulin levels were observed in MCHr KO ob/ob mice, indicating improved insulin sensitivity. MCHr KO ob/ob mice had higher locomotor activity, improved core body temperature regulation, and reduced corticosterone levels. Thus, MCHr may be involved in direct or secondary signalling cascades that lead to changes in insulin sensitivity, locomotor activity, and blood serum parameters.

In conclusion, GH and MCHr play important roles in the CNS in regulating energy balance, including effects on food intake, body weight, obesity, and circulating endocrine signals.

List of publications

This thesis is based on the following articles, which will be referred to by their Roman numerals:

I **Growth hormone overexpression in the central nervous system results in hyperphagia-induced obesity associated with insulin resistance and dyslipidemia.**
Bohlooly-Y M, Olsson B, Bruder CEG, Lindén D, Sjögren K, Bjursell M, Egecioglu E, Svensson L, Brodin P, Waterton JC, Isaksson OGP, Sundler F, Ahrén B, Ohlsson C, Oscarsson J and Törnell J.
Diabetes, 2005 Jan; 54(1):51-62

II **Growth hormone receptor deficiency results in blunted ghrelin feeding response, obesity, and hypolipidemia in mice.**
Egecioglu E, Bjursell M, Ljungberg A, Dickson SL, Kopchick JJ, Bergstrom G, Svensson L, Oscarsson J, Törnell J and Bohlooly-Y M.
Am. J. Physiol. Endocrinol. Metab. 2006 Feb; 290(2):E317-25

III **Importance of melanin-concentrating hormone receptor for the acute effects of ghrelin.**
Bjursell M, Egecioglu E, Gerdin AK, Svensson L, Oscarsson J, Morgan D, Snaith M, Törnell J and Bohlooly-Y M.
Biochem. Biophys. Res. Commun. 2005 Jan 28;326(4):759-65

IV **Melanin-concentrating hormone receptor 1 deficiency increases insulin sensitivity in obese leptin-deficient mice without affecting body weight.**
Bjursell M, Gerdin AK, Ploj K, Svensson D, Svensson L, Oscarsson J, Snaith M, Törnell J and Bohlooly-Y M.
Diabetes, 2006 Mar;55(3):725-33

Abbreviations

5-HT	serotonin
aa	amino acids
apo	apolipoprotein
AgRP	agouti-related protein
ARC	arcuate nucleus
BAT	brown adipose tissue
CART	cocaine- and amphetamine-regulated transcript
CCK	cholecystokinin
CNS	central nervous system
CRH	corticotrophin releasing hormone
DMH	dorsomedial hypothalamus
ffa	free fatty acid
GFAP-bGH mice	mice that overexpress bovine GH controlled by glial acid fibrillary protein promoter
GH	growth hormone
GHr	growth hormone receptor
GHr KO mice	growth hormone receptor knockout mice
GHRH	growth hormone releasing hormone
GHSr	growth hormone secretagogue receptor (ghrelin receptor)
GLUT	glucose transporters
HDL	high-density lipoprotein
hx rats	hypophysectomised rats
ICV	intracerebroventricular
IDL	intermediate-density lipoprotein
IGF-1	insulin-like growth factor 1
IL-6	interleukin-6
LDL	low-density lipoprotein
LHA	lateral hypothalamic area
LPL	lipoprotein lipase
α-MSH	alpha melanocyte-stimulating hormone
MAP	mitogen-activated protein
MC3-R	melanocortin-3 receptor
MC4-R	melanocortin-4 receptor
MCH	melanin-concentrating hormone
MCHr	melanin-concentrating hormone receptor
MCHr KO mice	melanin-concentrating hormone receptor knockout mice
MT-bGH mice	mice with general GH overexpression
NGE	neuropeptide GE
NEI	neuropeptide EI

ob/ob mice	leptin-deficient mice
PCR	polymerase chain reaction
POMC	proopiomelanocortin
PP	pancreatic polypeptide
PVN	paraventricular nucleus (in the hypothalamus)
PYY	peptide YY
QUICKI	quantitative insulin-sensitivity check index
RER	respiratory exchange ratio
TNF-α	tumour necrosis factor-alpha
VLDL	very-low-density lipoprotein
VMH	ventromedial hypothalamus
WT mice	wild-type mice

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Introduction

Recent research confirms a physiological system whose prime function is to maintain homeostasis between energy intake, expenditure, and storage. The system is highly complex and involves neuronal, endocrine, and various metabolic signals that act both in the periphery and in the central nervous system (CNS). Although energy intake and energy expenditure vary considerably from meal to meal and day to day, body weight in the short term remains steady. This reflects an active regulatory process that promotes energy homeostasis. Any distortion of this balance over time may lead to obesity or weight loss. Obesity results from ingesting more calories than is required at a certain time point. Biological defence mechanisms that ensure steady body weight may result in obesity when they are altered. The concept of a "negative feedback system" from adiposity has long been established. Different adiposity signals inform the CNS of any changes in body fat, and the CNS acts to balance and stabilise fat stores. Criteria for an adiposity negative feedback signal include these: that serum levels of the signal are in proportion to body fat content and the signals enters the CNS, that the signal promotes weight loss by acting at a neuronal level that involves energy homeostasis regulation, and that the signal inhibits neurons that increase food intake and body weight. Although nutrients (e.g. free fatty acids, glucose), cytokines (e.g. interleukin-6), and classical hormones (e.g. glucocorticoids) fulfil some of these criteria, leptin and insulin satisfy all these criteria [1]. But obesity is associated with peripheral and central insulin and leptin resistance [2-5]. Thus, natural protective signals and defence mechanisms do not function properly in obesity.

The prevalence of obesity has increased dramatically in the last 30 years. A new era in obesity research has produced more thorough knowledge of the signals and systems involved in the regulation of energy homeostasis.

Overweight and eating disorders constitute a vast impact on human health, linking several disease states that contribute to high mortality worldwide. Metabolic syndrome is a collection of risk factors: abdominal obesity, impaired glucose tolerance, insulin resistance, high plasma triglycerides, low HDL levels, and hypertension [6]. These are highly associated with cardiovascular disease. Obesity has been shown to correlate with, for example, hypertension, arteriosclerosis, coronary artery disease, and stroke. Lipid abnormalities act as additional risk factors for various cardiac outcomes (reviewed in [7]). Certain types of cancer are also associated with obesity, including endometrial, kidney, and colon cancer; oesophageal adenoma; hepatocellular carcinoma; and

postmenopausal breast cancer (reviewed in [8]). A large body of evidence correlates obesity with endocrine insensitivity disorders, where insulin insensitivity and type 2 diabetes are central. A strong positive correlation between obesity and diabetes risk has been established [2]. Other endocrine insensitivity disorders such as leptin insensitivity are common in obese subjects [9]. The global obesity epidemic requires a better understanding of the etiology of obesity and how obesity-related disorders might be treated.

Regulation of food intake and energy balance

Several hormonal, neuroendocrine, and neuronal signals that act in the CNS tightly regulate food intake and energy balance. Several signals – derived from peripheral tissues and organs – circulate as endocrine signals to inform the CNS about the current nutritional and energy balance. These signals could also be neuronal, for example, occur via vagus neurons, which mainly act in nuclei in the brain stem. The signals act in the CNS, which adequately responds by initiating neuronal, neuroendocrine, and/or endocrine activity to correct any imbalances. Peripheral food-intake signals can be divided into long-term adiposity signals (e.g. leptin and insulin) that exert prolonged control and short-term adiposity signals (e.g. ghrelin, peptide YY [PYY], glucagon-like peptide 1 [GLP-1], and pancreatic polypeptide [PP]) that act to initiate or terminate food intake. The blood brain barrier is considered to be a regulatory interface that controls the transport of signals like leptin, ghrelin, glucose, insulin, and insulin-like growth factor 1 (IGF-1) [10, 11].

Leptin, mainly derived from adipocytes, has been shown to circulate in concentrations proportional to body fat content [12, 13]. Food restriction lowers leptin levels, which is reversed by re-feeding, and both central and peripheral leptin administration lead to lower food intake and body weight [14]. Mice deficient in leptin (ob/ob) or the leptin receptor (db/db) are obese, hyperphagic, hyperinsulinemic, hyperlipidemic, and hypothermic [15]. The leptin receptor is primarily expressed in the hypothalamus in areas involved in control of food intake and energy balance, for example, the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the dorsomedial hypothalamus (DMH), and the ventromedial hypothalamus (VMH) [16]. Insulin, which is produced by β cells in the islets of Langerhans in the pancreas, also circulates in the bloodstream in proportion to body fat content [17] and is thought be in proportion to visceral fat mass. Hypothalamic nuclei involved in the regulation of food intake and energy balance also express insulin receptors [18], and injection of insulin to the CNS is known to decrease

food intake and body weight [19-21]. Ghrelin is mainly synthesised by endocrine cells in the stomach. Ghrelin levels have been found to increase before expected meals and rapidly decrease after food intake, which suggests a role in meal initiation [22]. Peripheral and intracerebroventricular (ICV) ghrelin injection increase acute food intake, whereas chronic ghrelin administration induces obesity [23]. Meal termination and satiety factors include cholecystokinin (CCK) from the gastrointestinal tract, which besides controlling gall bladder contraction, pancreatic secretion, and gut motility also inhibits food intake. CCK may inhibit food intake via neurons in the brain stem [24]. Endocrine cells in the distal intestine produces PYY, GLP-1, and oxymodulin, which all inhibit feeding [24, 25].

The hypothalamus and the brain stem are important regions for regulation of the energy balance. Neurons in the hypothalamus express receptors for several peripheral signals, for example, ghrelin, PYY, GLP-1, insulin, leptin, and adiponectin [26, 27]. The ARC mainly contains two subsets of nerve cell populations, neuropeptide Y/agouti-related protein (NPY/AgRP) producing and proopiomelanocortin/cocaine- and amphetamine-regulated transcript (POMC/CART) producing cells. NPY and AgRP neurons are considered to be first-order orexigenic neurons, whereas POMC/CART neurons are considered to be first-order anorexigenic neurons [1]. These neurons project to and interact with other second-order neuron populations in other hypothalamic nuclei, for example, the VMH, the DMH, the PVN, and the lateral hypothalamic area (LHA) [see section below “Signals within the hypothalamus”]. Second-order orexigenic peptides, which promote food intake (some also decrease energy expenditure), include orexin, melanin-concentrating hormone (MCH) and cannabinoids. Anorexigenic signals include α-melanocyte stimulating hormone (α-MSH), corticotrophin releasing hormone (CRH), and serotonin (5-HT) [1]. The POMC precursor is cleaved into α-, β-, and γ-MSH; adenocorticotrophic hormone (ACTH); and β-endorphin [28]. α-MSH reduces food intake and body weight and increases energy expenditure [29, 30]. NPY is thought to have direct orexigenic effects while AgRP is thought to be an endogenous antagonist for α-MSH actions on melanocortin-3 receptor (MC3-R) and melanocortin-4 receptor (MC4-R). All these bio-molecules act via the nucleus of solitary tract (NTS) or via endocrine signalling, and several of these neuromolecules affect the endocrine axes of the body, including the hypothalamic-pituitary-adrenal (HPA), hypothalamic-pituitary-thyroid (HPT), and hypothalamic-pituitary-gonadal axes. Although NPY and AgRP seem to be important orexigenic signals that originate from the ARC, genetic ablation of the genes that encode NPY and AgRP produce

only minor changes in the energy balance [31]. Thus, several compensatory mechanisms and signals may be involved, which highlights the complexity of energy balance regulation. The ARC is anatomically situated at the base of the hypothalamus, directly above the median eminence and close to capillaries that facilitate access to various circulating signals [26, 32]. But other nuclei of the hypothalamus also express receptors for nutritional signals (e.g. VMN and LHA), which may indicate that these nuclei are also important for nutritional sensing [33]. In fact, the VMN is essential for leptin regulation of the energy balance [34]. Besides, other extrahypothalamic regions of the brain, such as the NTS, express leptin receptors, and leptin administration to the NTS reduces food intake [35, 36].

