INTERACTIONS OF THE GROWTH HORMONE SECRETORY AXIS AND THE CENTRAL MELANOCORTIN SYSTEM

By

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This document is dedicated to my wonderful family.

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Ghrelin acts on the growth hormone secretagogue receptor (GHS-R) in the hypothalamus and pituitary to potently stimulate growth hormone (GH) secretion. Ghrelin also stimulates food intake and affects energy balance in part by activating neuropeptide Y (NPY) neurons containing the melanocortin receptor antagonist agouti related peptide (AGRP) in the arcuate nucleus. We therefore sought to further investigate the role of AGRP/melanocortin signaling in mediating the orexigenic effects of ghrelin by studying mice deficient in the melanocortin 3 (MC3R) and 4 (MC4R) receptors. We also sought to determine whether reduced ghrelin levels and/or an altered sensitivity to the GH-stimulating effects of ghrelin accompany the obesity syndromes of MC3R knockout (MC3R KO) and MC4R knockout (MC4R KO) mice. We measured various components of the GH axis in several age groups of male and female MC3R KO, MC4R KO and WT mice. Overall, MC4R KO mice displayed normal to elevated levels of the GH effector protein, insulin-like growth factor-I (IGF-I), in spite of reduced serum GH

levels. In addition, pituitary GH content was reduced in some age groups in both MC3R KOs and MC4R KOs however; no clear cut trend was established. The effects of peripheral ghrelin on food intake were reduced in both male and female MC3R KO and MC4R KO mice compared to wild-type controls. In addition, circulating ghrelin levels were reduced in MC4R KO mice, with significance observed in females. Female MC3R KO and MC4R KO mice exhibited a diminished responsiveness to the GH-releasing effects of peripheral ghrelin, and these effects were age-dependent. Our data indicate that deletion of the MC3R or MC4R results in a decreased sensitivity to ghrelin and verifies the involvement in the melanocortin system in ghrelin-induced food intake.

CHAPTER 1
LITERATURE REVIEW

Introduction

The prevalence of obesity has increased to epidemic proportions over the last 20 years. It is now estimated that approximately two-thirds of U.S. adults are either overweight or obese. Obesity is also a problem in many other countries with around 1.5 billion people worldwide overweight or obese [1]. Some of the factors contributing to this epidemic include the availability of high fat foods, overeating and a decline in physical activity. There is also evidence for genetic factors that may lead to obesity in certain individuals and ethnic groups. Obesity is associated with an increased incidence of many chronic diseases including type II diabetes, cardiovascular disease, stroke, osteoarthritis and some cancers [2] and can significantly decrease life expectancy [3]. In addition, the prevalence of obesity increases with age [4] and the incidence of obesity in children and teenagers is on the rise [5]. Body mass index (BMI) is a measure of obesity that is dependent on the height and weight of an individual. Guidelines presented by the World Health Organization (WHO) state that a BMI of 18.5-24.9 kg/m^2 is normal, a BMI over 25 kg/m^2 is considered overweight, and a BMI greater than 30 kg/m^2 indicates the individual is obese. Data from the WHO also demonstrates that the risk of mortality is dramatically increased in obese individuals [6] indicating a strong need for strategies to help manage or cure obesity. In many organisms, caloric restriction promotes longevity, even when started later in life, which implicates a important link between aging and metabolism [1].

Currently, there are no bonafide pharmacological interventions available to successfully treat obesity and only 2 drugs, sibutramine, a presynaptic reuptake inhibitor of norepinephrine and serotonin, and orlistat, a gastrointestinal lipase inhibitor, are FDA approved for the treatment of obesity [7]. With either of these drugs, the maximal weight loss achieved is only around 10% of the initial weight [7]. Consequently, healthcare providers and facilities are faced with the heavy burden of caring for patients with obesity and obesity-related diseases until effective treatments can be identified.

Although many of the underlying causes of obesity are a combination of genetic and environmental factors, a complex physiological system exists to tightly control body weight and energy balance over long periods of time despite daily fluctuations in caloric intake and energy expenditure. In obesity, these metabolic systems can be altered and cause a new, elevated body weight set point to develop. Studies in humans and rodents have suggested that during weight loss, the body tries to defend the elevated set point and preserve energy by reducing its metabolic rate [8]. Thus, weight loss and maintenance of reduced body fat after weight loss can become more difficult due to the altered set point, which favors the obese condition [9]. By furthering our understanding of these processes, better therapeutic strategies can be designed to help combat obesity and its related diseases.

Theories of Food Intake

There are a number of classical theories that have been developed to explain the complex regulation of food intake. The dual center hypothesis, proposed by Anand and Brobeck in 1951, was based on lesioning studies in the hypothalamus that identified two important regions for mediating food intake [10]. Bilateral lesions to the ventromedial nuclei (VMH) of the hypothalamus resulted in an increase in food intake and the

development of obesity [11] while lesions to the lateral hypothalamus (LH) completely abolished food intake [10]. Thus, the LH was called the "feeding center" and was proposed to control central hunger reactions while the VMH was called the "satiety center" and was proposed to exert inhibitory control over the "feeding center" [10]. Results from other lesioning studies identified several other hypothalamic sites important in feeding behavior including the arcuate nucleus (ARC), paraventricular nucleus (PVN), and dorsomedial nucleus (DMN) [12]. In addition to theories focusing on hypothalamus, other classical theories of food intake regulation suggest that single variables such as glucose, amino acids, heat generation, or adipose tissue stores are the major mediators of the endogenous drive to consume food.

The thermostatic theory, proposed by Brobeck in 1948 relates the regulation of body temperature to the control of feeding behavior. When rats were exposed to high temperatures, food intake decreased, whereas in cooler temperatures, food intake increases and direct calirometric effects of food prevents hypothermia. Brobeck summed up this theory by stating, "animals eat to keep warm and stop eating to prevent hyperthermia" [13]. This theory was refuted by Kennedy who felt that the decreased food intake and the reduction in body weight observed in rats exposed to high temperatures was due to dehydration and malaise rather than a direct influence of temperature. Therefore, Kennedy argued that it was unlikely that hypothalamic temperature centers and satiety centers were the same [14]**.**

In 1953, Mayer proposed the glucostatic theory of food intake, which suggested that plasma glucose concentrations are the key signals in the control of food intake [15]. This hypothesis states that glucoreceptors in the hypothalamus detect the rate at which

glucose is being utilized and initiate feeding when blood glucose levels are low. In addition, when glucose utilization rates are high, the glucoreceptors are able to detect this and inhibit feeding by activating satiety centers. Later studies determined that glucose alone was not able to initiate feeding and argued against glucose as the primary mediator of orexigenic drive [16].

The ability of rats to precisely maintain energy balance despite alterations in diet, activity level, or environmental factors led to the proposal of the lipostatic theory by Kennedy in 1953. This theory centered on the idea that a circulating factor derived from fat stores is necessary for regulating energy expenditure and food intake [14]. The discovery of the adipose hormone leptin and identification of inhibitory feeding pathways activated by leptin [17] strongly supports the lipostatic hypothesis of food intake. Thus, leptin is able to signal the hypothalamus as to the extent of fat stores and provides a mechanism for long-term regulation of energy status.

The aminostatic theory, proposed by Rogers and Leung in 1973, focuses on the influence of amino acids on the neuroregulation of food intake. Their work showed that rats decrease their food intake when they are fed a diet that is either low in certain amino acids or contains an excess of one amino acid, or a diet that is high in protein. These authors suggested that various brain regions are able to detect the amino acid composition in the diet and adjust feeding behavior based on the content of the diet [18].

These classical theories, along with more current research, indicate that a large number of central and peripheral hormones, peptides, and other factors are important in regulating energy homeostasis. Recent studies have focused on the role of the

hypothalamus and centered on the ARC as a primary hypothalamic area involved in the control of energy balance and food intake.

Hypothalamic Control of Energy Balance

The ARC contains many neuropeptides that act in regulating food intake and it is a critical hypothalamic area involved in integrating neuronal and peripheral signals such as leptin that are involved in energy homeostasis (***Figure 1-1***).

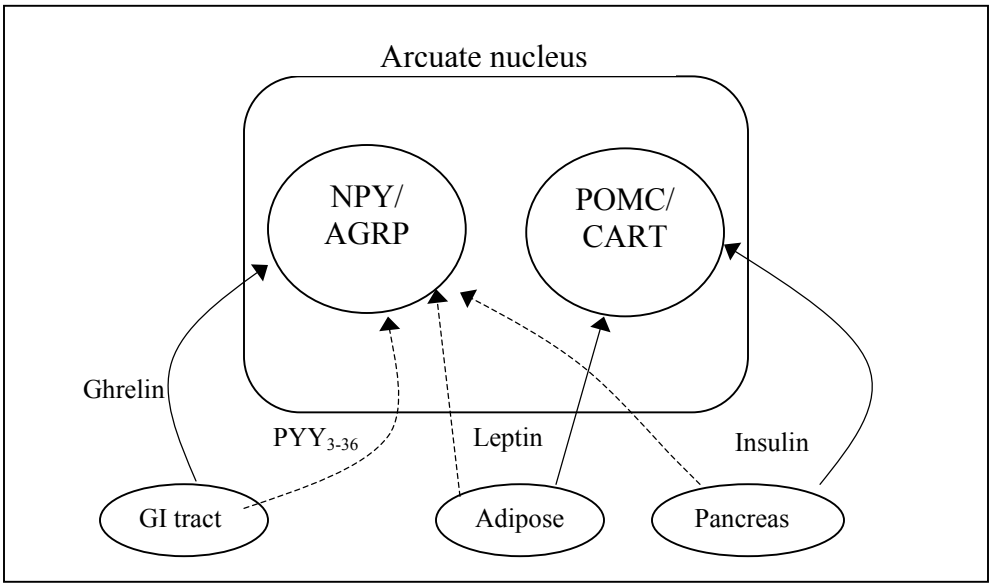

Figure 1-1. Major peripheral hormones involved in the control of food intake by action at NPY/AGRP and/or POMC/CART neurons in the ARC. Solid lines represent stimulatory pathways and dotted lines represent inhibitory pathways.

Its location at the base of the hypothalamus allows the ARC to detect and respond to circulating peptides and hormones outside the blood brain barrier [19]. Leptin, produced by adipose tissues, and insulin, produced in the pancreas, are important peripheral signals that relay information to the hypothalamus about body fat stores and energy status [7, 20]. When body fat stores are decreased, levels of leptin and insulin are reduced and this is sensed by the hypothalamus causing an increase in food intake [17]. Leptin activates the Janus kinase-signaling transducer and activator of transcription (JAK-STAT) pathway. This pathway is also used by proinflammatory cytokines

involved in anorexia as well as the insulin receptor substrate-phosphoinositol 3-kinase (IRS-PI(3)K) pathway used by insulin both centrally and peripherally [21]. Two major types of leptin and insulin responsive neurons are located in the ARC and are important in regulating appetite and food intake. Neurons containing POMC and cocaine- and amphetamine-regulated transcript (CART) are stimulated by leptin and insulin and inhibit food intake, whereas neuropeptide Y (NPY) and agouti-related peptide (ARGP)-containing neurons are inhibited by leptin and insulin and stimulate food intake [7, 22].