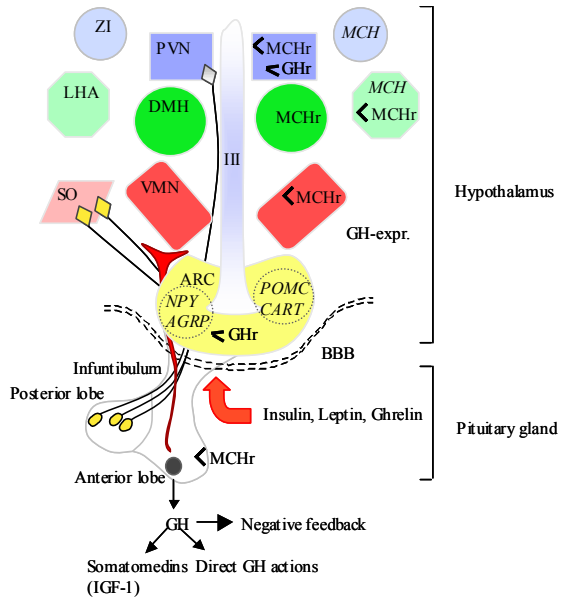

Figure 1. The hypothalamus, the pituitary gland, and endocrine pathways. The hypothalamic nuclei to the right of the third ventricle (III): ZI, zona incerta; PVN, paraventricular nucleus; LHA, lateral hypothalamic area; DMH, dorsomedial nucleus; SO, supraoptic nucleus; VMN, ventromedial nucleus; and ARC, arcuate nucleus. (BBB = blood brain barrier, GH = growth hormone, MCH = melanin-concentrating hormone, PYY = peptide YY, NPY = neuropeptide Y, AGRP = agouti-related protein, IGF-1 = insulin-like growth factor 1, POMC/CART = proopiomelanocortin/cocaine- and amphetamine-regulated transcript)

Signals within the hypothalamus

Neuronal interactions in the hypothalamus are complex. These include interactions within and between the hypothalamic nuclei. The ARC has extensive connections with the PVN, the DMH, the VMH, and the LHA.

The PVN is supplied with axons that project from ARC NPY/AgRP and POMC/CART neurons and from LHA neurons that express orexin. Nerve end terminals in the PVN are rich in NPY, α-MSH, 5-HT, noradrenalin, and opioid peptides. The PVN also contains oestrogen receptors that regulate transcripts for vasopressin and oxytocin [37, 38], which are hormones that are secreted from the posterior pituitary gland. CRH is expressed in PVN neurons that project to the median eminence and may act to inhibit NPY neurons in the ARC. The VMH has been implicated in food intake reduction and increased metabolism [39, 40], and high expression of leptin receptors are found in the VMH [41]. This nucleus has direct connection with the PVN, the LHA, and the DMH. The DMH nucleus has also been suggested to be involved in the control of ingestive behaviour and body weight [42]. The DMH contains α-MSH, MC4-R, MCH, orexin, and AgRP [43-48] and thus communicates with other hypothalamic nuclei. The LHA comprises a large distributed population of neurons, including subpopulations that express orexin and MCH. The LHA has many NPY terminals in contact with MCH and orexin cells. Both orexin and MCH neurons have wide projections to several parts of the CNS, which indicates involvement in a variety of functions. The MCH and orexin neurons also have reciprocal connections with each other. In general, orexin neurons have stimulatory effects on neurons in the LHA, whereas MCH depresses the synaptic activity of LHA neurons.

Interestingly, the body's nutritional status also affects these connections and synaptic activity. Fasting decreases excitatory and increases inhibitory synaptic contacts to POMC neurons while fasting has the opposite effect on NPY neurons [49]. This may involve leptin, since leptin deficiency results in similar synaptic alterations. Thus, fasting and overeating may, in the long term, alter the connectivity and activity of the neurons in the hypothalamic feeding centre in the process of adaptation to the novel situation. That might partly explain the why obese individuals find it difficult to loose body weight and anorexic individuals to gain body weight. The information in this section, *Signals within the hypothalamus*, is from references [[33, 42, 50-52].

Insulin biology and diabetes

Insulin is an anabolic endocrine signal in the periphery that promotes glycogenesis, lipogenesis, and protein synthesis. Blood glucose stimulates insulin secretion while adrenalin and somatostatin inhibit insulin secretion; other substrates such as ketones, free fatty acids (ffa), and amino acids (aa) and hormones like glucagon, cortisol, and GH also stimulate insulin secretion. Insulin secretion is also regulated autonomically by parasympathetic neurons (acetylcholine), which

stimulate insulin secretion, and sympathetic neurons (noradrenalin), which inhibit insulin secretion.

Glucose is mainly transported into cells by glucose transporter proteins (GLUT). By binding to its receptor, insulin stimulates translocation of GLUT4 to the cell surface and hence increases glucose transport across the cell membrane. But insulin also enhances glucose uptake in tissues that do not express GLUT4 (e.g. the liver) by several mechanisms, including activation of glucokinase, inhibition of glucose phosphatase, stimulation of glycogenesis and glycolysis, and inhibition of gluconeogenesis. Insulin also stimulates lipogenesis by increasing ffa production and inhibiting fat oxidation. By activating lipoprotein lipase (LPL) and inhibiting hormone sensitive lipase (HSL), insulin promotes lipogenesis and lipid storage in adipose tissue. Thus, insulin reduces circulating ffa. ffa are known to inhibit GLUT4 translocation; hence, when insulin lowers ffa in the bloodstream, the inhibiting effect of ffa on GLUT4 translocation is reduced, which indirectly promotes glucose uptake. Higher levels of ffa in the bloodstreams of obese subjects may thus contribute to reduce GLUT4 translocation and glucose intolerance.

Changes in insulin biology impair the metabolism of carbohydrates, fats, and proteins and may lead to the disease diabetes mellitus. Lack of insulin secretion typically causes insulin-dependent diabetes mellitus (IDDM, or type 1), whereas lower insulin sensitivity in body tissue and possibly lower insulin secretion causes non-insulin-dependent diabetes mellitus (NIDDM, or type 2). Type 2 diabetes is more common than type 1 diabetes; about 90% of the diabetic population has type 2. Higher blood glucose levels caused by lower insulin sensitivity stimulate β cells to produce more insulin to compensate for lower insulin sensitivity. In later stages, the hyperinsulinemia is not enough to normalise blood glucose, which leads to higher fasting and non-fasting blood glucose levels and higher insulin levels. High insulin production can eventually cause β-cell dysfunction, which may lower insulin secretion. Obesity is known to be a primary cause of insulin insensitivity and type diabetes [2]. More and larger adipocytes result in higher ffa secretion and higher secretion of adipocyte derived peptides – adipokines – which are associated with worsened insulin sensitivity [53]. Adipose tissue is considered the largest endocrine organ in the body and is highly active in secreting bioactive signals such as leptin, adiponectin, and resistin and inflammatory signals such as TNF-α and IL-6 [54].

Lipid biology and dyslipidemia

Lipids cannot circulate on their own in the bloodstream since they are hydrophobic. ffa are transported in the blood bound to the protein albumin in ffa-albumin complexes. Triglycerides and cholesterol esters are transported in the blood in particles called lipoproteins, which have a hydrophilic surface and a hydrophobic core that contains the lipids. Incorporated in the surface are different apolipoproteins (apoB, apoC, apoE) which serve to interact with different body tissues. The lipoproteins are classified by their density, that is, very-low-density (VLDL), low-density (LDL), intermediate-density (IDL), and high-density (HDL) lipoproteins. Chylomicrons (CMs) are the lowest density particles that carry lipids from the intestine to body tissue. Tissues in the body express LPL, an enzyme that catalyses triglyceride degradation, which releases ffa for the tissue to take up. The liver takes up the glycerol part of the triglyceride since it expresses glycerol kinase. CM and VLDL (produced by the liver) are rich in triglycerides and deposit much of their triglyceride load in tissues such as adipose tissue and muscle, and the lipoprotein particles thus raise their density. LDL contains much unesterified cholesterol and CE. HDL particles are small and are important for reverse cholesterol transport, that is, transport of cholesterol from peripheral tissue to the liver. After losing its triglycerides, CM becomes a CM remnant and VLDL is degraded to IDL, which is rapidly converted to LDL. The different lipoproteins contain different apolioproteins and thus interact with different tissues. The apoE receptor in the liver takes up the CM remnant, which expresses apoB-48, apoC, and apoE, while IDL and LDL, which express apoB-100, binds to the hepatic LDL receptor. After binding to the LDL receptor, the particles are degraded via endocytosis. One major difference between humans and mice is that human VLDL contains apoB-100 as the structural protein, whereas mice secrete VLDL containing either apoB-48 or apoB-100. Following hydrolysis in peripheral tissues, the apoB-48-containing lipoproteins are cleared from the bloodstream via the apoE receptor, whereas the apoB-100-containing lipoproteins can bind to both LDL receptors and apoE receptors in the liver. The turnover of apoB-48-containing lipoproteins in the bloodstream is higher compared with apoB-100-containing lipoproteins [55]. This results in fewer LDL particles. This, together with the fact that HDL is the major subclass of lipoproteins in mice, may explain why mice are less prone to developing cardiovascular disease compared with humans.

Dyslipidemia results from impaired regulation and metabolism of blood lipids and constitutes a high risk factor for cardiovascular diseases and type 2 diabetes. Low levels of LDL and high levels of HDL are known to

protect against cardiovascular disease; for example, drugs that lower LDL (statins) reduce the risk of myocardial infarction and prolong life [56]. Type 2 diabetes is known to be associated with obesity and impaired lipid metabolism. For instance, high levels of triglyceride-rich VLDL particles can, by different mechanisms, reduce HDL levels and thus increase the risk of disease. Measurements of cholesterol fractions, apolipoproteins, and receptors are important in the investigation of metabolic changes that may result in dyslipidemia and related disorders.

Growth hormone

The anterior part of the pituitary gland releases important endocrine signals to the body and is under control of the hypothalamus. All but two major anterior pituitary hormones exert their effects on peripheral glands, which include the gonadal, thyroid, adrenal, and mammary glands. Prolactin (PRL) and GH have no single target gland but exert their effect on several tissues and organs that express receptors for PRL and GH.

GH is a polypeptide of about 23 KD, constituting 191 aa in humans and 190 aa in rodents with about 66% aa homology. This peptide hormone was first isolated from bovine pituitary gland in 1944 [57] and later isolated in several other species. The gene encoding human GH (hGH) is located on chromosome 17 and closely related to chorionic somatomammotropin (CS or placental lactogen) and PRL, which is a group of homologous peptides with growth-promoting and lactogenic activity. Somatotrophic cells in the anterior part of the pituitary gland secrete GH in a pulsatile manner. Peaks of GH secretion typically last between 10 and 30 minutes, and the most pronounced peak occurs after sleep onset. In rodents, the interval between peaks is about 3 hours, but the level of GH between and at the peaks varies according to age and gender [58-60]. GH secretion is mainly controlled by signals from the hypothalamus, which are transported in the venous blood in the hypothalamic-hypophyseal portal vessels that surround the pituitary. Hypothalamic growth hormone releasing hormone (GHRH) from the ARC stimulates and somatostatin from the PVN inhibits GH secretion. Ghrelin, a peptide hormone mainly produced by endocrine cells in the stomach, also has a strong GH releasing effect [61]. GH secretion is also under control of a negative feedback system of circulating GH and IGF-1 [32]. Although these signals control the balance of GH release, GH secretion is also affected by a variety of other physiological stimulators such as exercise, hypoglycaemia, dietary protein, estradiol, and glucocorticoids [32].

Signal transduction of GH occurs via binding to the GH receptor (GHr), which results in receptor dimerisation that in turns leads to activation of several intracellular signals, including Janus tyrosine kinase (JAK) and signal transducers and activators of transcription (STAT) pathway activity [62, 63]. The majority of circulating GH is bound to growth hormone binding protein (GHBP), which is derived from the GHr. Although GH is mainly produced in somatotroph cells in the pituitary, production of GH and GHr also occurs in various parts of the CNS. GH is expressed in several limbic structures, for example, the amygdala, the hippocampus, and the hypothalamus [64, 65]. Neurons in the thalamus and hypothalamus express GHr [66, 67].

Effects of growth hormone on metabolism

Transgenic mice have been studied for more than 25 years, and the first transgenic mouse model overexpressed rat GH [68]. Early studies by Törnell *et al* [69] on hGH overexpressing transgenic mice revealed that hGH overexpression induced growth and mammary adenocarcinomas, which suggests GH involvement in cancer development in mice. GH regulates whole body metabolism and growth, both as a direct effect and via stimulation of IGF-1. Local infusion of GH in the epiphyseal plates of hypophysectomised (hx) rats directly stimulates longitudinal bone growth, which indicates direct GH effects or possibly effects of local IGF-1 production [70]. Liver specific IGF-1 deficient mice also have normal body growth compared to wild-type (WT) mice [71], which suggests a direct effect of GH on body growth. It is well known that GH affects body composition by increasing lean body mass and decreasing body fat in a variety of species. Several studies show that GH influences protein [72-74] and carbohydrate [75, 76] metabolism. GH increases protein deposition by enhancing aa uptake, DNA transcription, and RNA translation. In addition, GH decreases catabolism of proteins and aa, which prevents muscle tissue loss. GH action in carbohydrate metabolism includes decreased glucose uptake in skeletal muscle and adipose tissue, increased glucose production in the liver, and increased insulin secretion. Thus, GH attenuates insulin action and increases serum glucose levels while also stimulating compensatory insulin secretion. These effects are known as diabetogenic actions, and an overabundance of GH may cause symptoms similar to those of patients with type 2 diabetes.

Adipocytes also express GHr [77], and the direct effect of GH on adipose tissue is to enhance fat metabolism by stimulating triglyceride breakdown and oxidation and suppressing the ability of the tissue to accumulate circulating lipids [78-82]. But what effects GH has on circulating ffa is

still under discussion, and the studies that have reported higher levels of ffa after GH treatment have been performed on fasted animals [83].

In humans, low body weight is associated with elevated circulating GH levels [84], whereas obesity is associated with a reduction in GH secretion [72, 85, 86]. In addition, GH excess in, for example, acromegaly, is correlated with reduced body fat mass [87]. Animal models with genetic GH overexpression or GH administration have similar effects as those in humans, such as decreased fat mass [78, 88, 89], while animal models with a deficiency of GH are associated with increased fat mass [90], which is also seen in humans. In addition, GH administration affects obese leptin-deficient ob/ob mice by reducing fat mass [91]. Thus, data from both human and animal models strongly support a long-term fat-reducing effect of GH and a fat-enhancing effect of GH deficiency.

GH also affects lipoprotein metabolism. Hx rats have decreased HDL cholesterol and increased LDL cholesterol levels in serum in addition to increased apoB and decreased apoE levels [92-94]. But GH treatment in hx rats can normalise blood lipids by increasing HDL cholesterol and apoE and decreasing LDL cholesterol and apoB levels [94, 95]. In addition, GH treatment in hx rats increases LPL and hepatic lipase (HL) activity [96], which indicates yet other roles of GH in the blood lipid balance.

Melanin-concentrating hormone

MCH was discovered over 20 years ago as a mediator of skin colour change in fish [97]. But much data on rodents and humans support the involvement of MCH in the regulation of energy balance, endocrine balance, and food intake [98, 99].

MCH is a cyclic peptide of 19 aa and its aa sequence is fully conserved between mice, rats, and humans. The MCH gene (pMCH) encodes a 165-aa preproMCH peptide that is proteolytically processed to produce MCH and two other peptides: neuropeptide GE (NGE) and neuropeptide EI (NEI). pMCH also encodes alternatively spliced products, the MCH-gene overprinted polypeptide (MGOP). preproMGOP are cleaved into two peptides: MGOP-14 and MGOP-27. Finally, another product – antisense RNA overlapping MCH (AROM) – is encoded at the same locus as the pMCH gene on the opposite strand [100-104]. Whether NGE functions as a bioactive molecule is unknown, but in rodents, NEI behaves similarly to α-MSH in inducing grooming behaviour, increasing motor activity, and increasing LH levels [105, 106]. Furthermore, NEI may be involved in

the HPT axis and has been found in the pituitary gland [107, 108], although most NEI expression occurs in the hypothalamus [107].

Melanin-concentrating hormone receptor
Two G protein-coupled receptors for MCH – melanin-concentrating hormone receptor 1 (MCHr1) and 2 (MCHr2) – are found in primates, dogs, and ferrets, but only MCHr1 is found in rodents. Human MCHr1 and MCHr2 share 38% aa identity and differ in that the MCHr1 gene lacks intron in the coding region and activates multiple signalling pathways by coupling to G_i, G_o and G_q proteins while the MCHr2 gene has multiple exons and exclusively couples to one G-subunit, $G_{\alpha q}$ [109]. The rodent MCH receptor (also called SLC-1, or GPR24) is the orphan somatostatin-like receptor 1 and activation results in several intracellular effects, including suppression of forskolin stimulated cyclic adenosine monophosphate (cAMP), increased calcium ion mobilisation, and mitogen-activated protein (MAP) kinase activity [104, 109-112]. Interestingly, none of the pMCH-produced products found so far – NEI, NGE, MGOP, and AROM – are able to activate MCHr1 or MCHr2 [110, 113, 114]. In addition, neither somatostatin nor α-MSH are able to stimulate MCHr, which suggests that MCH is the specific ligand of these peptides for MCHr1 and neither somatostatin, α-MSH, nor NEI can block the effects of MCH on its receptors [110].

Melanin-concentrating hormone in autonomic and endocrine regulation
The expression of MCH and the MCHr may indicate involvement in the regulation of energy balance. MCH is highly expressed in the LHA and zona incerta in the hypothalamus and project broadly throughout the brain, including the cortex, amygdala, nucleus accumbens, olfactory structures, and various nuclei in the brain stem [115]. MCH is also found in rat plasma, which may indicate endocrine functions [116]. MCHr is mainly expressed in the brain, but mRNA for MCHr is also found in peripheral tissue, that is, in skeletal muscle and in the pituitary [110]. MCHr is also found in adipocytes and in insulin-producing cells in the pancreas [116, 117]. In the CNS, MCHr is widely distributed in the brain, including the cortex, hippocampus, amygdala, nuclei of the brain stem, and hypothalamus, for example, the ARC, LHA, PVN, DMH, and VMH [118, 119].

This widespread distribution of MCH and MCHr suggests multiple functions for this peptide system. For instance, polysynaptic pathways from brown adipose tissue (BAT) lead to neurons containing MCH and orexin in the lateral hypothalamus [120]. MCH decreases synaptic activity in certain gamma-aminobutyric acid (GABA) and glutamate

neurons extending from the LHA and lowers body temperature and levels of BAT uncoupling protein 1 (UCP-1) [121, 122] whereas MCHr-deficient mice have higher body temperature [123]. Interestingly, cold exposure increases MCH expression levels in rats [124].

MCHr KO mice have higher locomotor activity and heart rate [123, 125] and upregulated mesolimbic dopamine receptors and norepinephrine transporters, which indicates that MCHr might modulate mesolimbic monoamine functions [126]. Dopamine and dopamine receptors are known to alter locomotor activity [127].

MCH is also found in autonomic neurons that project into and control the pancreas [128]. In addition, MCH and MCHr were recently found in vagal afferents and interact with the satiety signal CCK [129]. MCHr KO mice are suggested to have increased sympathetic tone [123]. The above suggests that MCH has multiple autonomic and central functions, which corresponds with the wide distribution of MCH and MCHr. This is further supported by recent findings that MCH and MCHr are involved in motivated behaviours, anxiety, and depression [98]. MCH is also reported to be involved in several endocrine axes, including the HPA, HPT, and HPG axes. A stimulatory role in the HPA [130] and HPG axes (LH and gonadotropins) [131, 132] and a suppressive effect in the HPT [108] axis has been suggested. Furthermore, it was recently found that MCH can stimulate GH secretion, suggesting a role in hypothalamus-GH axis [133]. MCHr KO mice also have osteoporosis; thus, MCH may be involved in the regulation of bone mass [134].

Melanin-concentrating hormone in energy balance

Several studies suggest a central downstream role for MCH and MCHr in energy balance and feeding regulation. Fasting and leptin deficiency upregulates MCH and MCHr, while leptin administration downregulates MCHr [119, 135]. MCH can also stimulate leptin mRNA expression and secretion [116]. In addition, the melanocortin system inhibits MCH since MC receptor agonists reduce MCH expression, whereas in the obese agouti mouse model – that results from impaired melanocortin signalling – MCH is upregulated [136, 137]. MCH is believed to counteract the anorectic effects of α-MSH [138]. Both acute and chronic MCH administration increase food intake and body weight in rodents [139, 140]. Chronic MCH injection also causes obesity, and in combination with a high-fat diet, chronic MCH injection increases leptin and insulin levels in blood [121, 141]. Genetic overexpression of the pMCH gene leads to weight gain, hyperphagia, and obesity on a high-fat diet in combination with increased leptin levels, insulin resistance, and islet cell

hyperplasia [142]. Mice that lack the pMCH gene are lean and hypophagic despite reduction of leptin and POMC and have higher energy expenditure [143]. This was confirmed by other MCH ablation studies, which reported that MCH deficiency is beneficial for ageing-related insulin resistance and metabolic changes [144, 145]. MCHr KO mice have reduced body weight, fat mass, and leptin and insulin levels, and while they are hyperactive with increased energy expenditure, they are also hyperphagic. MCHr KO mice are also resistant to diet-induced obesity, and MCH injection in MCHr KO mice has no effect on food intake or body fat [125, 146]. Rats with altered MCHr signalling via selective agonists have increased feeding, body weight, and obesity whereas antagonist treatment resulted in lower food intake, body weight, and body fat [147]. MCHr antagonists may be a future target in obese subjects since they also reduce food intake, body weight gain, and body fat and lower obesity-induced hyperinsulinemia, hyperglycemia, hyperleptinemia, and hypercholesterolemia [148]. Two studies also found that loss of MCH in leptin-deficient mice results in lower body weight and obesity and improves glucose tolerance [149, 150].

Thesis aims

The general aim of this thesis was to study two endocrine signal systems, GH and MCH, and their involvement in the regulation of energy homeostasis. Articles I and II explore the GH system, whereas articles III and IV explore the MCH system. But these two systems are interconnected, and both these endocrine signals affect metabolism. The specific aims of articles I–IV were to:

I Investigate the function of GH in the CNS on metabolism by studying mice that overexpress GH in glia cells.

II Study the acute effects of ghrelin on food intake in GHr-deficient mice and examine the effects of GHr deficiency on lipoprotein biology and body composition.

III Study the role of MCHr in the acute effects of ghrelin on food intake, circulating hormones, and expression levels of GH and related peptides.

IV Investigate the importance of MCHr in the phenotype of ob/ob mice regarding food intake, body composition, and glucose tolerance by studying MCHr ob/ob double KO mice.

Materials and methods

This thesis is based on studies of mice with a genetic modification that leads to either overexpression or loss of a gene product. The effects of the genetic modifications have been studied both *in vivo* and *in vitro*. In addition, studies of genetically modified mice in combination with substance administration have been performed. Each article includes a detailed description of the experiments performed. The following section contains an overview of the methods and techniques used.

Genetically modified mice

The use of animals with specific genes knocked out or introduced (transgenics) has revolutionised medical research and is essential for analysing and understanding functions of gene products. By introducing a specific promoter sequence, it is possible to limit expression to a certain cell type in a tissue or organ.

In gene-addition (transgenic) mice, a transgene fragment that, for example, contains promoter and coding DNA regions is microinjected into the pronucleus of a fertilised egg the day after fertilisation. The transgene integrates at random in the genome of the developing embryo, which is transferred to the oviduct of a pseudopregnant female recipient. The resulting litter of mice is analysed for founder transgenics.

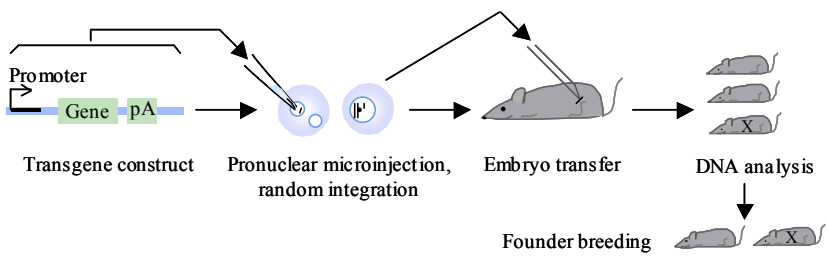

Figure 2. Gene-addition transgenics for general or tissue-specific gene overexpression.

Mice strains that carry spontaneous mutations can also be useful models, for example, leptin-deficient ob/ob mice, which are central to this thesis. In ob/ob mice, the coding sequences contains a C→T mutation, which leads to a change from an arginine to a stop codon [151]. To genotype the ob mutation, a combined polymerase chain reaction (PCR) and restriction digest approach was used, which relied on the fact that the ob mutation creates a new restriction enzyme site.

For targeted gene deficiency, that is, gene knockout (KO), a targeting construct was designed and introduced into pluripotent embryonic stem cells by electroporation. The most commonly used targeting vector is a replacement vector in which a segment of genomic DNA is replaced by an exogenous DNA fragment. This could also contain an antibiotic-resistance marker for selection purposes (e.g. neomycin resistance) flanked by two arms of homology to enable homologous recombination. Thus, the incorporation is targeted at a specific site of the genome. Embryonic stem colonies are selected, collected, and expanded for analysis to identify the correctly targeted clones, which are microinjected into a blastocyst, a 3.5-day-old embryo. The blastocysts are transferred to the uteri of pseudopregnant female recipients, and the resulting litters may include chimeric mice, which carry the targeted allele in the genome. Chimeras, as determined by coat colour, are bred to WT mice for germline transmission of the targeted allele, and the targeted mutation is confirmed by southern blot screening and PCR analysis.