In addition to leptin, peripheral signaling factors such as glucocorticoids, thyroid hormones, growth hormone, peptide YY_{3-36} and ghrelin interact with hypothalamic networks to influence energy balance and food intake [23]. Some of these signaling factors act rapidly and are involved in short-term meal regulation, while others are involved in regulating long-term energy homeostasis. Thus, the control of body composition and metabolism is highly complex and involves the interaction of many central and peripheral pathways.

Animal Models of Obesity

There are multiple animal models of obesity caused by various genetic mutations that are useful for studying obesity and understanding many of the factors involved in regulating energy balance (***see Table 1-1 below***). Important roles for leptin and insulin in maintaining energy homeostasis are supported by the obesity syndromes of leptin deficient *ob/ob* mice [17], animals with leptin receptor mutations, *db/db* mice [24] and *fa/fa* Zucker rats [25], and neuronal insulin receptor knockout mice [26]. In addition to insulin and leptin, animals with genetic alterations related to the hypothalamic melanocortin system also develop obesity. Targeted disruption of the proopiomelanocortin (POMC) gene in mice results in obesity and administration of the

POMC derived peptide α-melanocyte stimulating hormone (α-MSH) to these obese mice results in a dramatic reduction in their excess body weight [27]. Mutations in the POMC gene causing POMC deficiency have been described in humans and cause severe early-onset obesity [28]. Overexpression of the melanocortin receptor antagonists agouti-related peptide (AGRP) [29] or agouti [30] and deletion of the central melanocortin receptors [31, 32] also results in obesity. Lean transgenic mice overexpressing α-MSH are characterized by decreased weight gain and adiposity. When α-MSH is overexpressed in obese *db/db* mice, adiposity and body weight gain are also significantly reduced [33], further supporting a role for POMC derived peptides and the melanocortin system in regulating energy balance.

Table 1-1. Selected animal obesity models

Model	**Anomaly**	**Characteristics**	**Reference**
Ob/ob Mouse	Absence of leptin mRNA or expression of mutant protein	Hyperphagia, hyperglycemia, hyperinsulinemia, early-onset obesity, leptin tx cures obesity	[36]
Db/db Mouse	Mutation in leptin receptor	Hyperphagia, hyperglycemia, hyperinsulinemia, early-onset obesity, resistant to leptin tx	[37]
Fa/fa rat	Missense mutation of extracellular portion of leptin receptor	Juvenile onset obesity, growth and reproductive disorders	[38]
Koletsky rat	Leptin receptor null	Hyperinsulinemic, hypertensive, obese	[39]
A^y mouse	Agouti overexpression in the brain	Obese, yellow pigmentation,	[40]
POMC mouse	Deletion of POMC gene coding region, no POMC peptides produced	Obese, yellow pigmentation, adrenal insufficiency, increased body length	[27]
MC3-R KO mouse	Deletion of melanocortin 3 receptor	Maturity onset obesity, metabolic alterations	[32]
MC4-R KO mouse	Deletion of melanocortin 4 receptor	Maturity onset obesity, hyperphagia	[31]
NIRKO	Neuron specific deletion of insulin receptor	Hyperphagia, diet sensitive obesity, hyperleptinemia, mild insulin resistance	[26]

In addition to the animal models of obesity that result from genetic alterations, obesity can also be induced in animals by lesioning of the various hypothalamic regions as previously discussed, or through the feeding of high fat diets [34, 35]. These varying animal models give evidence of multiple factors and hormonal systems involved in regulating energy balance and body composition.

The Melanocortin System

One of the major systems involved in the control of energy homeostasis and food intake is the melanocortin system. The melanocortin system is involved in a large number of physiological processes and consists of agonist POMC derived peptides (α-, β-, γ-, melanocyte stimulating hormone (MSH) and ACTH) and endogenous antagonists agouti and AGRP. To date, there have been five G-protein coupled melanocortin receptors (MC1R-MC5R) identified which mediate the various effects of the melanocortin peptides [41] by coupling through G_s to raise intracellular cAMP [42]. The MC1R, found primarily on melanocytes, plays a role in pigmentation and hair coloration [43]. The MC2R is expressed in the adrenal cortex and on adipocytes and is involved in mediating the effects of ACTH on steroidogenesis [41]. The MC5R is located in a number of tissues including skin, skeletal muscle, adrenal gland, adipocytes, ovary and testis but its only established function is an involvement in regulating exocrine secretions [44]. The MC3R and MC4R are both expressed in the brain with particularly high levels in the hypothalamus and are involved in the regulation of food intake and energy homeostasis. In the hypothalamus, the MC4R is found in the PVN, dorsal hypothalamus and LH [41, 45]. The MC3R is found in the ARC and is widely expressed in peripheral tissues such as adipose tissue, skeletal muscle, placenta and the GI tract [41]. α-MSH is

an agonist at the MC3 and MC4 receptors and inhibits food intake, while AGRP antagonizes the action of α-MSH at these receptors and stimulates food intake. AGRP is also considered an inverse agonist because it suppresses the constitutive activity of the MC3R and MC4R [46]. Central infusions of MC4R agonists dose-dependently reduce food intake and are able to suppress food intake stimulated by fasting or NPY [47] while the MC3R is considered to act as an inhibitory autoreceptor on POMC neurons [19]. Animals with genetic modifications in agouti, AGRP, POMC, and the MC3R and MC4R genes exhibit alterations in energy homeostasis including hyperphagia and obesity. Also, mutations in the MC4R and POMC genes have been observed in a small percentage of human obesity [48]. In fact, over 40 different mutations in the MC4R gene have been identified in various individuals with early onset and severe obesity making mutations in the MC4R gene the most prevalent genetic cause of obesity currently identified [49]. The central melanocortin system is a downstream target of leptin and ghrelin [50], both of which interact in regulating metabolism and GH secretion. Thus, further study of the central melanocortin system is important to help understand its role in integrating both peripheral and central signals involved in controlling energy homeostasis.

Melanocortin 3 and 4 Receptor Knockout Mice

Genetic studies using knockout mice reveal different roles for the MC3R and MC4R in regulating body composition and energy metabolism (***see Table 1-2 below***). By 6 months of age, both male and female MC3R KO mice exhibit approximately double the fat mass and a 15-20% reduction in lean body mass as compared to wild-type littermates [32]. Although these animals have high fat mass, they do not increase their food intake nor gain excessive weight, suggesting that their obesity is caused by changes in

metabolism [51]. The phenotype of these animals resembles the metabolic changes that occur in aging, including reduced lean body mass and increased fat mass, without distinct changes in food consumption. In addition, both male and female MC3R knockout mice have reduced bone lengths and are shorter than wild-type controls [32].

Animals deficient in the MC4R KO display maturity-onset (12-20 weeks) obesity characterized by hyperphagia, hyperinsulinemia, and hyperleptinemia similar to that observed in the obese *agouti* mouse. Obesity in this model is thought to be primarily a result of hyperphagia, as MC4R knockout mice exhibit a 46% increase in food consumption as compared to wild-type littermates [31]. This is likely due to an interruption of leptin and/or α-MSH signaling in the hypothalamus. However, it has also been demonstrated that metabolic defects such as an increased efficiency in storing calories as fat play a role in the development of obesity in the MC4R KO mouse [52]. MC4R KO mice are hyperinsulinemic and hyperglycemic, but have normal corticosteroid levels [53]. Interestingly, MC4R knockout mice are significantly longer than controls, opposite of that observed in the MC3R knockout mouse. The alterations of somatic growth in MC3R and MC4R deficient mice suggest possible involvement of neurons expressing these receptors in GH regulation. NPY induced food intake is not altered in MC4R KO mice [54], suggesting that signaling through the MC4R is not involved in mediating the effects of NPY on food intake. However, the inhibitory effects of leptin on food intake are not observed in obese MC4R KO mice, suggesting that this receptor plays a role in mediating the effects of leptin [54]. In addition to being hyperphagic, MC4R KO mice have an elevated respiratory exchange ratio, which is a measurement of the volume of CO_2 produced per amount of O_2 consumed. Also, these mice do not exhibit

the decrease in food intake or increase in metabolic rate in response to the synthetic melanocortin agonist, MTII, as seen in WT mice or diet-induced obese mice [55], further supporting the idea that the MC4R is important for energy regulation.

MC3R and MC4R double knockout mice are significantly more obese than mice with deletion of either receptor individually, suggesting that these two receptors have non-redundant functions and regulate independent pathways [56]. In addition, these knockout mice do not respond to the orexigenic effects of ghrelin [57]. Thus, further characterization of MC3R KO and MC4R KO mice will help to determine the functions of these receptors and their role in regulating energy homeostasis and possibly their involvement in growth hormone secretion.

Table 1-2. Comparison of MC3R KO and MC4R KO mice. Selected characteristics listed are in comparison to WT littermates. [31, 32]

MC3R KO Mouse	**MC4R KO Mouse**
Obesity onset: 6 months	Obesity onset: 3-5 months
Increased fat mass	Increased fat mass
Reduced lean body mass	Normal lean body mass
Reduced linear growth	Increased linear growth
Hyperleptinemic	Hyperleptinemic
Mild hyperinsulinemia (males)	Hyperinsulinemia
Normal to decreased food intake	Increased food intake

Growth Hormone and Insulin-Like Growth Factor I

Growth hormone (GH) is a 22 kDa, 121 amino acid peptide secreted in a pulsatile fashion from somatotroph cells of the anterior pituitary gland [58]. GH has a wide range of biological functions and acts on almost all tissues both directly via binding to its own receptor and indirectly via its stimulation of insulin-like growth factor-I (IGF-I) [59]. It circulates in part bound to high-affinity, low-capacity binding proteins (GHBP) which act

to form circulating GH reservoirs, prolong the half-life of circulating GH, and modulate the activity of GH at GH receptors [60]. IGF-I mediates many of the effects of GH and is primarily produced in the liver in response to GH activation of the GH receptor [61]. There is also a family of IGF binding proteins, (IGFBP-1 to -6) which regulate the actions of IGF-I and can influence GH secretory status [62, 63]. These proteins act as carrier proteins for and prolong the half-life of IGF-I, and may have direct effects on cell function independent of IGF-I [64].

GH has a large number of biological effects, which involve multiple organs and physiological systems. One of the major actions of GH is its ability to stimulate longitudinal bone growth and skeletal muscle growth and pulsatile GH secretion is necessary for normal postnatal growth and development [65]. IGF-I has some effects on longitudinal growth but GH is thought to be the primary mediator of these effects [66]. In addition, GH is a potent anabolic hormone and is an important regulator of body composition. It causes an increase in lean mass by inducing a positive nitrogen balance and stimulating protein synthesis in muscle [64, 67] and has a lipolytic effect on adipose tissue resulting in a decrease in fat mass [68]. Other actions of GH include inhibition of insulin action and IGFBP-1 and IGFBP-2 synthesis, stimulation of IGFBP-3, mediation of thyroid hormone synthesis and function, and immunomodulation [64].

The effects of GH are mediated by its receptor, the GH-R, located in many different tissues and cell types. The GH-R is a member of the class I cytokine receptor superfamily [69] and GH-induced receptor dimerization is the first step in GH-R activation [70]. The primary signaling pathway involved in GH-R activation is the JAK-STAT pathway and SOCS proteins are involved in turning off GH-R signaling.

Activation of the GH-R by GH in tissues such as liver, skeletal muscle and bone is thought to be required for the synthesis and secretion of IGF-I [70].

Regulation of Growth Hormone Secretion

The regulation of pulsatile growth hormone secretion has long been attributed to the interplay of the hypothalamic peptides growth hormone releasing hormone (GHRH) and somatostatin (SRIH) [71]. GHRH is released from ARC neurons and stimulates GH secretion by activating its receptors on pituitary somatotrophs, which causes an increase in cAMP levels [72]**.** SRIH is produced primarily in the periventricular nucleus of the hypothalamus and inhibits GH secretion directly by inhibiting cAMP and hyperpolarizing somatotroph cells [73] and indirectly by blocking the activation of GHRH neurons [74]. GHRH and SRIH are secreted 180° out of phase with each other and display surges every 3-4 hours. Secretion of GH from the anterior pituitary occurs when SRIH concentrations are low and GHRH concentrations are high [75]. In addition, synthetic peptidyl and non-peptidyl compounds called growth hormone secretagogues (GHS) have been shown to potently stimulate GH secretion and potentiate the action of GHRH by acting at a distinct receptor, the GHS-R [76], found in the pituitary and hypothalamus [77]. In the ARC, this receptor is primarily located in GHRH, SRIH and NPY/AGRP containing neurons [78]. The presence of this receptor suggested that there might be an additional pathway important for the regulation of GH secretion other than GHRH and SRIH. Recently, ghrelin was identified as an endogenous peptide capable of inducing GH secretion by acting through the GHS-R [79], supporting the role for this pathway in the regulation of GH.

There are also several negative feedback pathways that are important in regulating GH secretion. GH can influence its own production and release by modulating the release of GHRH and SRIH from the hypothalamus [80] and by direct inhibition of pituitary somatotrophs [81]. Transgenic mice overexpressing GH in the hypothalamus have a dwarf phenotype and are characterized by decreased hypothalamic GHRH levels, reduced pituitary GH content and low circulating IGF-I levels [81]. This supports the presence of a short loop feedback mechanism of GH on its own release. Circulating IGF-I can also inhibit GH secretion via a negative feedback loop to the pituitary and hypothalamus [64, 82].

In addition to the primary regulators of GH (GHRH, SRIH and GHS/ghrelin), a large number of secondary neural, metabolic and hormonal factors can influence the complex regulation of GH secretion. The orexigenic peptide NPY has an inhibitory effect on GH secretion when administered centrally to rats [83, 84] and short loop negative feedback by GH is thought to involve stimulation of GH-R containing NPY neurons in the ARC. NPY is then released from its nerve terminals in the periventricular nucleus, which connect with SRIH neurons, and stimulates SRIH release [85]. Leptin increases GH levels diminished by fasting [85] and administration of leptin antiserum to rats causes a decrease in GH levels suggesting that leptin is necessary for normal GH secretion [86].

The patterns of pulsatile GH secretion and target tissue sensitivity to GH have been shown to vary between male and female animals with similar observations in humans [87]. In male rats, GH secretion occurs in discrete pulses accompanied by low interpeak levels whereas in female rats, GH secretion is characterized by high interpeak

levels and less pulsatility [66]. It has been suggested that growth patterns and differences in body composition between males and females may be directly related to the sexually dimorphic patterns of GH secretion [88]. Sex differences in liver GH receptors and receptor binding exist and circulating concentrations of GH binding proteins are greater in females in both rats and humans which may decrease the availability of circulating GH in females [88]. Sex steroids can greatly influence the individual effects and interactions of GHRH, SRIH, and GHS/ghrelin in regulating GH [89]. Testosterone and estrogen positively regulate GH secretion and are important in maintaining GH secretion throughout the lifespan [90]. Estrogen augments the maximal effect of GHS in stimulating GH release in rats, and in older women, estradiol treatment amplifies the GH secretory response to increasing doses of a GHS, growth hormone releasing peptide-2 (GHRP-2) [80]. In addition, stomach cells expressing ghrelin mRNA coexpress mRNA for the estrogen receptor-α, indicating a role for estrogen in the regulation of ghrelin secretion and/or production in the stomach [91]. Testosterone administration to males causes an increase in mean plasma GH and GH secretion rate by increasing GH pulse amplitude [88]. Taken together, these results suggest that sex steroids are important regulators of GH secretion.

The breakdown of adipose tissue causing the release of glycerol, free fatty acids (FFA) and ketone bodies is caused by GH, and a negative feedback system between GH and FFA clearly exists [92]. Elevations in FFA reduce both basal and stimulated GH release, and pharmacological reductions in FFA are associated with increased GH secretion [93-95]. Other influences on GH secretion include exercise, stress, fasting, glucocorticoids, thyroid hormone, and various neurotransmitters. To further complicate

the matter, many of the effects of these influences on GH secretion vary among species [80]. However, the effect of obesity and aging on GH secretion is dramatic and affects many species, including rodents and humans, similarly.

Growth Hormone Secretion in Obesity

In obesity, circulating levels of GH are extremely low, its half-life is reduced, and the amplitude of GH pulses can be nearly undetectable [96, 97]. Negative correlations exist between body mass index, body fat percentage and the half-life of GH as well as the amplitude and frequency of GH pulses [98-100]. The primary mechanism of this reduction is likely a decrease in GH release from the pituitary somatotrophs [92]; however, the causes of this decline are less clearly identified [101, 102]. It has been suggested that alterations in hypothalamic function such as SRIH hyperactivity, GHRH hypoactivity or GHS-R ligand hypoactivity as well as elevated peripheral signals such as FFA, leptin, and insulin could all contribute to the decline in GH secretion in obesity [92, 103]. In fact, circulating GH levels are reduced in obese patients who are insulin resistant and hyperinsulinemic. This increase in insulin levels may suppress IGF binding protein levels and lead to an increase in free IGF-I, which would cause negative feedback on GH secretion [104].

Obese patients show an impaired responsiveness to the GH-releasing effects of GHRH alone but show an increase in GH secretion in response to various GHS such as growth hormone-releasing peptide-6 (GHRP-6) and hexarelin. The combined administration of GHRH and GHRP-6 elicits a greater GH response than any other stimulus alone however the overall GH response to various secretagogues is less than that of lean patients [96]. Recently, ghrelin was shown to potently stimulate GH secretion when administered alone or in combination with GHRH to obese patients [105].

In obese Zucker rats, circulating levels of GH are low and GHRH mRNA and protein levels are decreased [106]. Obese Zucker rats and high fat diet-induced obese rats show an impairment of the GH axis with female rats being better preserved than male rats. This suggests that the alterations of the GH axis in obesity may be gender-related and possibly influenced by gonadal function [107]. Administration of GH to obese rats causes a decrease in body weight [101, 102] and a normalization of GH secretion after weight loss in humans [103] supports a role for GH in obesity. When rats are fed a moderately high fat diet, only about half of the animals will become obese and these animals are considered obesity prone. The other half gain weight similar to animals on a normal diet and are considered obesity resistant [34]. Studies measuring GH levels in these animals revealed that the obesity-prone animals had a deficit in GH secretion that occurred before the onset of obesity. Therefore, impaired GH secretion may contribute to the susceptibility to obesity in these animals [108].

Growth Hormone Secretion in Aging

In addition to obesity, it has been well characterized that both spontaneous and stimulated GH secretion declines with aging in both humans and animals [109]. The amplitude of GH pulses is reduced with aging in both humans and rats, but the frequency of pulses is not affected [110]. The age-related reduction in GH secretion in humans is correlated with increased percentage of total body and visceral fat, decreased physical fitness, and decreased testosterone concentrations in men, or the menopause in women [80]. Studies in humans have indicated that the age-associated GH decline may be due to an increase in SRIH tone which parallels the increase in adiposity that occurs in aging [59]. Many studies reveal that there is in fact an age-related decrease in hypothalamic SRIH content [111] and a decline in SRIH gene expression [112] while others do not

detect a difference in SRIH immunoreactivity in the hypothalamus between young and old rats [113]. However, perifusion studies demonstrate that there is actually an age-related alteration in the major form of SRIH released from the hypothalami of aged rats. A more potent form of SRIH, SRIH-28, was released from aged hypothalami suggesting that this form of SRIH may be important for inhibiting GH function in aged animals [114]. Pituitary function appears to be intact in aged humans and animals while alterations in hypothalamic function are more likely to mediate the changes in the GH axis in aging [109]. In addition, hypothalamic GHRH mRNA levels and hormone content are reduced in aged rats [113, 115].

As in obesity, there is a greater sensitivity to GHS in aging compared to other GH-releasing stimuli including GHRH [109]. This suggests that diminished GH secretion in aging and obesity may result from a decrease in a GHS-R agonist, such as ghrelin. Although the response to GHS in aging is greater than those of other stimuli, the overall response to either GHRH or GHS in aged humans is blunted compared to young individuals, and this effect has been related to the increase in body fat that occurs with aging [59, 116]. However, normalization of the GH response to GHRH and GHS in aging occurs when SRIH influence is reduced by coadministration of arginine [117]. This gives further support to an increase in somatostatin tone as a major mechanism of the GH decline in aging. In addition, the GH response to hexarelin, a GHS, is increased by coadministration of GHRH indicating that these two pathways act synergistically to regulate GH secretion [118].

Not all aspects of the GH decline in obesity and aging are similar however. In particular, IGF-I levels are diminished in aging, whereas in obesity, studies have shown

increased, normal or diminished levels of IGF-I [96, 97]. Resistance to the effects of leptin occurs in both obesity and aging [4] and diminished ghrelin levels occur in obesity [119] however, the status of ghrelin in aging is currently unclear. Because leptin and ghrelin both affect GH secretion and food intake and act on common pathways, further studies are needed to help elucidate their roles in the metabolic changes that occur with aging and obesity.

Ghrelin

Ghrelin was isolated from rat stomach and identified as the endogenous ligand for the GHS-R. This 28 amino acid peptide contains a unique n-octanoyl modification at Serine 3 residue that is necessary for its biological activity [79] (Figure 1-2) and is the only peptide known to contain this modification. It has been suggested that this modification may help ghrelin cross the blood brain barrier by adding a hydrophobic property to the peptide [120].