The transgene and knockout mice studied were backcrossed to C57Bl6/J (Harlan, AD Horst, the Netherlands). The offspring were genotyped by PCR strategies with gene- and construct-specific primers (see the separate publications for the sequences).

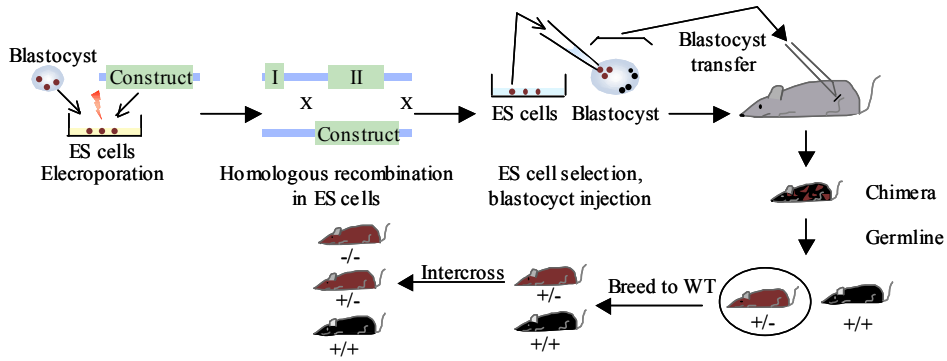

Figure 3. Gene knockout by targeted homologous recombination (ES = embryonic stem, WT = wild-type).

Considerations

Different genetically modified mouse models are used to study the function and importance of gene products. In medical research, these models can be used to study a variety of experimental setups, including

target identification and validation. Treatment experiments to study, for example, endocrine pathways, ligand-receptor relations, and drug metabolism and toxicity are also possible. By giving mice diets that vary in fat and carbohydrate content, it is possible to investigate the response in, for example, metabolism, a shifted energy balance, and energy source and to study genes that are involved in energy oxidation processes. The mouse model is a good model since man and mouse have most genes and gene products in common. In addition, practical advantages include short breeding times, availability of inbred mice strains, abundant data in the literature, and several *in vivo* systems designed for mice. Mice are also, in principle, the only animal model that could be genetically modified by targeted deletions. Human genes, for example target genes or other relevant genes that have no mouse counterpart can be introduced to investigate a hypothetical human response to compounds.

But it must be born in mind that by interfering with the genome, phenotypes can occur due to natural biological responses. These may include compensatory mechanisms where lack of a gene product results in changes in the expression of other genes and signals as natural compensatory mechanisms. In addition, deletion and replacement of parts of the genome may inadvertently delete or affect other regulatory elements or important sites that have yet to be annotated and could change the expression of upstream or downstream genes. It may also be argued that the phenotype which results from a modification of the genome, for example, inactivation of a gene by targeted deletion, does not necessarily correspond to inactivation of that gene by drugs in humans. There may be large differences between lack of a gene and antagonising a gene product for therapeutic purposes. The most prominent differences include a total absence versus a lower level or signal, specificity of a drug (agonist or antagonist), and lack of a gene product from the embryonic stage versus modification in adulthood.

Administration routes

Before ICV surgery, the mice were anaesthetised with an initial dose of 4% isoflurane (Baxter, Kista, Sweden) followed by a maintenance dose of 2% and placed in a steriotactic frame (Stoelting, Wood Dale, IL, USA). This was done to implant a permanent, 31-gauge, stainless steel guide cannula (Eicom Corp., Kyoto, Japan) above the dorsal third ventricle (0.94 mm posterior to the bregma, 1.0 mm below the outer surface of the skull). The stereotactic coordinates were determined according to a mouse brain atlas [152]. The guide cannulas were held in position with dental cement (Heraeus Kulzer, GmbH, Hannau, Germany) attached to two stainless steel screws driven into the skull. A stainless steel obtruder

(Eicom Corp.) was inserted into the guide to maintain cannula patency. The animals were allowed seven days postoperative recovery. ICV injections were made during a short, 30-second period of anaesthesia with 2% isoflurane. Substance solution was injected with a stainless steel injector that was inserted in and projected 1.5 mm below the tip of the guide cannula; the injector thus projected into the cavity of the third ventricle. A Hamilton syringe (VWR International AB, Stockholm, Sweden) was connected to a plastic tube and used for the injections, which were performed over a 20-second period. A maximal volume of 1 µl was injected.

Intraperitoneal (IP) injections were performed in unanaesthetised animals with a typical volume of 100 µl substance solution.

Intravenous (IV) injection was performed under midazolam anaesthesia (0.14 mg/mouse, Dormicum®, Hoffman-La-Roche, Basel, Switzerland) in combination with IP injection of fluanison (0.9 mg/mouse, Janssen, Beerse, Belgium) and fentanyl (0.02 mg/mouse; Hypnorm®, Janssen). Substance solution was given in the tail vein. Volume load was 10 µl/g body weight.

Oral administration (PO) was done in gavage using oral mouse probes (Scanbur AB, Sollentuna, Sweden). A volume of 200–300 µl was administered.

Considerations
Choice of administration route depends on the aims of a study.

The ICV route is an excellent method for administering bioactive substances to the CNS and close to the site of action. The substance is injected into the third ventricle and the bioactive molecules diffuse to the hypothalamus. The ICV route is preferred for molecules with a short half-life and can be used to study acute effects in the CNS. But the test substance will mix with the cerebrospinal fluid (CSF) and may be transported to other parts of the CNS. Substances in the CSF can also diffuse into the bloodstream. Furthermore, the procedure involves invasive surgery and injections under anaesthesia that may affect the body weight and general condition of the mice. It is therefore important that the design of all experiments include control mice that receive only the vehicle solution, so that any affects due to surgical procedures will be discovered.

IP injections are fast and easy to perform, cause the animals only minor stress, and do not typically affect their general conditions. No anaesthesia is needed. The substances normally diffuse into vessels in the abdomen and are transported to the body tissues.

IV administration is obviously the best route for a fast injection of substances into the bloodstream. A speedy introduction of a substance in the bloodstream may be important for studies of clearance and endogenous responses over time. In mice, injection is often made in the tail vein, usually under anaesthesia.

PO administrations resemble the natural route for assimilation of nutrients and drug substances via the gastrointestinal tract. Individual doses or quantities can be administered by gavages in a fast, non-invasive manner in unanaesthetised animals. Differences in both intestinal assimilation and metabolic rate after assimilation must be considered in PO experiments.

Body weight, body length, and food intake
Studies of body weight and length progress from an early age, and measurements of the effects of starvation on body weight, are central to this thesis. Typically, body weight was checked on a weekly basis whereas body length was measured a few times during development. Since body length measurement involves light sedation, the measurements may affect weight gain and thus should not be done frequently. It must also be noted that handling the animals may affect different measurement parameters such as body weight and food intake, which are associated with effects on different physiological and endocrine pathways. The studies were designed to minimise potential stress; the animals were allowed to acclimatise to their new environment, and controls after surgical procedures were made. Mice were housed with about the same number per cage.

To measure individual food intake, cages (23 x 16 cm^2) were prepared with a similar amount of diet and dried at 80^oC for 1 hour to equalise humidity. Thereafter, the cages were kept in the animal room to equalise moisture. In certain experiments, the mice were starved for up to 12 hours overnight before food intake measurement to assess the effects of fasting on body weight and to correct for eating before measurement. At the beginning of the experiment, the cages and mice were weighed separately, and the mice were put in their cages for a designated experimental time. The mice were single housed for at least 3 days before the experiment to customise them to the novel situation. Acute food

intake was measured over 3 hours whereas long-term food intake was measured over 24 or 48 hours. Food and water were available *ad libitum* during food intake measurements. After measurement, the mice were returned to their original cages and all faeces were collected. The cages were re-incubated at 80^{o}C to dry out water spill and urine and reweighed after 6 hours in the animal room.

Indirect calorimetry

Oxygen consumption (VO_2), carbon dioxide production (VCO_2), food intake, water intake, and spontaneous horizontal and rearing activity were measured using an open circuit calorimetry system (CLAMS, Columbus Instruments International Corp., Columbus, OH, USA). The animals were placed in calorimeter chambers with access to food and water *ad libitum* for 48 hours at either room temperature or a temperature in the thermoneutral zone (29.5°C). Basal metabolism is assessed in the thermoneutral zone, which is the range of temperatures at which the mice do not use energy to actively maintain its body temperature. An air sample was withdrawn from each cage for 5 seconds every 9 minutes, and O_2 and CO_2 content was measured with a Zirconia O_2 sensor and a spectrophotometric CO_2 sensor. These values were used to calculate VO_2 and VCO_2. Data from the first time period (typically the first 2–4 hours) were not used in the results analysis because the animal was acclimatising to the novel environment. Data from subsequent hours were used in 2-hour bins. Energy expenditure (kcal/kg/hr) was calculated with this equation:

$$(3.815 + 1.232*RER)\times VO_2$$

where RER is the respiratory exchange ratio (volume of CO_2 produced per volume of O_2 consumed [both ml/kg/min]) and VO_2 is the volume of O_2 consumed per hr per kg mass of the animal. Constant values were derived from this function:

$$y = 3.815+1.232x$$

where y is heat (kcal) per litre O_2 and x is RER. The function is based on studies of oxidation of food mixtures that contain different amounts of fat and carbohydrates. A VO_2 of 4000 ml/hr/kg for a 25 g mouse with an RER of 0.85 would thus be:

y (heat/lit) = (3.815+1.232*0.85)*4000 ml/hr/kg =
(19448.8/1000)*0.025kg = 0.486 kcal/hr

Since 6 units of CO_2 are produced and 6 units of O_2 are consumed when carbohydrates are oxidised, RER is equal to 1. RER for the oxidation of most lipids is about 0.7; in general, about 16 units of CO_2 are produced and 23 units of O_2 are consumed.

Bomb calorimetry
After the indirect calorimetry measurement or food intake measurement, all faeces were collected, dried at 55°C overnight, and stored in airtight containers at -20°C until assayed. Gross energy content of the faecal boli was determined in a bomb calorimeter (C 5000, IKA® Werke GmbH & Co. Staufen, Germany).

Considerations
The indirect calorimetry system is designed to measure food intake (calculations of calorific intake are possible), water intake, locomotor activity, and respiratory metabolism over time. Thus, energy intake and expenditure are monitored. By collecting and measuring energy in faeces, energy assimilated over the measurement period can be determined. In addition, it is possible to make indirect calorimetry measurements in the thermoneutral zone. Thus, the energy spent at this ambient temperature correlates with basal metabolism, which includes all body processes that require energy except for the body temperature regulation. One of these processes is locomotor activity, which is closely monitored. An advantage with this system is that these parameters could be studied over several days, which includes the active phase at night and the resting phase during the day. This allows good data to be collected from the different phases, which vary significantly, and the long period of measurement attenuates the stress of novel environments and a normal situation for the mice can be studied. But the cages were smaller than the mice were used to and contained no nesting materials, which may have affected the behaviour of the mice. Food intake measurement in the indirect calorimetry system is not as correct as in the pre-weight cages protocol since the food spillage that occurs in the indirect calorimetry system is not a source of error in the pre-weight cage protocol. But in the pre-weight cages, the mice could be coprophagic or chew nesting materials, which is not possible in the indirect calorimetry cage.

Body temperature
Rectal core temperature was measured using a rectal probe (ELFA, Järfälla, Sweden) in conscious mice. In some experiments, the surrounding temperature was lowered to 6°C to study effects of cold exposure on body temperature.

Dual energy X-ray absorptiometry
Before dual energy X-ray absorptiometry (DEXA) measurements, the animals were sedated with isoflurane (Forene, Abbott Scandinavia AB, Solna, Sweden), and body length (nose-rump) was measured. Body fat (g) and (%), lean body mass (g) and total bone mineral density (g/cm^2) and bone mineral content (g) were determined by densitometry using a PIXImus imager (GE Lunar, Madison, WI, USA). During measurement, the entire animal is exposed to a small, cone-shaped beam of both high- and low-energy X-rays. The ratio of energy attenuation in the luminescent panel separates bone, lean tissue, and fat tissue. Field calibration and calibration versus the quality control phantom were made before imaging.

Computed tomography
Computed tomography (CT) was performed with the STRATEC pQCT XCT (software version 5.4B; Norland Medical Systems, Fort Atkinson, WI, USA) operating at a resolution of 70 μm as previously described [153, 154]. Sections were made at the same level in all mice, that is, 5 mm proximal to the crista iliaca.