The ghrelin gene is expressed primarily in the stomach, specifically in the X/A like cells of the oxyntic gland [121]. However, ghrelin gene expression has also been observed in many other tissues including pituitary [122], hypothalamus [79, 123], intestine [121], kidney [124], heart [125], and placenta [126]. A recent report by Cowley *et al.* [123] describes a unique pattern of distribution of ghrelin in the hypothalamus of rats and mice. Ghrelin immunoreactive neurons were observed in the internuclear space between the PVN, ARC, VMN, and DMN as well as in the ependymal layer of the third ventricle. Expression of ghrelin is also found in somatotrophs, lactotrophs and thyrotrophs suggesting that ghrelin may act in a paracrine fashion in the pituitary [127].

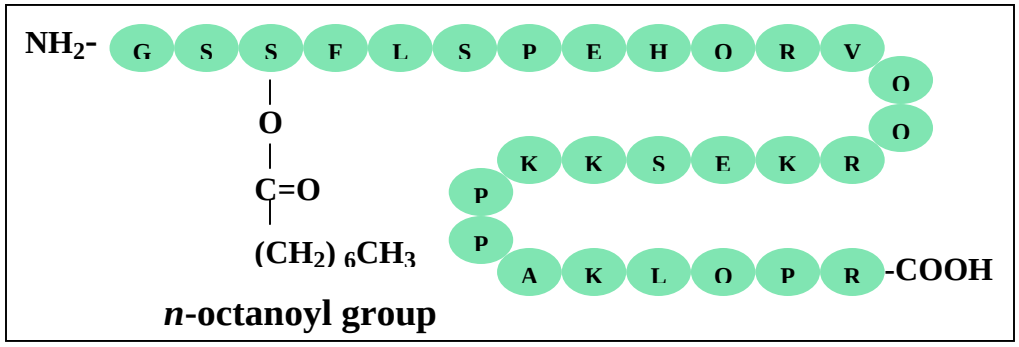

Figure 1-2. Amino acid sequence of human ghrelin with serine 3 modification. In rat ghrelin, the arginine and valine at positions 11 and 12 are replaced by lysine and alanine, respectively [128].

Ghrelin circulates in the blood at femtamolar concentrations with the majority released from the stomach [129] and its secretion has been shown to be pulsatile [130]. However, removal of the stomach results in only a 65% reduction in ghrelin levels indicating that other tissues do contribute to the maintenance of ghrelin levels [131]. In human plasma, approximately 90% of circulating ghrelin is in the inactive desoctanoylated form, while the remaining 10% is biologically active and has a short half-life [43]. In rats, the half-life of ghrelin is around 30 minutes and mean ghrelin concentrations are higher in animals with high amplitude GH peaks [132]. Although ghrelin was originally characterized as a potent GH-releasing peptide, subsequent studies have revealed additional roles for ghrelin. These include involvement in regulating energy homeostasis and inducing adiposity, cardiovascular actions, and effects on gastrointestinal functions. Additionally, ghrelin has been shown to potently stimulate food intake presumably by activating neuropeptide Y (NPY)/AGRP neurons in the ARC nucleus [133]. Because of the effects of ghrelin on GH secretion and food intake, it has been suggested that ghrelin serves as a link between the energy needs of the body and the regulation of the growth process.

Ghrelin Receptors

Ghrelin receptors, or GHS-Rs, are located in many areas throughout the body but are found primarily in the pituitary and the ARC and VMN of the hypothalamus [134]. In the ARC, GHS-R mRNA is colocalized with neurons containing GHRH and SRIH [135], and 94% of NPY/AGRP-positive neurons in the ARC contain GHS-R mRNA [136]. The highest specific binding for the synthetic GHS-R ligand, ^{125}I-hexarelin, was found in the human hypothalamus and pituitary gland [137]. The GHS-R receptor is a 364 amino acid, G- protein coupled receptor, which is highly conserved among humans, rat, pig, and mouse [138]. Two isoforms of this receptor, GHS-R1a and GHS-R1b, have been identified as a result of alternative splicing [139]. GHS-R1b is not activated by ghrelin or other GHS and its biological function is currently unclear. Thus, the GHS-R1a is considered the primary receptor for ghrelin and synthetic GHS moieties, however, the existence of other receptor isoforms cannot be ruled out.

Pituitary GHS-R mRNA levels are greater in female rats than males, whereas there is no difference in hypothalamic GHS-R mRNA levels between male and female rats [140]. In aging, a decline in the GHS-R occurs in the hypothalamus but not in the pituitary, and GHS-R levels in the pituitary are negatively regulated by circulating GH concentration [109]. Short-term ghrelin or GHS administration causes a rapid down-regulation of pituitary GHS-R in several species tested to date [141]. Dexamethasone administration to rats causes increased GHS-R mRNA levels in the pituitary and adrenalectomy causes a decrease in pituitary GHS-R mRNA levels, indicating a role for glucocorticoids in the regulation of GHS-R gene expression [142]. In addition, thyroid hormones also increase pituitary GHS-R mRNA levels, in part by increasing half-life of the message [143]. Feeding status can also influence GHS-R mRNA levels as fasted rats

exhibit an 8-fold increase in hypothalamic and a 3-fold increase in pituitary GHS-R mRNA levels [144]. Deletion of the GHS-R in mice results in an inability of ghrelin to stimulate GH secretion and food intake, indicating that this receptor is necessary for mediating the primary effects of ghrelin [145].

Regulation of Ghrelin Levels

The production of stomach ghrelin and its release into the circulation are regulated by many nutritional and hormonal factors. The levels of circulating ghrelin and stomach ghrelin mRNA levels are modified by short-term changes in energy status as they increase during fasting [144, 146] and before meals [147] and decrease following meals [144, 148], suggesting a role for ghrelin in meal initiation and satiety. The premeal increase in ghrelin levels occurs when serum leptin levels are low and the decrease following meals corresponds with an increase in leptin secretion [149, 150]. Studies in rodents demonstrate a nutrient requirement for reduction in ghrelin levels as dextrose administration causes a reduction in ghrelin levels whereas filling the stomach with water does not affect ghrelin levels [151]. Ghrelin levels are also influenced by chronic states of altered energy balance as they are elevated in anorexic patients [43] and decreased in obesity [119], a condition usually associated with hyperleptinemia.

The primary hormonal stimulators of ghrelin secretion are insulin, glucose and SRIH [152]. Fasting ghrelin levels are negatively correlated with insulin levels and meal-induced suppression of ghrelin is correlated with the post-meal rise in insulin [153] in addition to leptin. Insulin infusion during euglycemia in humans causes a rapid decrease in ghrelin levels, and ghrelin levels remain suppressed during subsequent periods of hyper- and hypoglycemia, indicating a role for insulin in regulating ghrelin levels independently of glucose concentrations [154]. Studies in various mouse models of

altered GH secretion and/or action reveal that stomach mRNA levels and serum concentrations of ghrelin are not affected by chronic changes in the peripheral GH-IGF-I axis [155]. However, stomach ghrelin gene expression in mice displays an inverted U-shaped curve when measured from prenatal stages to old age. This expression profile resembles growth curves and secretions of GH and IGF-I throughout the lifespan. At two months of age, male and female mice have similar levels of gastric ghrelin mRNA levels, whereas in 19 month-old mice ghrelin levels exhibited a significant sexual dimorphism in that stomach ghrelin levels in aged female mice were 2.2-fold greater than those of aged male mice [156]. Therefore, variations in ghrelin levels between male and female mice may contribute to the sexual dimorphism of GH secretion, especially in aging and the profile of ghrelin throughout the lifespan suggests that ghrelin may play an important role in growth and metabolism.

Because of the role of ghrelin in stimulating food intake and adiposity, it was originally thought that ghrelin levels would be increased in obesity. However, the opposite was found in that circulating ghrelin levels are actually diminished in obesity [119]. In addition, alterations in macronutrient effects on circulating ghrelin levels have been observed in obese patients. Obese women fed a high carbohydrate meal do not show a meal-induced suppression of ghrelin levels as observed in lean women, whereas a fat-rich meal does not suppress ghrelin levels in either lean or obese women [157]. This suggests that ghrelin-induced satiety mechanisms may be altered in obese patients. Upon weight loss in humans, circulating ghrelin levels are increased and positively correlate with the extent of weight loss [158].

Twenty-four hour blood sampling in lean men revealed that ghrelin secretion is pulsatile with a nocturnal increase in ghrelin levels that is greater than the surge in ghrelin seen before a meal. However, sampling in obese men revealed that ghrelin secretion is pulsatile as seen in lean men, but the nocturnal increase in ghrelin levels is not seen in obese men [159]. This suggests that blunted nocturnal ghrelin secretion may be an important feature of human obesity that may influence the altered GH secretion in obesity. In rats, ghrelin secretion is also pulsatile and displays an ultradian rhythmicity. The number of peaks and interpeak intervals of ghrelin are similar to those of GH but no direct correlation exists between ghrelin levels and GH secretion. However, ghrelin pulses are correlated with episodes of food intake in the dark phase in rats [132] suggesting a further link between ghrelin and energy balance.

Ghrelin and Growth Hormone Secretion

The discovery of ghrelin gives definitive proof to the involvement of the GHS-GHS-R signaling system in the control of GH secretion. Indeed, in the short time since its discovery, numerous studies have confirmed the role of ghrelin as a potent GH secretagogue both *in vitro* and *in vivo*. Peripheral administration of ghrelin potently stimulates the release of GH in many species, including rats and humans [79, 160-162] while central administration has been shown to elicit GH secretion only in certain studies [163-165]. Intraperitoneal administration of ghrelin to rats or mice rapidly stimulates GH release with peak values observed around 15 minutes post injection and values back to baseline by 60 minutes [163]. A similar time course is observed for IV and ICV administration of ghrelin to rodents [79, 163, 165] as well as for IV administration in humans [162].

Ghrelin stimulates GH release by direct action on pituitary cells [79] and indirectly via its action on GHRH neurons [131]. *In vivo*, ghrelin is a more potent stimulus of GH secretion than GHRH, whereas *in vitro*, the opposite is true [161]. In addition, GHRH signaling is thought to be important for full action of ghrelin because administration of GHRH antiserum has been shown to block GH responsiveness to ghrelin *in vivo* [166]. Co-administration of GHRH with ghrelin in humans causes a synergistic increase in GH secretion, rather than an additive increase in GH levels [167]. Studies involving co-administration of GHRH and synthetic GHS such as hexarelin or GHRP-2, demonstrate a synergistic effect on GH secretion similar to that observed with ghrelin and GHRH [168] In addition, co-administration of hexarelin with ghrelin does not cause a further increase in GH secretion [167]. Taken together, these studies suggest that ghrelin stimulates GH secretion through pathways independent of GHRH and similar to those used by synthetic GHS. Infusion of GHRH increases the expression of both the ghrelin gene and the GHS-R gene in the pituitary, which may influence GH secretion through autocrine or paracrine mechanisms [169]. An intact ARC nucleus is required for full action of ghrelin in stimulating GH secretion. Neonatal administration of monosodium glutamate (MSG) results in a virtually complete destruction of ARC, and MSG-treated rats show diminished responsiveness to both peripheral and central administration of ghrelin with no altered sensitivity to GHRH [170]. An infusion of SRIH in humans at a dose that abolishes the GH response to GHRH blunts the GH-releasing effects of ghrelin, although to a lesser extent than GHRH [171]. In rats, ghrelin, unlike GHRH, is able to stimulate GH during periods of high SRIH tone [172] and the GH-releasing activity of ghrelin is not dependent on inhibiting SRIF release [166]. In

humans, injections of ghrelin have been shown to cause increases in prolactin, ACTH, and cortisol [129], and these responses are not modified by administration of SRIH [171]. Thus the inhibitory effects of SRIH on GHRH and ghrelin are specific to their effects on GH release and do not affect other hormonal systems.

Ghrelin and Energy Regulation

In addition to its GH-releasing properties, ghrelin was found to be a potent orexigenic peptide and to be involved in the regulation of energy homeostasis. Both central [173] and peripheral [174] administration of ghrelin stimulates food intake, adiposity [151] and insulin secretion [175]. The lowest dose of ghrelin that stimulates food intake in rats results in a plasma concentration similar to that observed after a 24 hour fast, suggesting a physiological role for ghrelin in the control of food intake [176]. In addition, ghrelin-induced food intake is not attenuated following repeated peripheral administration [176]. Ghrelin is directly able to increase adiposity in rats by stimulating the differentiation of preadipocytes into adipocytes and antagonizing lipolysis [177].

Several studies have indicated that the effects of ghrelin on food intake are primarily mediated by the activation of NPY/AGRP neurons in the ARC [178]. Peripheral and central administration of various synthetic GHSs, such as growth hormone releasing peptide-2 (GHRP-2), has been shown to induce *c-fos* expression and electrical activity in these neurons [179]. Systemic ghrelin administration to both fasted and fed rats increased the number of cells expressing Fos and Egr-1 proteins in the arcuate nucleus with greater effects occurring in fasted rats [180]. In mice, a single peripheral ghrelin injection in mice specifically induced Fos expression in the ventromedial arcuate nucleus and about 90% of the Fos-immunoreactive cells expressed NPY mRNA [174]. Electrophysiological studies demonstrated that ghrelin increased the spontaneous activity

of ARC NPY neurons and caused the release of NPY, AGRP and GABA. A secondary effect was to hyperpolarize POMC neurons, thus decreasing α-MSH release [123]. The relative importance of NPY versus AGRP in mediating the orexigenic and adipogenic effects of ghrelin is still unclear, however. Chronic administration of GHRP-2 to NPY-deficient mice results in an increase in fat mass and hypothalamic AGRP, similar to that observed in wild-type mice [181]. Central administration of ghrelin to rats increases AGRP mRNA levels in the ARC without affecting ARC NPY, POMC, GHRH mRNA levels or SRIH mRNA levels in the periventricular nucleus [164]. However, chronic central administration of ghrelin does increase NPY mRNA levels in the ARC in addition to AGRP [182]. Ghrelin stimulates food intake in both NPY and AGRP single knockout mice, but when both neuropeptides are knocked out in the same animal, ghrelin-induced food intake is abolished [57], indicating that both NPY and AGRP are important for mediating the orexigenic effects of ghrelin. In addition, ghrelin-induced food intake is suppressed by central administration of anti-AGRP IgG [173]. Taken together, these data suggest that AGRP signaling may play an important role in mediating the effects of GHS-R agonists, including ghrelin, on appetite. In addition to its effects on food intake, chronic ghrelin administration increases body weight and adiposity in rodents. This is due in part to an increase in respiratory quotient, amounting to a decrease in fat utilization [151]. Chronic central administration of ghrelin is able to antagonize the effects of centrally administered leptin on food intake, adiposity, and plasma concentrations of glucose and insulin, and leptin [183] indicating that leptin and ghrelin influence energy balance through similar pathways.

Although the hypothalamus is important in mediating the actions of ghrelin on energy balance and food intake, the pituitary axes are also involved as hypophysectomized, thyroidectomized and andrenalectomized rats do not exhibit ghrelin-induced adiposity or food intake. In addition, circulating ghrelin levels are about 3-fold higher in hypophysectomized rats compared to normal, suggesting a feedback loop between the pituitary and stomach in regulating ghrelin secretion [184]. Thus, ghrelin may be an important hormonal signal that acts to integrate energy regulation and growth by modulating the GH axis in response to nutritional status. The intact GH response to ghrelin administration in obese patients coupled with diminished ghrelin levels in obesity suggests that ghrelin signaling may be an important component in the altered GH secretion of obesity.

Summary and Objectives

The regulation of energy balance and body composition is a complex process that involves the interactions of many central and peripheral systems. GH is important in building lean body mass and breaking down fat mass, and its secretion is markedly suppressed in both obesity and aging. The central melanocortin system is important in regulating food intake and metabolism, and MC3R and MC4R KO mice exhibit interesting alterations in energy homeostasis. Ghrelin has been shown to potently stimulate GH secretion and to induce food intake, at least in part through activation of the melanocortin antagonist, AGRP.

Thus, the major objectives of this work are to:

- further investigate the role of AGRP/melanocortin signaling in mediating the effects of ghrelin on food intake

- evaluate the role of the MC3R and MC4R in ghrelin-induced growth hormone secretion in young and old animals
- characterize the GH secretory axis in MC3R KO and MC4R KO mice throughout their lifespan.

This will help to better understand the mechanisms by which ghrelin affects metabolism, energy balance and GH secretion. Further characterization of MC3R KO and MC4R KO mice will help also to elucidate the role of the melanocortin system in regulating energy homeostasis and body composition.

CHAPTER 2
GENERAL METHODS

Animals

Male and female MC3R KO, MC4R KO and WT mice were obtained from the breeding colony of Dr. Carrie Haskell-Luevano, Associate Professor, Department of Medicinal Chemistry, University of Florida College of Pharmacy and used for these studies. The MC4R KO mice were originally provided by Dr. Dennis Huszar (Millennium Pharmaceuticals) and the MC3R KO mice were originally provided by Dr. Lex Van der Ploeg (Merck Research Laboratories). Mice were housed in standard group housing (3-4 animals per cage), except during feeding studies which required individual housing, with 12 hour light cycle and food and water available *ad libitum.*

Age Study

Several age groups (4-6, 8-9, 11-13 and 17-19 months) were selected in order to characterize the GH-IGF-I axis throughout the lifespan of male and female MC3R KO, MC4R KO and WT mice. Body weight was measured prior to sacrifice by decapitation. Animals were sacrificed between 0900-1100 to minimize diurnal fluctuations of the hormones of interest. Trunk blood was collected, centrifuged at 12,500 x g for 5 minutes and serum was separated and stored at –80°C until use in GH and IGF-I radioimmunoassays (RIA). Pituitaries were removed from the base of the skull and the posterior and intermediate lobes were removed and discarded. The anterior pituitary was then saved until use in the GH RIA for measurement of GH content. The hypothalamus was dissected away using forceps and stored until processing for measurement of GHRH

and SRIH content by RIA. A portion of skeletal muscle was cut away from the left quadriceps, and a piece of liver was removed. The liver and skeletal muscle samples were stored until extraction and assay by the IGF-I RIA. All tissues removed were snap frozen on dry ice and stored at –80°C until use in assays. A portion of the tail was collected and used to verify genotypes by PCR.

Genotyping

In order to verify the genotypes of the animals, a portion of the tail was collected upon sacrifice and frozen until isolation of genomic DNA using the phenol: chloroform extraction procedure. DNA was amplified by PCR using procedures as described for MC3R KO mice [32], and MC4R KO mice [185]. The genotypes of MC3R KO mice were identified by PCR using the following primers: MC3R forward primer (5'-GATGAGAGAAGACTGGAGAGAGAGGGTC-3'), MC3R reverse primer (5'-GAAGAAGTACATGGGAGAGTGCAGGTT-3'), and NEO1 (5'-TACCGGTGGATGTGGAATGTGTGC-3'). The amplification conditions used were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 seconds and 68°C for 1 min, and final extension at 68°C for 10 min. PCR products were separated on a 2% agarose gel by electrophoresis and genotypes identified by the presence of a 514 bp band for the WT allele and a 294 bp band for the MC3R KO allele. To genotype MC4R KO mice the following primers were used: MC4F3 forward primer (5'-GGAAGATGAACTCCACCCACC-3'), MC4 reverse primer (5'-GACGATGGTTTCCGACCCATT-3'), and PGK-R3 reverse primer (5'-TTCCCAGCCTCTG AGCCCAGA-3'). To amplify the DNA, these conditions were used: denaturation at 94°C for 1 min, 39 cycles of 92°C, 64°C and 72°C, for 45 seconds

each, followed by final extension for 10 min at 72°C. PCR products were separated as for MC3R genotyping and genotypes were identified by the presence of a 313 bp band for the WT allele and a 405 bp band for the MC4R KO allele.

Radioimmunoassays (RIA)

GH Iodination

Mouse GH antigen (NIDDK, National Hormone and Peptide Program) was iodinated using the chloramine T method with 0.05M sodium phosphate buffer as the reaction buffer. Five microliters (0.5 mCi) of ^{125}I-Na was added to a 5 μg aliquot of mouse GH. The iodination reaction was initiated by the addition of 10 μl of 1.5 mg/ml chloramine T, causing an oxidation reaction which allows ^{125}I-Na to attach primarily to tyrosine residues in the peptide. After 60 seconds, 50 μl of 3.75 mg/ml sodium metabisulfite, a reducing agent, was added to terminate the reaction. The reaction mixture was then diluted with 100 μl of 10% BSA solution. The entire mixture was then loaded onto a 1.0 x 50 cm column (Bio-Rad) packed with Bio-Gel P60 (Bio-Rad). Thirty-drop fractions were collected and 10 μl of each sample was then counted on the Apex Automatic Gamma Counter (ICN Micromedic Systems Model 28023, Huntsville, AL with RIA AID software, Robert Maciel Associates, Inc., Arlington, MA) to determine peak ^{125}I incorporation fractions. The peak tubes were then diluted using GH RIA buffer and stored at 4°C until use in assay.

GH RIA

Pituitary and serum GH concentrations were determined by RIA using reagents supplied by the NIDDK's National Hormone and Peptide Program and Dr. A.F. Parlow. Pituitaries were sonicated in 0.1 M borate buffer, pH 9.0 and centrifuged. Supernatant

was collected and stored at -20°C until day of assay. Each sample was assayed in duplicate using 10 µl or 25 µl of serum, depending on amount available, or 25 µl of pituitary supernatant. Values were determined based on a standard curve of mouse growth hormone reference preparation. The GH RIA had a sensitivity of 1.0 ng/ml and a range of detection from 2.5 ng/ml to 100 ng/ml. Values for serum GH are reported in ng/ml. Pituitary GH content was normalized to pituitary protein content and values are expressed as ng/µg protein. Pituitary protein content was measured using a Micro BCA protein assay. All samples from a single study were run in the same assay.