Magnetic resonance imaging and spectroscopy
A magnetic resonance imaging (MRI) system (Varian, Palo Alto, CA, USA) that incorporated a 4.7-T magnet (Oxford Instruments, Oxford, UK) and pulsed field gradients capable of 200 mT m^{-1} with a rise time of 0.3 ms was used. A quadrature birdcage radio frequency tranceiver with a 103-mm internal diameter and sufficient radio frequency homogeneity to encompass the entire cadaver was employed. Image acquisition employed a multi-slice 2D spin-echo technique (TR=5s;TE=11ms; 41 contiguous transverse slices; 2-mm slice thickness; matrix 128 × 128; field of view 50 × 50 × 82 mm^3). Both fat- (C^1H_2 and C^1H_3) and water- (^1H_2O) suppressed MR images were taken. The image matrix included the entire animal. Two phantoms were also included in the image field. These were two 4.2-mm internal diameter tubes containing water and olive oil. Fat- and water-suppressed images were obtained by applying a Gaussian saturation pulse to the fat and water resonance.

Magnetic resonance spectra (MRS) were obtained without the phantoms, using an identical coil. Eight averages were acquired, and repetition time was 17 sec. Tissue water was used as an internal reference at a chemical shift of 4.8 ppm. Spectra were integrated using VNMR software (Varian). The integrals of the water signal ($I_{3.5\text{-}6.5}$: signal between 3.5 and 6.5 ppm), and the CH_2 and CH_3 signals from fat ($I_{0\text{-}3}$: signal between 0 and 3 ppm), in arbitrary units, were converted to estimated mass, in grams, using the simplifying assumption that mice are composed entirely of water (mw 18,

2/2 protons resonate between 3.5 and 6.5 ppm) and tripalmitin (mw 807, 93/98 protons resonate between 3.5 and 6.5 ppm).

Considerations
These imaging techniques were used to explain differences in body composition with a central focus on body fat and lean tissue. DEXA and CT are both based on 2-dimensional X-ray measurements to assess differences in the densities of the body tissues. CT data are generated when an X-ray beam rotates around the animal, creating a slice picture at a certain level. This was done on one representative animal from each group of mice. DEXA data arise from several X-ray radiation intensities, and quantitative values for fat mass, lean mass, and bone content and density are collected. The advantage with DEXA is that several animals could be scanned and average values calculated for different groups. DEXA scanning is the imaging technique that was most commonly used in this thesis. The MRI technique is based on signals from hydrogen nuclei in fat and water in a strong uniform magnetic field. The technique is typically used with non-calcified tissue, not bone tissue. Mathematical calculations make it possible to quantify fat mass by comparing signals from the molecules in fat (C^1H_2 and C^1H_3) and water (^1H_2O). Computer software produced 3-dimensional pictures that illustrate certain body fat depots.

Blood serum analysis
These radioimmunoassay (RIA) kits were used: RI-13 K (Linco Research Inc., Missouri, USA) to measure insulin, ML-82 K (Linco Research Inc.) to measure leptin, RPA 548 (Amersham Biosciences AB, Uppsala, Sweden) to measure serum corticosterone levels, GHRT-89HK (Linco Research Inc.) to measure total ghrelin levels, and RIA DSL-2900 (Diagnostic Systems, Inc., Webster, TX, USA) to measure IGF-1.

Glucose was measured with a photometric assay kit (HK 125, ABX Diagnostics-Parch Euromedecine, Montpellier, France). Serum triglycerides and total cholesterol were measured with cholesterol oxidase phenol 4-aminoantipyrine peroxidase (CHOD-PAP) (TG/GB, no. 12146029216, Cholesterol no. 2016630, Roche Diagnostics GmbH, Mannheim, Germany). Non-esterified fatty acid (NEFA) levels were analysed with a NEFA C Assay kit (Cat. no. 999-75406, Wako Chemicals GmbH, Neuss, Germany).

Cholesterol distribution profiles were measured using the method previously described [155]. Briefly, a size exclusion high-performance liquid chromatography system (SMART) with column Superose 6 PC

3.2/30 (Amersham Pharmacia Biotech, Uppsala, Sweden) was used to separate lipoproteins in a 10-µl sample over 60 minutes. The area under the curve represents the cholesterol content. The peaks in the profiles were designated VLDL, LDL, and HDL for simplicity, even though separation is determined primarily by the size of the lipoproteins. Serum apoB was measured in an electroimmunoassay as previously described [78].

Western and southern blot analysis
Protein levels were measured with western blotting as described previously [155] using the enhanced chemiluminescence protocol (Amersham Pharmacia Biotech, Buckinghamshire, UK) and quantified using a Fluor-S Multi-imager and Quantity one software (BIO-RAD, Hercules, CA, USA). In brief, protein is separated on an SDS-PAGE electrophoresis and transferred to HybondTM-P Polyvinylidene difluoride membrane. Primary and secondary antibody is added to the membrane in a blocking buffer. The membrane is exposed to hyper photo film.

DNA levels were measured with southern blotting as described previously [78]. In short, DNA was digested with restriction enzyme, run on an agarose gel, and blotted on a membrane. A ^{32}P-labled gene-specific probe was hybridised to the membrane, which was developed in a computer scanner.

Glucose tolerance test
Glucose tolerance was analysed after oral or IV glucose load in fasted or non-fasted mice. In some studies, fasting glucose and insulin were measured before glucose administration. Blood samples were taken at different time points in the different studies.

In the IV glucose tolerance test (IGTT), the animals were anaesthetised, D-glucose (1 g/kg; British Drug Houses, Poole, UK) was injected in the tail vein, and blood samples were taken from the retrobulbar capillary plexus. Levels of glucose and insulin were analysed as described above under blood serum analysis.

In the oral glucose tolerance test (OGTT), D-glucose (2 g/kg; VWR International Ltd. Poole, England) was administered via oral gavage and blood samples were taken from the tail vein. Glucose levels in OGTT were measured using an Accu-chek device and plasma calibrated test strips (Roche Diagnostics GmbH). Insulin levels were determined with an

ultra-sensitive insulin enzyme-linked immunosorbent assay (ELISA) Kit (Crystal Chem Inc., Downers Grove, IL, USA).

To further investigate insulin sensitivity, quantitative insulin-sensitivity check index (QUICKI) calculations were done:

$1/[\log(I_0)+\log(G_0)]$

where I_0 is the fasting insulin (ng/µl) and G_0 is the fasting glucose (mM). QUICKI has previously been shown to correlate well to insulin sensitivity in humans [156].

Immunohistochemistry
Tissues were immersed overnight in Stefanini's fixative (2% formaldehyde and 2% picric acid in phosphate buffer, pH 7.2) and further rinsed repeatedly in sucrose-enriched (10%) buffer, frozen on dry ice, and stored at -70 °C. Thereafter, 10-µm thick sections were cut in a cryostat and mounted on chrome-alum-coated slides. Indirect immunofluorescence was used to assay the sections for proinsulin and glucagon. Details of the antibodies and methods were described previously [157]. For each hormone, five sections taken at different levels of each pancreas (n=6) were examined.

Tissue analysis
Frozen livers were homogenised in isopropanol (1 ml/50 mg tissue) and incubated at 4ºC for 1 h. The samples were centrifuged at 4ºC for 5 min at 2500 rpm and the triglyceride concentration in the supernatant was measured as described for serum level measurement.

Quantitative expression level analysis
Total RNA from dissected organs was prepared for analysis using TRIzol® Reagent (Invitrogen™, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. In short, tissues were homogenised in Trizol reagent and chloroform (Merck KgaA, Darmstadt, Germany) was added for a three-phase solution, separated by centrifugation. The aqueous phase containing the RNA was kept, and isopropanol was added to precipitate RNA (Merck KgaA). The RNA was pelleted, washed with RNase free ethanol (Merck KgaA) and later dissolved in RNase free H_2O. Concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and an aliquot was loaded on a nuclease free agarose gel to validate RNA quality. To eliminate genomic DNA contamination in the samples, the RNA samples were treated with DNase before cDNA

synthesis using an Ambion DNA-free™ kit (Ambion Inc., Austin, TX, USA). Minus-RT controls were used to check for DNA contamination. cDNA was synthesised using Superscript™ II RNase H- Reverse Transcriptase and random hexamer primers (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After synthesis, the cDNA samples were stored at -20°C until analysis.

mRNA levels were quantified using Taqman® real-time PCR (Roche Molecular Systems, Inc., Branchburg, NJ, USA) with both FAM/TAMRA and VIC/TAMRA labelled fluorescence probes and SYBR® Green PCR fluorescence technology (Applied Biosystems, Warrington, UK). All samples were run in triplicate and normalised using mouse acidic ribosomal phosphoprotein PO (M36B4) as an endogenous control. Primers were optimised and linear amplification was confirmed using cDNA dilution series. In certain studies, pipetting was performed on a Tecan Genesis RSP 200 Robot (Tecan, San Jose, CA, USA) and analysed in an ABI 7900 HT (Applied Biosystems). Non-template controls were included on each plate. Data were analysed in SDS 2.1 (ABI Prism, Applied Biosystems). Sequences for the primers and probes used in the Taqman® PCR are reported in the separate articles.

Genome-wide expression analysis (Affymetrix)
RNA preparation and cDNA synthesis was performed as described above. Two duplicate cDNA syntheses were done, followed by cRNA labelling and finally hybridisation against the MG-U74Av2 mouse genome chip from Affymetrix, using protocols supplied by the manufacturer. Washing and staining were done with a Fluidics Station 400 according to the EukGE-WS2 protocol (Affymetrix, Santa Clara, CA, USA). The chips were then directly scanned twice with a GeneArray® scanner.

Signal intensity of the arrays was analysed using the Microarray suite 5.0 from Affymetrix. The intensities were scaled using a target signal factor of 150. The intensity file from each array was then imported into GeneSpring 4.2.1 for further analysis (Silicon Genetics, Redwood City, CA, USA). Initially, genes that were scored as absent on at least 22 of 24 arrays were sorted out. Genes that were significantly different when grouped by genotype were filtered out using a parametric test without the assumption of equality of variance (Welch ANOVA) and a *p*-value cut-off of 0.05. This analysis was followed by multiple testing correction analysis. The Benjamini and Hochberg test was used, which controls the false discovery rate defined as the proportion of genes expected to be identified by chance relative to the total number of genes called significant. Genes with significant changes of expressions between the

transgenic group and the WT group were further filtered using a ratio of ±1.25 (25%) or ±2 (100%) as thresholds. These genes were then sorted according to function, protein family, or metabolic pathway.

Statistical analysis

Values are presented as group mean ±SEM. Multiple comparisons were analysed with the one-way ANOVA followed by Bonferroni's *post hoc* test. Pair-wise comparisons were made using Student's *t*-test. In some experiments, more than two groups were investigated over time. In these cases, a type of two-way ANOVA (a standard repeated measures model) was used with time and group as main effects and with a time and group interaction term included in the model. Group comparisons were made using simultaneous confidence intervals of Tukey type and sometimes *post hoc* Holm-Bonferroni adjustments. The analyses were conducted using the statistical software SAS Proc Mixed (SAS Institute Inc., Cary, NC, USA). The level of significance was set at $p<0.05$.

Summary of results and comments

Article I

Peripheral effects of GH include promoting bone and muscle growth and inducing lipolysis [32]. Although GH is mainly produced in somatotroph cells in the anterior pituitary gland, GH is also produced in various parts of the CNS [64, 65]. GHr is also expressed in several parts of the brain, for example, in the hypothalamus [66, 67]. Thus, GH may exert effects on autonomic complex processes, such as regulation of food and water intake that are controlled in, for example, the hypothalamus. To investigate the CNS effects of GH on body composition and metabolism, transgenic mice that overexpress bovine GH (bGH) controlled by a glia cell specific promoter, glial acid fibrillary protein promoter (GFAP-bGH), were studied.

Both male and female GFAP-bGH mice had higher body weight compared to control mice. A significant difference was observed at age 25 and 60 days for GFAP-bGH transgenic female and male mice, respectively. Similar data were found in another GFAP-bGH founder line. The increase in body weight was correlated to higher fat depots, as analysed by different imaging techniques and dissection. MRI and CT analysis revealed that visceral and subcutaneous fat depots were increased in GFAP-bGH transgenic mice compared to WT controls. Both the absolute and relative weights of the dissected fat depots, including retroperitoneal, reproductive, and BAT, were significantly higher in GFAP-bGH mice. In addition, the weights of different organs, for example, the brain, heart, and liver, differed significantly between transgenic and WT mice, which suggests that CNS overexpression of GH affects the weight of multiple organs. Liver biopsies were analysed for triglyceride content, but no hepatic steatosis was found in the transgenic mice. The major effects of central GH overexpression were the increases in adipose tissue.