IGF-I Iodination

Recombinant human IGF-I (Bachem, Torrance, CA) was iodinated using the chloramine T method with 0.5M sodium phosphate buffer as the reaction buffer. Ten microliters (1.0 mCi) of ^{125}I-Na was added to a 2.5 µg aliquot of IGF-I. The iodination reaction was initiated by the addition of 10 µl of 1.0 mg/ml chloramine T. After 45 seconds, 200 µl of 10% BSA solution was added to the mixture to terminate the reaction. The entire mixture was then loaded onto a 1.0 x 30 cm column (Bio-Rad) packed with Sephadex G-50 (Sigma) using IGF-I assay buffer as the column buffer. Thirty-drop fractions were collected and 10 µl of each sample was then counted on the Apex Automatic Gamma Counter (ICN Micromedic Systems Model 28023, Huntsville, AL with RIA AID software, Robert Maciel Associates, Inc., Arlington, MA) to determine peak ^{125}I incorporation fractions. The peak tubes were then diluted using IGF-I assay buffer and stored at 4°C until use in assay.

IGF-I RIA

Serum IGF-I was measured by RIA following extraction by the acid/ethanol procedure as previously reported [186]. Liver and skeletal muscle IGF-I levels were also measured using this RIA following extraction. Tissues were homogenized in 0.5 N HCl at a concentration of 200 mg/ml and homogenate was incubated on ice for 1-2 hours. A sample of the homogenate was taken for protein measurement using the Micro BCA protein assay. After incubation, samples were centrifuged and 300 μl of the supernatant was loaded onto a Sep-Pak C18 cartridge for extraction. Prior to loading the sample, the cartridge was first activated with 2-propanol, methanol and 4% acetic acid. The samples interacted with the column for 3 minutes, then the cartridge was washed with 4% acetic acid and IGF-I was eluted with methanol. The eluant was dried in a Speed-Vac and stored at -80°C until assay. Liver samples were reconstituted with 1 ml IGF-I assay buffer and skeletal muscle samples were reconstituted with 0.5 ml IGF-I assay buffer overnight at 4°C. Serum and tissue samples were assayed using 50 μl in duplicate and values were expressed in terms of the BACHEM IGF-I reference preparation. The IGF-I RIA had a sensitivity of 0.1 ng/ml and a range of detection from 0.1 ng/ml to 20 ng/ml.

GHRH RIA

Hypothalami from 4-6 months and 17-19 month age groups were extracted prior to use in GHRH and SRIH RIAs. Briefly, hypothalami were sonicated in 1 ml 2.0M acetic acid and then boiled for 5 minutes. Samples were frozen and thawed to clarify, then centrifuged for 10 minutes at 4°C. The supernatant was removed and split into 2 aliquots: 1 for the GHRH RIA and the other for the SRIH RIA. The samples were

lyophilized using a speed-vac and reconstituted with 1 ml of the appropriate assay buffer the night before the assay was run.

Hypothalamic GHRH content was measured using a mouse GHRH RIA as previously described [187]. Unknowns were assayed in duplicate and values were expressed in terms of the mouse GHRH standard (Phoenix Pharmaceuticals, Inc; Belmont, CA). The antibody to mouse GHRH was kindly provided by Dr. L.A. Frohman and was added to the tubes at a dilution of 1:50,000. The 125-I-mGHRH tracer (Phoenix Pharmaceuticals, Inc) was added to the reaction 20 hours after initial setup and the tubes were incubated for 48 hours at 4°C. On day 4, the precipitating antibody was added and the tubes were centrifuged for 30 minutes at 3000 rpm. The supernatant was aspirated and the resultant pellet was counted on a gamma counter. This RIA had a range of detection from 0.04-10 ng/ml and the data was expressed as ng/hypothalamus.

SRIH Iodination

Tyr'SRIH (Bachem; Torrance, CA) was iodinated using the chloramine T method, using 0.05M sodium phosphate buffer as the reaction buffer. Ten microliters (1.0 mCi) of ^{125}I-Na was added to a 10 μg aliquot of Tyr'SRIH. Ten microliters of a 1.0mg/ml solution of chloramine T was added for 15 seconds to initiate the iodination reaction. To terminate the reaction, 200 μl of 10% BSA was added to the mixture. The reaction mixture was applied to a Sephadex G-25 column prepared in 0.087 M acetic acid/0.1% BSA and 60 drop fractions were collected. A 10 μl aliquot from each fraction was removed and counted to determine peak incorporation. The peak tubes were diluted in SRIH RIA buffer, aliquoted, and stored at –80°C until use in the RIA.

SRIH RIA

Hypothalamic SRIH was measured using a rat SRIH RIA as previously described [188]. A portion of the extracted hypothalamus (as described for GHRH RIA above) (400 μl) was lyophilized and reconstituted using 1 ml of SRIH RIA buffer the night before the assay. Unknowns (100 μl) were assayed in duplicate and values were expressed in terms SRIH reference preparation. Assay tubes were incubated for 24 hours at 4°C and on day 2 a 1:1 mixture of normal rabbit serum and goat anti-rabbit IgG was added to precipitate the reaction. On day 3, the assay tubes were centrifuged for 30 minutes at 3100 rpm. The supernatant was removed and the resultant pellet was counted on a gamma counter for 1 minute. This RIA had a sensitivity of 2.5 ng/ml and data was expressed as ng/hypothalamus.

Ghrelin RIA

Serum ghrelin levels were analyzed using a total ghrelin RIA kit (Linco Research, Inc., St. Charles, MO), which measures both the octonylated and des-octonylated forms of ghrelin in human, rat, mouse, and canine samples. The assay sensitivity was 100 pg/ml and the range of detection was from 100 pg/ml to 10,000 pg/ml when using a 100 μl sample size. Due to higher levels of ghrelin in rat and mouse serum, a sample size of 25 μl was used for unknowns and the volume was made up to 100 μl using supplied assay buffer. The tubes were incubated overnight (20-24 hours) at 4°C. On day 2, ^{125}I-ghrelin was added and the tubes were incubated overnight (22-24 hours) at 4°C. On day 3, normal rabbit IgG and cold precipitating reagent (goat anti-rabbit IgG) were added and tubes were incubated for 20 minutes at 4°C. All tubes except total counts were centrifuged for 20 minutes at 3200 rpm at 4°C (Beckman Centrifuge Model J-6B,

Fullerton, CA). Supernatant was removed using a vacuum aspirator and tubes were inverted on absorbent paper for approximately 60 seconds. Excess supernatant from lip of tubes was removed by vacuum aspiration. Resultant pellets were counted on gamma counter for 1 minute.

Animals for Feeding and GH Release Studies

Male and female mice MC3R KO, MC4R KO, and WT mice obtained from the breeding colony of Dr. Carrie Haskell-Luevano were used for these studies. Mice were group-housed (3-4 per cage) and received food and water *ad libitum* until the start of the feeding studies. Each genotype was divided into 2 groups: a control group receiving an i.p. injection of sterile saline or a treatment group receiving an i.p. injection of ghrelin at a dose of 400 μg/kg. This dose is based on previous studies reported in the literature utilizing mice and peripheral injections of ghrelin to measure both food intake and GH secretion [174, 189-191].

Peptide Preparation

Rat ghrelin (BACHEM; Torrance, CA) was dissolved in 1 ml sterile saline and diluted to a concentration of 80 μg/ml. The diluted peptide was separated into 1.5 ml aliquots and stored at -80°C. Immediately before the start of the experiments, one aliquot of the peptide solution was thawed and kept on ice for the duration of the experiments. Human GH-releasing hormone (hGHRH) (BACHEM; Torrance, CA) was dissolved in 1 ml sterile water to give a concentration of 0.5 mg/ml. The peptide was stored in 50 μl aliquots at -80°C. Just prior to the start of experiments, hGHRH was diluted with 2.45 ml of sterile saline to obtain a working concentration of 10 μg/ml. Again, the peptide solution was kept on ice for the duration of the experiment.

Measurement of Food Intake (Old mice: 14-15 months)

Two days before the day of injection, animals were placed in individual cages (standard mouse cages with corncob bedding were used) and allowed to acclimate for 24 hours with food and water *ad libitum*. The day before the injection, several large pellets of food were weighed and placed in the food hopper. Body weight was also measured. On the morning of the injection (around 10:00 a.m.) the remaining food was weighed to determine pre-injection 24-hour food intake. The bedding was also examined for small pieces of food that may have fallen into the cage. Throughout all of these experiments, this was not usually a problem as we used large pellets, which minimized small pieces of food breaking off and falling into the cage. The mice were weighed and then given an i.p. injection of saline or ghrelin (400 μg/kg) in a volume of 0.1 ml/20g BW. Immediately following the injection, the animal was returned to its cage containing a pre-weighed amount of food. Food intake was then measured at 30 minutes, 1, 2, 4, and 24 hours post-injection by weighing the amount of food remaining in the hopper and any pieces that may have fallen into the cage. After the 24-hour measurement, the mice were weighed and then returned to group housing, and allowed several weeks before the start of the GH studies.

Measurement of Food Intake (Young mice: 3-4 months)

Due to results obtained in feeding studies with old mice, some modifications were made for the feeding studies in young mice. Animals were weighed while in group housing to establish a "normal" body weight for each animal. Mice were then placed in individual cages and allowed to acclimate until their body weight returned to "normal", as measured in group housing. During this period, daily food intake was also measured. Once animals were acclimated, (5-7 days) feeding studies were conducted as described

above for old animals. Following completion of the light phase study, animals were given a 3-day drug washout period and the study was repeated during the dark phase. The injections were given just prior to the start of the dark phase and food intake was measured at 30 minutes, 1, 2, 4, and 24 hours post-injection. Animals were randomly assigned into treatment groups for both light and dark phase feeding studies. After the 24-hour dark phase measurement, the mice were weighed and returned to group housing. At least a 3-day washout period was allowed before the start of the GH studies.

GH Stimulation

These experiments were initiated and completed between 0900-1200h to minimize diurnal variations in GH levels between animals. Mice were anesthetized using a ketamine (100 mg/kg), xylazine (15 mg/kg) mixture administered i.p before the first blood sample was taken. Immediately following the first blood sample, the animals were injected with either rat ghrelin (400 μg/kg, i.p.) or saline. Blood samples were collected at 15, 30, and 60 minutes post injection by saphenous venipuncture. Serum was collected after centrifugation and frozen at –20°C until measurement of GH by RIA. Following the last blood sample, animals were allowed to recover from anesthesia and returned to animal care. Three to four weeks later, this experiment was repeated using the same protocol except that GHRH (50 μg/kg, i.p.) was injected instead of ghrelin. The dose of GHRH selected was based on previous studies reported in the literature [191, 192]. Following the completion of the GHRH study animals were again allowed to recover from anesthesia. Three days following this experiment, animals were sacrificed by decapitation and trunk blood, hypothalami and anterior pituitaries were collected, snap frozen on dry ice and stored at -80°C until further analysis.