Higher body weight and obesity were correlated with increased total and relative food intake. In line with this, ICV administration of bGH to C57bl/6J mice resulted in increased food intake. Thus, GH has an orexigenic effect in the CNS of mice. In accordance with higher food intake, hypothalamic mRNA expression of the orexigenic peptide AgRP was elevated in male and female GFAP-bGH mice, and mRNA levels of NPY were also elevated compared with control mice, but the differences were significant only in female mice. Other neuropeptides involved in food intake regulation, such as MCH, MCHr, MC4-R, and POMC, were unchanged between the mice of different genotypes.

To assess the effects of central GH overexpression in general metabolism, indirect calorimetry was analysed. No changes in resting metabolism, in neither RER nor energy expenditure, were found between GFAP-bGH and WT mice.

Obesity is correlated with worsened insulin sensitivity [2]. The IVGTT was used to measure glucose tolerance in GFAP-bGH transgenic mice. Basal non-fasting serum insulin levels in GFAP-bGH mice were severely increased and further elevated after glucose administration compared to control mice, but glucose levels were normal in the transgenic mice. To further investigate insulin biology, the morphology of the islets of Langerhans was analysed. This analysis revealed marked islet hyperplasia and α-cell disorganisation in the GFAP-bGH mice.

Serum analyses of endocrine parameters, metabolites, and lipids were performed. Serum levels of total cholesterol were higher and levels of total triglycerides lower in GFAP-bGH compared to WT mice. In a cholesterol lipoprotein profile, small LDL/large HDL fractions were higher for the transgenic mice. Serum levels of apoB were lower, whereas hepatic levels of LDL receptor did not differed significantly between transgenic and WT mice, suggesting higher HDL in the blood samples of GFAP-bGH mice. Thus, lower serum apoB might not be due to higher LDL receptor levels. Leptin has previously been found to be correlated with increased fat mass [12, 13], and we found leptin was higher in GFAP-bGH than in WT mice.
A gene expression analysis further showed that central bGH overexpression affected expression of several hepatic signals involved in metabolism. The level of GHr expression was increased whereas IGF-1 levels were not significantly different.

In conclusion, mice that overexpress bGH under control of the GFAP promoter have higher body weight; are obese, hyperphagic, hyperinsulinemic, and hyperleptinemic; and have several alterations in expression levels of signals regulating food intake and metabolism. Thus, GH actions in the periphery differ from GH actions in the CNS.

While ghrelin is known to increase food intake and promote obesity, it also strongly stimulates GH secretion, which has lipolytic effects in the periphery. It might be speculated that central GH is involved in ghrelin-mediated hyperphagia and obesity. Thus, central ghrelin treatment of a GH or GHr deficient model could be used to study ghrelin involvement in GH-mediated obesity.

Article II

To further investigate the findings in article I – that GH is important in the CNS for food intake, obesity, and regulation of metabolism – mice deficient in the gene encoding GHr (GHr KO) were treated with ghrelin by ICV administration. GHr KO mice were previously found to be growth retarded and have decreased body weight and body length [158, 159]. In addition, GHr KO mice have increased serum levels of GH; decreased serum levels of IGF-1, glucose, and insulin [158, 160]; and unchanged levels of leptin [161].

The peptide hormone ghrelin is mainly produced by endocrine cells in the stomach [61, 162]. Ghrelin receptor (GHSr) is highly expressed in the hypothalamus and less so in somatotroph cells in the pituitary [163, 164]. Peripherally, GHSr expression has been detected in adipose tissue [61, 165]. Ghrelin has a strong GH-releasing effect [61], and central and peripheral ghrelin administration induce body weight gain and adiposity by increasing food intake in rodents [166-168].

GHr KO mice with and without central ghrelin administration were examined to study the importance of functional GH signalling for the acute effects of ghrelin and for the regulation of body composition and lipoproteins.

An ICV injection of ghrelin was found to stimulate food intake in fasted and non-fasted WT mice, but this effect was blunted in GHr KO mice. GHr, and thus functional GH signalling is required for ghrelin's acute effect on food intake. In line with this, ghrelin treatment increased the hypothalamic expression level of orexigenic AgRP in WT mice but not in GHr KO mice. A similar tendency was found for hypothalamic NPY expression, but the difference was non-significant. When no ghrelin was administered, relative food intake was higher in GHr KO than in WT mice. Expression of other hypothalamic food-intake signals, for example, of POMC, CART, Orexin, MCH, and GHSr, were measured, but no differences between GHr KO and WT mice and between ghrelin-treated GHr KO and WT mice were found.

The GHr KO mice in this study had decreased body weight and body length. In a body composition analysis, GHr KO mice had 43% reduction in relative lean mass and a 2.4-fold increase in fat percentage compared with WT mice. Bone content was also reduced in GHr KO mice. Relative weights of the brain, retroperitoneal adipose tissue, and BAT were disproportionately larger in GHr KO mice compared with WT controls.

In line with increased fat pads, we found that serum leptin levels were dramatically increased 4.8 fold.

Lower serum levels of glucose and insulin and increased serum levels of corticosterone were found in GHr KO mice. Glucocorticoids alter carbohydrate metabolism, increase gluconeogenesis, and reduce insulin sensitivity [169], but despite increased corticosterone levels and obesity, GHr KO mice are insulin sensitive. Both GH and glucocorticoids reduce peripheral glucose consumption, which increases blood glucose. Lack of GH signalling may thus alter this function, but several other factors affect that blood glucose, for example, glucagon and adrenaline, were not investigated in this study.

Serum levels of total cholesterol, triglycerides, and apoB were lower in GHr KO than in WT mice, and a cholesterol lipoprotein analysis revealed that mainly LDL and HDL fractions were lower in GHr KO. Lower levels of lipid particles with apoB were found in obese GHr-deficient mice.

In conclusion, GHr is important for the stimulatory effect of ghrelin on feeding and the hypothalamic expression of first-order orexigenic signals. GHr KO mice are growth retarded but obese with higher serum leptin levels and a short-term, 3-hour higher food intake than WT mice. Interestingly, low serum insulin and glucose and high corticosterone levels were found in GHr KO mice. Moreover, cholesterol and especially LDL and HDL were lower in obese GHr KO mice. Thus, obesity caused by GHr deficiency does not lead to insulin insensitivity or hyperlipidemia.

Measurements in articles I and II detected no changes in the orexigenic peptide MCH as a consequence of central GH overexpression, GHr deficiency, or central ghrelin treatment. Nevertheless, MCHr expression in several nuclei of the hypothalamus and in the pituitary gland may indicate MCHr involvement in ghrelin-mediated feeding, GH-mediated feeding, or GH expression. A study of the effects of central ghrelin in a model of impaired MCH signalling could reveal the involvement of MCH and MCHr in ghrelin-mediated feeding or GH regulation.

Article III
In article II, it was found that GHr is important for the acute effects of ghrelin on food intake. In this third article, the importance of MCHr for the acute effects of ghrelin on food intake, endocrine regulation, and GH expression was investigated. This was done by examining MCHr KO mice given ICV administration of ghrelin.

MCH affects energy balance regulation by increasing food intake and body weight and inducing obesity (reviewed in [104]). The expression of MCHr in the hypothalamus and pituitary gland also suggests that the MCH/MCHr system is involved in GH regulation [118, 170].

Both MCHr KO and WT mice had increased acute food intake after ghrelin administration. Thus, MCHr may not be essential for ghrelin effects on food intake. To explore the importance of MCHr for the effects of ghrelin on GH expression, GH mRNA levels in the pituitary gland were measured. As expected, WT mice had a marked 2.6-fold increase in GH mRNA after ghrelin administration, but no such increase was found in MCHr KO mice. Thus, MCHr may indeed be important for ghrelin-mediated GH expression. A recent article confirms that MCH can stimulate GH secretion [133], which is in line with our data. Furthermore, expression level analysis was done to measure any changes in hypothalamic MCH, MCHr, GHSr, and GHr, but no changes due to ghrelin injection or genotype were found. Thus, changes in GHSr cannot explain the difference in response after ghrelin injection between MCHr KO and WT mice. No MCHr expression was found in MCHr KO mice, as expected. ICV delivery of ghrelin increased circulating insulin levels only in WT mice; MCHr KO mice did not respond with any change in serum insulin. Ghrelin treatment did not affect leptin levels in any genotype, but MCHr KO mice had lower leptin levels than WT mice, in line with previous findings [125]. Serum IGF-1 levels were also lower in MCHr KO than in WT mice but unaffected by ghrelin treatment. But ghrelin stimulated corticosterone in both MCHr KO mice and WT controls. ICV administration of ghrelin led to higher circulating ghrelin levels 30 minutes after injection. To investigate whether central pathways caused these effects on insulin and corticosterone, peripheral ghrelin was administered via IP injection in doses known to affect food intake [171]. But no effects on these circulating hormones due to peripheral ghrelin were observed. Thus, MCHr is important for the CNS effects of ghrelin on serum insulin levels but not for the CNS effects of ghrelin on corticosterone levels, and MCHr KO mice have lower serum leptin and IGF-1 levels than WT mice.

In conclusion, MCHr is not important for the acute effects of ghrelin on food intake, but it is important for the stimulatory effects of ghrelin on GH expression in the pituitary gland. ICV effects of ghrelin on serum insulin levels require MCHr. In addition, MCHr KO mice have lower IGF-1 in serum than WT mice, which may be due to decreased fat depots, a finding also supported by lower levels of leptin.

Leptin and MCH interact, possibly via common pathways, to affect energy homeostasis [116, 119, 135, 149, 150]. Low leptin levels promote feeding; MCHr KO mice have low leptin levels and, surprisingly, higher food intake [125, 146]. This is surprising since MCH is orexigenic and impaired MCH signalling should reduce food intake. And, despite higher food intake, MCHr KO mice have low body weight and fat mass [125, 146]. Moreover, leptin deficiency leads to hyperinsulinemia [172] while MCHr deficiency leads to lower insulin levels [146]. Studies of a model lacking MCHr and leptin could possibly explain the importance of these systems regarding food intake, body weight, and metabolism.

Article IV

In article III, it was found that MCHr is important for ghrelin-induced GH expression and insulin release. MCHr deficiency results in low body weight and lower body fat, despite increased food intake, possibly because of increased locomotor activity and energy expenditure [125, 146]. Leptin deficiency results in higher food intake and lower locomotor activity and energy expenditure [150, 172]. MCHr KO mice also have lower serum levels of leptin and insulin [125, 146]. Several reports suggest that the MCH/MCHr system is involved in insulin biology. These includes findings that MCH is present in autonomic neurons which project to and control the pancreas [128], that MCHr are found in insulin-producing cells in the pancreas and MCH can stimulate insulin secretion [117], and that MCH injection in rats worsens insulin sensitivity independent of body weight [173].

In article IV, the aim was to investigate the role of MCHr in the obese leptin-deficient mouse model. This was done by studying MCHr KO ob/ob double knockout mice regarding body weight, food intake, body composition, and glucose tolerance.

MCHr KO ob/ob mice and ob/ob mice had similar body weight development and significantly higher body weight after 6 weeks compared to WT mice. Yet at age 30 weeks, there was no difference in body weight between MCHr KO ob/ob and ob/ob mice; average body weight of both genotypes was over 60 grams. No differences in food intake between MCHr KO ob/ob and ob/ob mice were found, but food intake in both groups was higher than in WT mice. Faeces energy was analysed for differences in intestinal nutritional uptake, but no differences between the genotypes studied were found. A body composition analysis revealed slightly but significantly lower body fat and higher lean mass in MCHr KO ob/ob mice compared to ob/ob mice, but the double knockout mice had markedly higher fat mass compared to WT mice. Both MCHr

KO ob/ob and ob/ob mice had higher total cholesterol, compared to WT mice, but it was mainly the LDL and HDL fractions that were increased. Both obese mice models had hepatic steatosis and increased hepatic expression of stearoyl-CoA desaturase-1 (SCD-1), a key enzyme in the biosynthesis of monounsaturated fatty acids that is upregulated in ob/ob mice [174]. Indirect calorimetric measurements revealed similar RER and energy expenditure (EE) in MCHr KO ob/ob and ob/ob mice, and both groups had higher RER and lower EE that WT mice. But locomotor activity in MCHr KO ob/ob mice was higher than in ob/ob mice. In a cold exposure experiment, cold tolerance in MCHr KO ob/ob mice was found to be greater than in ob/ob mice and worse than in WT mice. The obese models also had lower BAT expression of UCP-1, but expression was significantly lower only for ob/ob mice compared to WT mice. Interestingly, a glucose tolerance test revealed normal glucose clearance and 50% reduction in fasting insulin and insulin response in the MCHr KO ob/ob mice compared to ob/ob controls, and a QUICKI calculation indicated that insulin sensitivity in MCHr KO ob/ob mice was improved compared with the ob/ob controls. In line with MCHr involvement in insulin sensitivity, MCHr KO mice had lower glucose and insulin responses after glucose administration compared to WT mice. Glucocorticoids affect glucose homeostasis by, for example, lowering peripheral insulin sensitivity [169]. In line with improved glucose tolerance, MCHr KO ob/ob mice had over 40% lower serum corticosterone levels compared with ob/ob controls.