Micro BCA Protein Assay

Protein content in liver and skeletal muscle samples was determined by the micro BCA protein assay (Pierce, Rockford, IL). Fifty microliters of 1.5N NaOH was added to a 50 μl sample of the tissue homogenate and samples were digested for 1-2 days until clear. The samples were assayed in duplicate at a dilution of 1:10,000 to fit within the range of the assay: 0.1-20 μg/ml. Unknown values were determined by comparison to a standard curve prepared from albumin. All unknowns were pipetted in duplicate and standards in triplicate. A working reagent was prepared using a mixture of the kit reagents MA, MB, and MC at a ratio of 25:24:1 and 150 μl of working reagent was added to each well of a 96 well plate. 150 μl of the diluted standard or sample was added to the appropriate well and the plate was incubated at 37°C for 30 minutes. The plate was cooled to room temperature and read on the SLT 400 AC plate reader at 575 nm and data was analyzed using a four parameter standard curve fit.

RNA Isolation

Tubes containing pituitaries and hypothalami were removed from the -80°C freezer and placed on dry ice. Tissues were then sonicated in 1 ml (hypothalamus) or 0.5 ml (pituitary) TRI REAGENT™ (Sigma; St. Louis, MO) and allowed to incubate at room temperature for 5 minutes. Following incubation, 200 μl chloroform per 1 ml starting TRI REAGENT™ was added, tubes were shaken for 15 seconds, and allowed to stand at room temperature for 2-15 minutes. The resultant mixture was centrifuged at 12,000 x g for 15 minutes at 4°C which separates the mixture into 3 phases: lower organic phase containing protein, interphase containing DNA and upper aqueous phase containing RNA. The aqueous phase was transferred to a fresh tube and 500 μl isopropanol per 1 ml

starting TRI REAGENT™ was added and the mixture was incubated for 5-10 minutes at room temperature. The RNA was pelleted by centrifugation at 12,000 x g for 10 minutes at 4°C. The supernatant was discarded and the RNA pellet was washed by adding 1 ml of 75% ethanol per 1 ml starting TRI REAGENT™. The tubes were vortexed and centrifuged at 12,000 x g for 5 minutes at 4°C. The ethanol supernatant was discarded and pellets were air-dried for 5-10 minutes. Pellets were resuspended in 50 µl DEPC treated water (Ambion, Austin, TX) and incubated in a 60°C water bath for 10 minutes to facilitate dissolution of the pellet. Samples were quantified by adding 2 µl RNA to 98 µl HPLC water and reading the absorbance at 260 nm. A 280 nm absorbance reading was also taken and the 260/280 ratio was used as a measure of RNA quality. After quantification, 5 µg of each RNA sample was DNase treated using a DNase treatment kit (Sigma). This reaction was done in a volume of 50 µl, which included 5 µg of RNA diluted in DEPC treated water, 5 µl 10X reaction buffer and 5 µl DNase I. The reaction was incubated at room temperature for 15 minutes and stopped using 5 µl of stop solution. The samples were then placed in a thermocycler for 10 minutes at 70°C. Following DNase treatment samples were requantified by measuring the absorbance at 260nm.

2-Step Real-Time RT-PCR

RNA samples obtained after isolation and DNase treatment were converted to single stranded cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Five hundred nanograms of RNA were reverse transcribed and the reaction volume was 30 µl. Thermocycling conditions were as follows: 25°C for

10 minutes, 48°C for 30 minutes and 95°C for 5 minutes. The resulting cDNA samples were stored at -80°C until real-time RT-PCR was performed.

Real-time RT-PCR reactions were conducted using primers and reagents from Applied Biosystems. Taqman Gene Expression Assays containing predesigned primers and probes based on the mouse GHS-R sequence (Genbank accession #NM177330 and AK049671) and eukaryotic 18s ribosomal RNA for the internal control (Genbank accession #X03205) were used. These assays utilize TaqMan DNA minor groove binding probes that are fluorescently labeled with FAM™ dye for detection. The total reaction volume was 25 µl and contained Taqman universal PCR master mix, Taqman gene expression assay mix containing primers and probes and cDNA. For GHS-R detection, 75 ng of cDNA was used and 10 ng of cDNA was used for the 18s wells. Reactions were run in triplicate in optical grade 96 well plates on an ABI Prism 7000 PCR machine. Expression levels of GHS-R in hypothalamus and pituitary were calculated using the $2^{-\Delta\Delta Ct}$ method [193] using 18s RNA as an internal control. To perform this calculation, the difference in cycle threshold (ΔCt) between the GHS-R and the corresponding 18s threshold for each sample was calculated. Mean ΔCts were then calculated for each genotype and tissue type. The ΔΔCt was then calculated by subtracting the mean ΔCt for WT mice from that of each genotype, and this was done separately for each sex and tissue type. Numbers were then normalized to the WT group within sex by calculating the fold change in expression ($2^{-\Delta\Delta Ct}$).

Statistical Analysis

All data shown are mean ± SEM. Statistical analyses were performed using SigmaStat 3.0 software. Serum IGF-I , pituitary GH, hypothalamic GHRH and SRIH

content, BW, and liver and skeletal muscle IGF-I content were analyzed by two-way ANOVA for the factors of genotype and age group. Post-hoc comparisons were done using the Tukey test. This test is used for pairwise comparisons and it is more conservative than other tests such as the SNK or Fisher's LSD test. Because GH is secreted in a pulsatile fashion, the variances between groups are unequal. In addition, the limit of detection of the GH RIA is 2.5 ng/ml making the data not normally distributed. Because of these two conditions, unequal variance and lack of normal distribution, the data would require nonparametric analysis. We therefore transformed the GH data using a natural log transformation to allow for parametric analysis. After transformation, differences in GH levels among genotypes within each age group using two-way ANOVA with post-hoc comparisons using the Tukey test. Differences in ghrelin levels were analyzed by two-way ANOVA for the factors of genotype and sex with post-hoc comparisons using the Tukey test. Differences in GH levels in response to injection of ghrelin were analyzed by two-way ANOVA for the factors of genotype and treatment at each time point with post-hoc comparisons using the Tukey test. Cumulative food intake at each time point post injection is analyzed using two-way ANOVA for the factors of genotype and treatment with post-hoc comparisons using the Tukey test. Differences in GHS-R levels in the hypothalamus and pituitary were determined by one-way ANOVA for the factor of genotype. We did not make any comparisons between sexes or between tissue types. The delta Ct values were used for the statistical analysis and data was expressed in terms of the delta Ct values and fold change compared to WT within sex for either hypothalamus or pituitary.

CHAPTER 3
CHARACTERIZATION OF THE GROWTH HORMONE SECRETORY AXIS IN MC3R KO AND MC4R KO MICE

Introduction

The central melanocortin system plays an important role in the control of food intake, energy homeostasis, and metabolism. Studies utilizing knockouts of the melanocortin 3- and 4 receptors (MC3R KO, MC4R KO) have revealed distinct functions of the two receptors that act to modulate overall body composition [45]. MC3R KO mice exhibit maturity-onset obesity characterized by elevated fat mass and reduced lean body mass as compared to their wild-type littermates [32]. However, these mice do not increase their food intake nor gain excessive weight suggesting that their obesity is caused by changes in metabolism [51]. In addition, both male and female MC3R KO mice exhibit reduced bone lengths and are shorter than wild-type controls [32]. Mice deficient in the MC4R (MC4R KO) also develop age-onset obesity similar to that observed in the MC3R KO mouse [31]. However, obesity in this model is thought to be a result of hyperphagia, as MC4R KO mice exhibit a 46% increase in food consumption as compared to wild-type littermates. These mice have increased fat mass with no change in lean body mass and are hyperleptinemic and hyperinsulinemic. Interestingly, MC4R KO mice are significantly longer than controls which is opposite of that seen in the MC3R KO mouse [31].

Growth hormone (GH) has a wide range of biological functions and acts on almost all tissues both directly and indirectly via its stimulation of insulin-like growth factor-I

(IGF-1) [59]. GH has repeatedly been shown to stimulate linear growth and to affect body composition by increasing lean body mass and decreasing fat mass. In obesity, circulating levels are extremely low and the amplitude of GH pulses can be nearly undetectable [96]. Because of the interesting phenotypes of both the MC3R KO and MC4R KO mice, we sought to examine the various components of the GH secretory axis in these animals. We wanted to investigate whether the alterations in body composition of the MC3R KO and MC4R KO mice characterized by elevated fat mass and reduced or normal lean body mass are a result of changes in GH secretion or from alterations in various components of the GH axis. We also investigated the possible role of GH in the longer body length observed in the MC4R KO mouse and/or the shorter body length as seen in the MC3R KO mouse. The opposite effects of MC3R or MC4R deletion on somatic growth suggest that there may be some differential effects of activation of these receptors on the GH axis. In addition, we evaluated the effects of age on the GH-IGF-I axis of these animals by utilizing animals of various age groups.

Materials and Methods

Animals

Wild-type (WT), MC3R KO, and MC4R KO mice were obtained from the breeding colony of Dr. Carrie Haskell-Luevano. Four age groups were selected to monitor the parameters of interest: 4-6, 8-9, 11-13, and 17-19 months old, in large part due to availability of these valuable animals. Body weight was measured prior to sacrifice by decapitation. Trunk blood was collected, centrifuged and serum was separated and stored at -80°C until use in GH and IGF-I RIA. Hypothalami, anterior pituitaries, and liver and skeletal muscle samples were removed, snap frozen on dry ice, and stored at -80°C until assayed.

Radioimmunoassays (RIA)

GH RIA

Pituitary and serum GH concentrations were determined by RIA using reagents supplied by the NIDDK's National Hormone and Peptide Program and Dr. A.F. Parlow. Pituitaries were sonicated in 0.1 M borate buffer, pH 9.0 and centrifuged. Supernatant was collected and stored at -20 °C until use in assay. The mouse GH RIA has a sensitivity of 2.5 ng/ml and a range of detection from 2.5 ng/ml to 200 ng/ml. The serum GH RIA intra-assay coefficient of variation was 3.56% and the pituitary GH RIA intra- and inter-assay coefficients of variation were 2.68% and 2.83%, respectively.

IGF-I RIA

Serum IGF-I was measured by RIA following extraction by the acid/ethanol procedure. Tissue IGF-I levels were measured in liver and skeletal muscle following extraction. Values were expressed in terms of the BACHEM IGF-I reference preparation. The IGF-I RIA has a sensitivity of 0.1 ng/ml and a range of detection from 0.1 ng/ml to 20 ng/ml. The serum IGF-I RIA intra-assay coefficient of variation was 6.04%. The tissue IGF-I RIA intra-assay coefficient of variation was 3.55% and the inter-assay coefficient of variation was 4.0%.