In conclusion, MCHr KO mice on an ob/ob background have similar body weight, food intake, EE, RER, and blood lipid profile compared to ob/ob mice. A slight increase in lean mass and decrease in fat mass was observed in MCHr KO ob/ob animals, which were also hyperactive, compared with ob/ob controls. Interestingly, although similar in body weight, MCHr-deficient ob/ob mice had significantly higher glucose tolerance than ob/ob controls, which may suggest higher insulin sensitivity. Thus, MCHr deficiency on an ob/ob background does not result in changed body weight but may be important for peripheral insulin sensitivity and increased locomotor activity in the obese state.

General discussion

This thesis comprises a series of investigations on changes in the energy balance and metabolism of genetically modified mice with altered regulation and signalling in the GH and MCH systems. Both systems are highly involved in the regulation of energy homeostasis parameters and are major actors in the regulation of autonomic functions. Findings in our studies and in the literature support that the GH and MCH systems are interconnected and that they affect food intake, body weight, obesity, and several other metabolic processes. The limbic system, the thalamus, and the hypothalamus is a major control unit of autonomic processes in the body. One of the autonomic processes is the regulation of energy homeostasis, which includes the essential functions of food (energy) intake, energy expenditure, and energy storage. The hypothalamus seems to be the major site of action for these processes. All articles in this thesis focus upon this central regulation and its impact on the periphery regarding metabolism.

GH in the central nervous system

GH is a well-defined anabolic signal, which promotes muscle and bone growth at the cost of increased lipolysis. GH effects occur in several peripheral tissues that express GHr or are carried out via IGF-1. Outside of main expression in the pituitary gland, GH and GHr are expressed in the limbic system, including the hypothalamus [64-67]. Although this central GH expression may be lower than in the pituitary, the importance and significance of central GH is not fully understood.

In article I, we found that central overexpression of bGH in transgenic mice (GFAP-bGH) caused the mice to develop higher body weight and fat mass. GFAP-bGH mice were hyperphagic and had higher levels of orexigenic AgRP signal expression in the hypothalamus. ICV administration of bGH to normal mice led to acutely higher food intake. Mice with general GH overexpression (MT-bGH) have higher body weight but unchanged relative energy intake and increased energy intake on a high-fat diet [175]. MT-bGH mice have lower body fat in accordance with the lipolytic effects of GH [78]. This contrasts with what was found in GFAP-bGH mice, which were severely obese and had higher subcutaneous, visceral, and brown fat depots and higher leptin levels. In addition, GFAP-bGH mice had lower serum triglyceride levels, hypercholesterolemia, and increased fractions of “small LDL/large HDL” lipoproteins. Interestingly, MT-bGH mice also have lower serum triglyceride, higher total cholesterol, and higher LDL/HDL [78]. GH is known to stimulate insulin secretion, and MT-bGH mice have higher

circulating insulin, which leads to lower blood glucose [78]. Leanness in MT-bGH mice is accompanied by increased energy expenditure, higher body temperature, and a higher RER, which indicates that lack of lipids for oxidation results in greater carbohydrate oxidation [175]. Obesity in GFAP-bGH mice may be caused by increased food intake since energy expenditure was unchanged. Although more lipids were available, no effect on RER was observed in GFAP-bGH mice. These results indicate that overexpression of GH in the CNS leads to hyperphagia-induced obesity. Thus, central GH may exclusively affect autonomic regulation of food intake. The orexigenic peptides AgRP and NPY were increased, which indicates involvement of CNS GH in the regulation of first-order neurons in food-intake regulation. Obesity is known to worsen insulin sensitivity and eventually lead to type 2 diabetes [2]. GFAP-bGH mice had markedly higher insulin levels, greater amounts of insulin-producing cells, and normal glucose levels compared with WT mice. The most probable explanation for this is the hyperphagia-induced obesity that may reduce insulin sensitivity and increase insulin production and secretion. GH stimulates insulin secretion; thus, higher levels of circulating GH or other signals that stimulate insulin, for example, corticosterone, may increase insulin levels, but this was not investigated in this thesis. A previous study found that GFAP-bGH mice have increased corticosterone levels [176]. Since GH is active in the CNS, GH may also be active in the autonomic (parasympathetic) control of insulin secretion. But the pancreas in embryos (E19.5) from GFAP-bGH mice developed normally regarding size of endocrine tissue and proinsulin- and glucagon-producing cells. Thus, increased insulin levels and increases in insulin-producing cells are a postnatal effect of GH overexpression in the CNS. It is interesting to note that MT-bGH mice have higher insulin levels, which lead to lower glucose, but not increased lipogenesis whereas GFAP-bGH mice have higher insulin, normal glucose, and massive lipogenesis. Further investigations are needed to determine whether one role of GH in the CNS is to modify insulin secretion and whether increased insulin in GFAP-bGH mice also contributes to the obesity observed.

The reason for obesity is not lower locomotor activity since a previous report found no changes between GFAP-bGH and WT mice [176]. Interestingly, the typical stimulatory effect of GH on IGF-1 is not present in GFAP-bGH mice, thus indicating that CNS GH is not involved in this regulation and the metabolic effects observed in GFAP-bGH mice are not due to changes in IGF-1.

Abolished GH signalling, body lipid biology

GH is known to signal via the GHr, a receptor found both in the CNS and in several tissues in the periphery [66, 67]. In article II, mice lacking GHr were found to be growth retarded in accordance with previous literature [159, 177, 178]. Since the effect of GH is abolished, these mice have lower lean mass (related to body length) and higher fat mass (percentage of body), besides higher leptin levels. In article II, it was found that acute food intake is increased in GHr KO mice, which is supported by previous findings [179]. Interestingly, this is fairly similar to the data for GFAP-bGH mice, higher food intake and obesity. But GFAP-bGH mice are not growth retarded. In addition, GHr KO mice have lower serum triglyceride and cholesterol levels, lower LDL/HDL levels, and lower apoB-containing lipid particles. apoB-containing lipids are in general considered to be atherogenic. Obese GHr KO mice are insulin sensitive, as evidenced by lower insulin levels and blood glucose. GH is known to stimulate insulin secretion and at the same time be diabetogenic, reducing insulin sensitivity. Thus, abolished GH signalling leads to lower insulin secretion and increased insulin sensitivity. As in GFAP-bGH mice, high corticosterone levels were found in GHr KO mice. This may be a compensatory mechanism to correct for lower insulin secretion, but the higher corticosterone levels do not seem to increase insulin secretion in GHr KO mice. Although obese, the mice had lower circulating lipid levels, which may affect insulin sensitivity. Further studies to measure serum levels of ffa may explain this finding. Glucocorticoids are known to stimulate gluconeogenesis, decrease glucose use, and increase circulating glucose. Also, corticosterone promotes ffa mobilisation from adipose tissue. None of these effects are observed in the GHr KO mice model, which indicates that functional GH signalling may affect certain glucocorticoid functions. Functional GHr is thus essential for several bodily functions, including normal growth, body composition, blood lipid, and hormonal balance.

Abolished GH signalling and ghrelin-mediated feeding

The peptide hormone ghrelin is a potent GH secretagogue and increases food intake by acting in the hypothalamus [61, 167]. In article II, it was found that functional GHr is important for the effects of ghrelin since the acute effect of ICV ghrelin on food intake was blunted in GHr KO mice. In line with this, levels of the first-order orexigenic neuropeptides AgRP and NPY were not increased as expected in GHr KO mice. Combining findings in articles I and II support that GH in the CNS increases food intake and that ghrelin may mediate this. A brain-specific GH KO or GHr KO mouse model could be used to further study the interaction of brain GH and ghrelin.

MCHr deficiency and ghrelin effects on food intake and GH expression

MCH, GH, and ghrelin are orexigenic peptides that act in various hypothalamic nuclei. These peptides may be interconnected, not only by the adjacent expression pattern but also since they exert effects on food intake and metabolism. The stimulatory effects of ghrelin on GH secretion and food intake are well established [61, 167]. But whether and to what extent MCHr is involved in the ghrelin-mediated effects was not known. MCHr is expressed in several of the hypothalamic nuclei involved in the regulation of food intake and in the pituitary gland, which releases GH [110, 118]. Ghrelin receptor (GHSr) is also highly expressed in the hypothalamus and pituitary [23]. But it is suggested that ghrelin-mediated food intake involves AgRP/NPY and orexin, not MCH [180, 181]. Our data are in line with the literature, since we found that ghrelin-induced food intake does not require functional MCHr. In addition, no changes in hypothalamic mRNA levels of MCH were found after ghrelin injection. Nevertheless, the strong ghrelin-induced pituitary expression of GH in WT mice was completely abolished in MCHr KO mice, which suggests that MCHr is involved in ghrelin-mediated GH expression. It is not known how MCHr deficiency causes insensitivity of ghrelin on GH expression. A difference in GHSr in the hypothalamus or pituitary gland would not explain this; neither ghrelin administration nor MCHr deficiency caused changes in GHSr expression. Since hypothalamic MCH expression was not affected by ghrelin injection, ghrelin might not act to increase GH expression directly via the MCH route. Ghrelin is known to release GH via dual mechanisms involving both the hypothalamus and pituitary gland. Direct effects on somatotroph cells occur via the GHSr, whereas ghrelin also alters GH release via GHRH (and maybe also somatostatin) in the hypothalamus (reviewed in [23]). It may thus be suggested that centrally injected ghrelin primarily alters GHRH (and somatostatin) to influence GH expression and secretion, at least according to the acute 30-minute experimental protocol used in our study. In that case, MCHr deficiency may be involved in the GHRH/SS regulation. It would be interesting to measure GHRH and SS in the MCHr deficient mice, with and without ghrelin administration.

As concluded in articles I and II, GH mediates food intake in the CNS via NPY/AgRP and GHr is required for ghrelin's stimulatory effects on food intake. In article III, we showed that MCHr is required for ghrelin's effect on pituitary GH expression but not for ghrelin's effect on food intake. Thus, the blunted pituitary GH expression in MCHr KO mice is not important for the food intake properties of GH in the CNS. Further studies on CNS expression of GH by ghrelin would be interesting to

support that ghrelin-mediated feeding occurs, at least partly, via GH and the GHr.

Ghrelin effects on circulating hormones in MCHr-deficient mice

Ghrelin is known to influence several different endocrine systems, both directly and through its actions on GH secretion, feeding, and induction of obesity. Interestingly, data from ghrelin KO mice suggest alternative pathways that could compensate for lack of ghrelin, since the mice have normal food intake, body weight, and body composition [182]. GHSr KO mice also have normal food intake and body composition but slightly reduced adult body weight [183]. But GHSr KO and ghrelin KO mice have reduced IGF-1 levels, thus these mice lines seem to have affected GH-IGF-1 axes. Although insulin and leptin levels in the ghrelin/GHS-r KO mouse models are normal, long-term ghrelin treatment stimulates adiposity and leptin levels [184]; depending on experimental conditions, ghrelin is reported to have both stimulatory and inhibitory effects on insulin secretion [185], but IV administration of ghrelin led to increased insulin secretion [186]. Ghrelin also has a well-documented positive effect on the HPA axis [187]. We found that ICV ghrelin had an acute stimulatory effect on insulin and corticosterone secretion, whereas IGF-1 and leptin levels were unchanged. When administered to MCHr KO mice, ghrelin stimulated corticosterone but not insulin, which suggests that MCHr is important for ghrelin's CNS-stimulatory effects on insulin secretion. Interestingly, MCH and MCHr are known to alter insulin regulation since MCHr KO mice have lower insulin levels [146], genetic MCH overexpression results in obesity and hyperinsulinemia [142], MCHr are expressed in insulin-producing cells, and MCH can stimulate insulin [117]. Autonomic nerves that innervate and control pancreas functions contain MCH, and both GHSr and MCHr are present in nodose ganglia (vagus), where about 75% of GHSr-expressing cells also express MCHr [128, 188]. We also injected ghrelin peripherally, which had no effect on insulin levels. Thus MCHr may be essential for the CNS and neuronal stimulatory effect of ghrelin on insulin. It is interesting that ghrelin, the "adiposity hormone", increases the levels of such functionally opposing peptides like insulin and corticosterone (and GH). In humans, both GH and cortisol stimulate insulin secretion while simultaneously increasing blood glucose and promoting lipolysis. Insulin lowers blood glucose and increases lipogenesis. Ghrelin may act to acutely increase food intake and energy storage (insulin), but it also promotes lower glucose consumption (GH, cortisol), which in an evolutionary perspective may be advantageous. These endocrine signals have opposing effects and thus, a fine balance may exist for short-term "energy absorptive" phases that involve several players with different half-lives and potencies.

MCHr deficiency and circulating hormones

The leaner phenotype of MCHr KO mice was accompanied by lower leptin levels [125, 146]. Interestingly, significantly lower IGF-1 levels were found in MCHr KO mice, but no differences in hepatic IGF-1 mRNA expression was found between MCHr KO and WT mice. Lower serum IGF-1 levels could be a result of lower adipose tissue mass since adipose tissue is the second largest producer of IGF-1 [189, 190]. In a glucose tolerance test, we found that fasting insulin was lower in MCHr KO mice and that insulin levels after glucose administration were lower over 60 minutes. Glucose levels were normal or, if anything, tended to be lower. Thus, MCHr deficiency may lead to improved insulin sensitivity since lower levels of insulin are required to maintain normal glucose homeostasis. IGF-1 is known to function as an insulin receptor agonist, having some 100x lower affinity to the receptor. But serum IGF-1 levels are about 100x higher compared to insulin, suggesting that IGF-1 exerts some "insulin effects" via the insulin receptor. Lower insulin and IGF-1 levels in MCHr KO mice may indicate a low anabolic state. It would be interesting to measure insulin receptor expression or expression levels of other related receptors.

MCHr in leptin deficiency

MCH and leptin interact to regulate food intake and energy balance. Leptin deficiency upregulates MCH and MCHr while leptin administration decreases the expression of MCH and MCHr [119, 135]. Adipocytes, which produce leptin, express MCHr, and MCH stimulates leptin secretion in isolated adipocytes [116]. Leptin reduces feeding by activating the anorectic pathway in the hypothalamus (POMC/CART neurons) [191, 192] and deactivating orexigenic pathways (NPY/AgRP neurons) [193, 194]. In contrast, MCH promotes eating and obesity [139-142]. Hyperphagia and reduced energy expenditure make ob/ob mice obese [172], whereas MCHr KO mice have lower body weight and fat mass despite hyperphagia due to higher energy expenditure [123, 125, 146]. The phenotype of ob/ob mice that lack MCH [149, 150] includes lower body weight and body fat and reduced hepatic SCD-1 expression, but different results concerning the development of hepatic steatosis have been found. In addition, higher energy expenditure and locomotor activity and altered thermoregulation have also been found in MCH KO ob/ob mice [150]. Interestingly, lower glucose levels and unchanged insulin levels have been found in MCH KO ob/ob mice compared with ob/ob mice.

Since MCH and MCHr KO mice differ, we aimed to explain the role of MCHr in ob/ob mice by creating a double MCHr KO ob/ob mouse line. Interestingly, MCHr is not important for the higher body weight and food intake observed in ob/ob mice. In addition, the lower energy expenditure and higher RER and blood lipid levels typically observed in ob/ob mice are not affected by MCHr deficiency. But lack of MCHr in this obese ob/ob mouse model resulted in markedly lower fasting insulin, lower insulin response, and normal glucose clearance. A QUICKI, previously shown to correlate with insulin sensitivity [156], was determined and was higher for MCHr KO ob/ob mice than ob/ob mice, which suggests improved insulin sensitivity. MCHr KO ob/ob mice need only about 50% of the insulin that ob/ob mice need to maintain normal glucose levels. The molecular explanation for this improved sensitivity to insulin due to MCHr deficiency has not been determined.

Firstly, MCHr is expressed in skeletal muscle and adipose tissue, which are tissues expressing the insulin sensitive GLUT4 transporter. Secondly, insulin sensitivity can be improved by exercise in humans [195], and MCHr KO mice (both on normal and ob/ob background) are hyperactive. Thirdly, corticosterone is involved in glucoregulation by, for instance, reducing insulin sensitivity [169], and MCHr KO ob/ob mice had a reduction of 40% in serum corticosterone compared to ob/ob mice. Finally, translocation of GLUT4 to the cell surface and glucose uptake are affected by ffa, and MCHr KO ob/ob mice had lower serum ffa than ob/ob mice ($p<0.05$, Student's *t*-test).

Taken together, the lower insulin levels and suggested improvement in insulin sensitivity may be a result of several parameters that result from MCHr deficiency. Further experiments can investigate whether MCHr is involved in intracellular mechanisms involved in insulin biology, for example, which affect GLUT4 translocation. Alternatively, lack of MCHr in obesity may cause a multiple cascade of events that results in this improved insulin sensitive phenotype.

Concluding remarks

This thesis is based on studies showing that peptides in the CNS play a crucial role in the regulation of the energy balance, including energy intake and metabolism. The hypothalamus is a key structure for this regulation and expresses GH, GHr, MCH, and MCHr. These two systems may play different roles in the periphery but are both orexigenic signals in the CNS and are not separate since ghrelin was found to be one signal that links MCH and GH. In the CNS, GH signals increases in food intake, body weight, and obesity, and that signalling may involve and be

mediated by ghrelin. Indeed, functional GH signalling via the GHr is required for ghrelin's CNS effects on food intake. Central GH overexpression or ICV ghrelin injection did not alter MCH or MCHr, and lack of GHr had no effect on MCH expression. Nevertheless, MCHr is involved in ghrelin's effect on GH expression.

GH and MCH actions in the CNS lead to changes in the periphery, including regulation of blood lipids, expression levels of key peptides, and endocrine balances (e.g. leptin, insulin, corticosterone). These peptides may act via autonomic signalling to the periphery. GH acts as a peripheral endocrine signal, but receptors for MCH are expressed in peripheral tissue and MCH is found in serum. GH is known to stimulate insulin and GH overexpression in the CNS results in higher serum insulin levels, which may be a result of reduced insulin sensitivity due to hyperphagia-induced obesity. Abolished GH signalling also reduces insulin levels and, if anything, improves insulin sensitivity. Interestingly, MCH is also involved in insulin regulation, and MCHr deficiency may have a positive effect on insulin sensitivity.

The data in this thesis may be a foundation for further knowledge on the control and regulation of energy homeostasis. In the future, these systems may be potent therapeutic targets in the treatment of disorders caused by obesity.

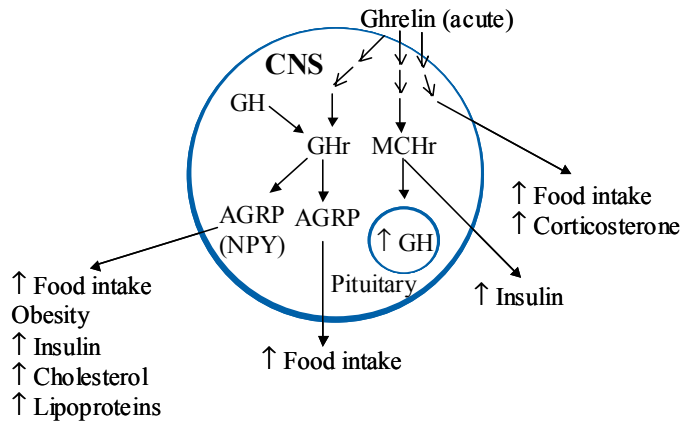

Figure 4. Signalling pathways in the central nervous system (CNS) and pituitary gland and physiological changes in the body based on findings in this thesis (GH = growth hormone, GHr = growth hormone receptor, AGRP = agouti-related protein, NPY = neuropeptide Y, and MCHr = melanin-concentrating hormone receptor).

Summary and conclusion

This thesis comprises four studies that investigated the role of GH and MCHr in metabolism. In short, major findings indicate the following:

- GH overexpression in mouse CNS causes hyperphagia-induced body weight gain and obesity. This is accompanied by higher cholesterol, with a marked increase in LDL/HDL lipoproteins, lower insulin sensitivity (since higher insulin levels are required for normal glucose levels), and islet hyperplasia.

- GHr is required for ghrelin's acute effect on increased food intake. Thus, ghrelin signalling that promotes feeding involves GH signalling and GHr.

- GHr is required for normal growth, body composition, carbohydrate metabolism, and blood lipids, according to findings in GHr KO mice. Compared with WT mice, GHr KO mice had lower body weight and body length, lower lean mass, and higher fat mass. In addition, they had lower serum glucose, lower insulin levels, and higher corticosterone levels. Regarding the blood lipids, lower total cholesterol, lower serum apoB levels, and LDL and HDL lipoprotein particles were found.

- MCHr is not essential for mediating the stimulatory effects of ghrelin on food intake but it is involved in ghrelin-stimulated GH expression in the pituitary.

- ICV administration of ghrelin does not acutely affect serum IGF-1 levels, but MCHr KO mice have lower serum IGF-1, perhaps due to lower amounts of adipose tissue. ICV administration of ghrelin acutely increases serum insulin levels in WT mice, but this effect is blunted in MCHr KO mice.

- MCHr is not important for the effects of leptin deficiency on body weight, food intake, energy expenditure and RER, blood cholesterol, and lipoprotein levels. Loss of MCHr in ob/ob mice results in normal glucose regulation in combination with a marked reduction in serum insulin levels, reduced corticosterone, and a tendency toward lower ffa levels, higher locomotor activity, and improved cold tolerance.

Acknowledgements

I would like to express my deepest gratitude to all the kind people that helped, assisted, and guided me throughout my Ph.D. studies. I would especially like to recognize the following individuals:

My two supervisors, **Mohammad Bohlooly** and **Jan Törnell**.
Mohammad has been extremely engaged and has put much time, effort and energy into all of my projects during my time as a Ph.D. student. His enthusiasm, knowledge, and positive attitude have no limits and he has been inspiring and helpful in all possible ways.
Jan has been very supportive and has given me a complete freedom to choose and perform projects of all kinds. His encouragement for me to attend classes and conferences, his great generosity of all kinds and his great scientific knowledge, all scientific advice and good criticism have been much appreciated and invaluable.

During my time as a Ph.D. student, I have been in daily contact with the wonderful people of ATCG at AstraZeneca. **Everyone there** has helped me a lot, and I would never ever have been able to learn and do so much without You all!!
When arriving at AstraZeneca, **Michael Snaith** was the person who introduced me to molecular biology. During my time at ATCG, he has spent a significant amount of time with me to explain and discuss science and has never hesitated to help, which is much appreciated.
I greatly appreciate the close contact, the nice and supportive discussions during dissections, and the extreme amount of help from the members of the phenotyping group; **Anna-Karin Gerdin** and **Marie Jönsson** (and Mohammad "utan resurser"). **Vivien Hennig** has been very helpful, among others with administrative matters and **Meint Schuelke** has helped a lot with all kinds of bio informatic issues. **Johan Johansson** and all the wonderful people at the animal facilities are much acknowledged for their assistance, always being very helpful and nothing is ever a problem. **Johan Jirholt** has been very enthusiastic and helpful with all kinds of discussions regarding among others genetics, science and cars.

I have had the pleasure of collaborating with several people at other departments within AstraZeneca. **Jan Oscarsson** has been extremely helpful and with his enormous knowledge, he has helped with scientific guidance of all kinds. I am also very greatful for the opportunity to collaborate – and I have had a great deal of fun during dissections (pre-glucose treatment, bocka av vävnader…) – with among others, **Daniel Lindén**, **Andréa Ahnmark** and **Lena William-Olsson**. Also, **David**

Morgan, **Christopher Lelliott**, **Karolina Ploj** and **Åke Jägerwall**, (to mention a few) have been very supportive and fun as colleagues. The "**Blod-gruppen**" and **Lennart Svensson** have given me much help and support in performing experiments, interpreting results and discussing science, which is very much appreciated. The animal personnel of LAR and **Liselotte Hallengren** have been generous in letting me have animals in their facilities. The department of Biostatistics has been very supportive regarding mathematical and statistical matters.

I have not spent very much time doing research at Göteborg University. Nevertheless, I have met several nice people at the section for endocrinology, with whom I have had much fun during courses and conferences. My scientific buddy at the university is **Emil Egecioglu**, whom I have had lots of fun with and has helped me much. Both **Håkan Billig** and **Lena Olofsson**, have been very helpful with administrative matters. Also, **John-Olof Jansson**, **Suzanne Dickson**, **Göran Bergström** and **Erik Hanse** have been important contacts at the university.

I would also like to acknowledge Gail Conrad-List for fast and excellent proof reading of the thesis.

All friends outside science – musicians, party friends, windsurfing buddies – are very much appreciated for letting me out from science every now and then!!

A sincere and deep thank you to my wonderful family; my mother **Annika**, my father **Gunnar**, my sister **Cecilia** – together with **Amanda** and **Samuel** – and my brother **Magnus** and his sambo **Anna**!! You mean so much to me and your support, help and generosity is invaluable.

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