GHRH RIA

Hypothalami were extracted before use in the GHRH RIA. The samples were sonicated in 2.0 M acetic acid and subsequently boiled for 5 minutes. The samples were then briefly frozen, thawed, and centrifuged. The supernatant was split into two tubes, one for GHRH analysis and the other for SRIH analysis. The samples were then lyophilized and reconstituted with 1 ml assay buffer the night before the RIA. Hypothalamic GHRH content was measured using a mouse GHRH RIA as previously

described [187]. The antibody to mouse GHRH was kindly provided by Dr. L.A. Frohman and the tracer was purchased from Phoenix Pharmaceuticals, Inc. This RIA has a range of detection from 0.04-10 ng/ml and the data was expressed as ng/hypothalamus. All samples were run in a single assay and the intra-assay coefficient of variation was 3.72%.

SRIH RIA

Hypothalami were extracted as described for the GHRH RIA. Hypothalamic SRIH content was measured using a rat SRIH RIA as previously described [188]. This RIA has a sensitivity of 2.5 ng/ml and data was expressed as ng/hypothalamus. All samples were run in a single assay and the intra-assay coefficient of variation was <5%.

Statistics

Values are expressed as means ± SEM. Differences between groups were analyzed using two-way ANOVA followed by the Tukey test for post-hoc comparisons. $p<0.05$ was considered significant.

Results

Mouse Body Weights

Both male and female MC4R KO mice exhibit significantly higher body weights at 4-6, 8-9, and 11-13 months of age when compared to wild-type (WT) controls as shown in ***Figure 3-1***. By 17-19 months of age, the body weights of WT animals have increased, and the body weight of MC4R KO mice have decreased such that there is no longer a difference between the two groups. The body weights of MC3R KO are generally not greater than WT controls except in 8-9 month old female mice.

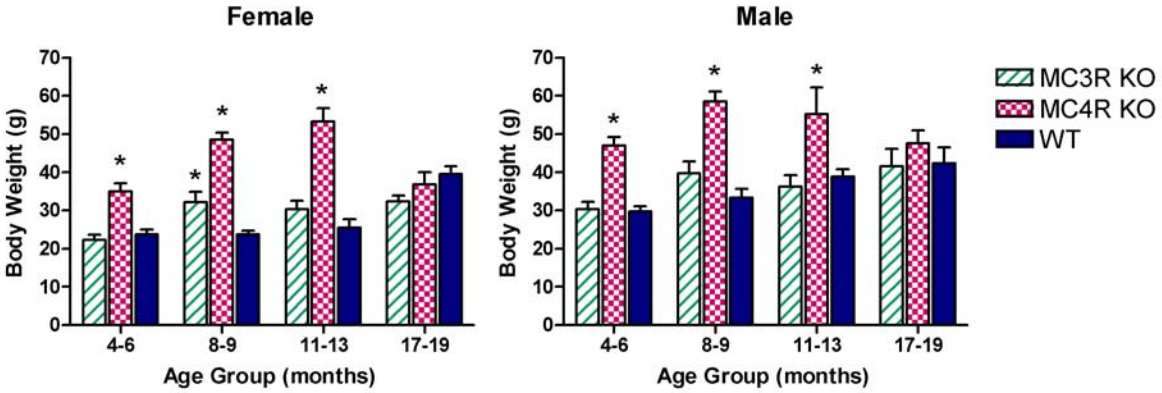

Figure 3-1. Mouse body weights. n= 5-10 per group except 11-13 month MC4R KO males where n=3. Bars represent mean ± SEM *p<0.05 vs WT within age group

Serum GH Levels

Both male and female MC4R KO mice displayed trends towards lower GH levels in all age groups as shown in ***Figure 3-2***. ANOVA revealed a main effect of genotype in both male ($F(2, 71 = 8.52$; $p<0.001$) and female ($F(2, 74 = 12.65$; $p<0.001$) mice which was due to a significant difference between MC4R KO and WT. MC3R KO mice were not significantly different from WT. There was no main effect or interaction involving age in either male or female mice.

Serum IGF-I Levels

In female mice, there was a significant interaction between genotype and age group ($F(6,75 = 3.56$; $p=0.004$). MC4R KO females exhibited higher IGF-I levels at 8-9 months and lower levels at 17-19 months as compared to age-matched wild type controls as shown in ***Figure 3-3***. ANOVA revealed only a main effect of genotype in male mice ($F(2, 64 = 6.73$; $p=0.002$) which was due to a difference between MC4R KO mice and WT mice. The differences in IGF-I levels between male MC4R KO and WT mice does

not depend on the age of the animal. Both male and female MC3R KO mice showed no difference in circulating IGF-I levels from WT in any age group tested.

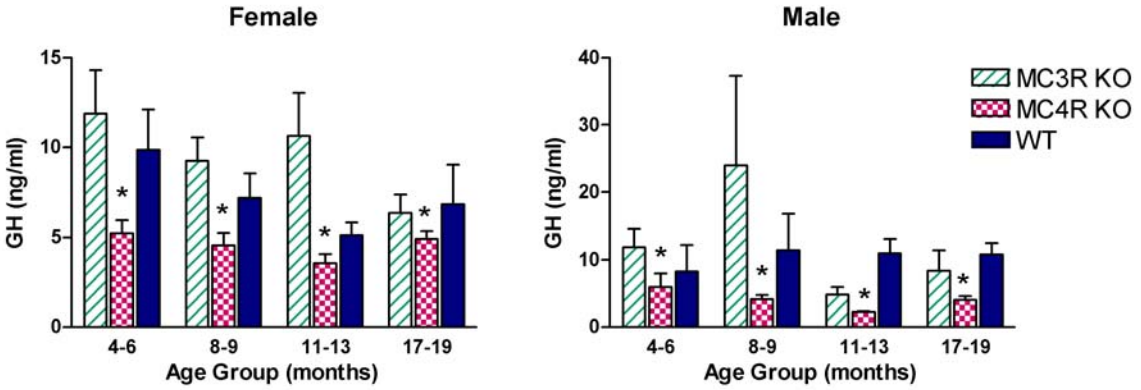

Figure 3-2. Serum GH levels. n= 5-10 per group except 11-13 month MC4R KO males where n=3. Bars represent mean ± SEM *p<0.05 vs WT (main effect not involving age).

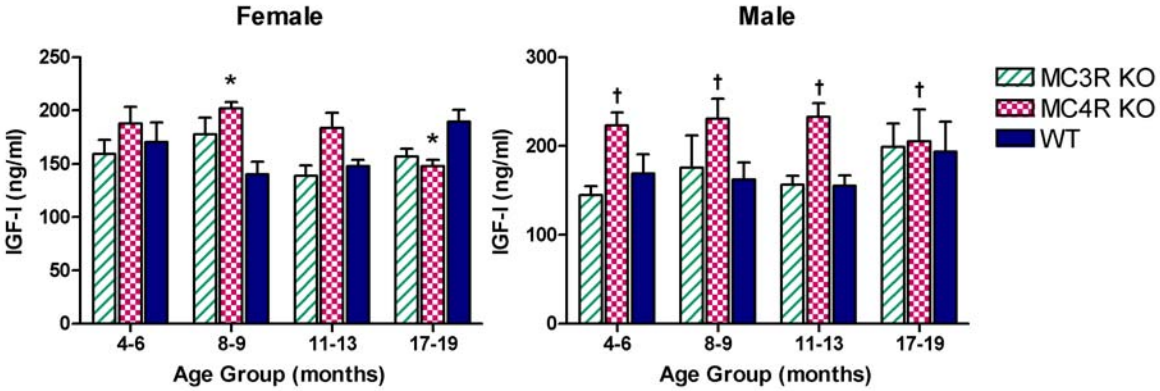

Figure 3-3. Serum IGF-I levels. n= 5-9 per group except male 11-13 month MC4R KO and 17-19 month WT where n=3. Bars represent mean ± SEM. *p<0.05 vs WT within age group. †p<0.05 vs WT (main effect not involving age).

Pituitary GH Content

Levels of pituitary GH content in the various age groups are shown in ***Figure 3-4***. In male mice, ANOVA revealed a main effect of genotype ($F(2, 64 = 6.71$; $p=0.002$) which was due to a difference between WT and MC4R KO mice. There was no main effect or interaction involving age in male mice. In female mice, ANOVA revealed a

main effect of genotype ($F(2, 74 = 8.48$; $p<0.001$), a main effect of age group ($F(3, 74 = 7.35$; $p<0.001$), and a significant interaction between genotype and age group ($F(6, 74 = 3.23$; $p=0.007$). Female MC3R KO mice (4-6 and 11-13 months old) and MC4R KO mice (4-6 months old) have significantly reduced pituitary GH content compared to WT controls. In addition, pituitary GH content declines with age in female WT mice whereas there is no age-associated decline in either female MC3R KO or MC4R KO mice. There was no difference in pituitary GH content among any genotype in 17-19 month old female mice.

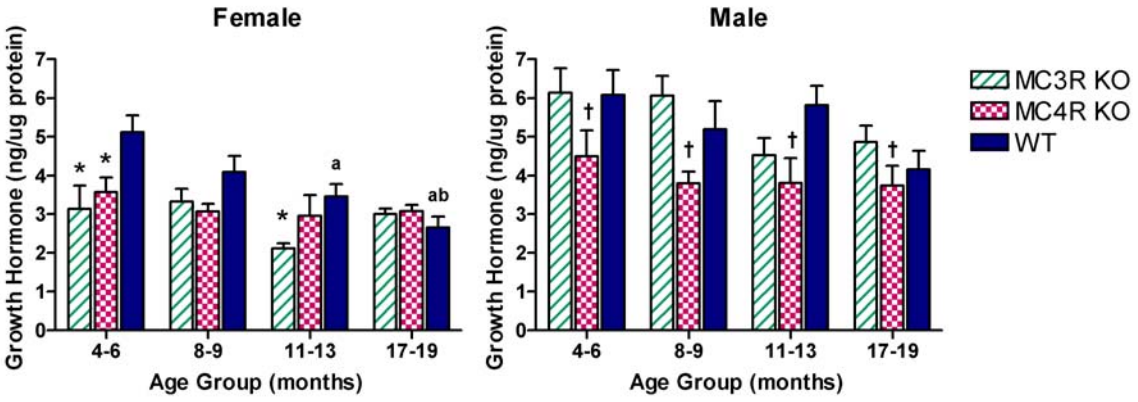

Figure 3-4. Pituitary GH content. n= 5-9 per group except male 11-13 month MC4R KO and 17-19 month WT where n=3. Bars represent mean ± SEM *$p<0.05$ vs WT within age group. †$p<0.05$ vs WT (main effect not involving age). [a]$p<0.05$ vs 4-6 month old WT. [b]$p<0.05$ vs 8-9 month old WT.

Liver and Skeletal Muscle IGF-I Content

To further characterize the IGF-I status of MC3R KO and MC4R KO animals, we measured liver and skeletal muscle IGF-I content in the youngest (4-6 months) and the oldest (17-19 months) age groups. The content of IGF-I in these tissues was expressed as either ng IGF-I/g tissue or pg IGF-I/ mg protein to normalize the data. The liver results are shown in ***Figure 3-5*** and the skeletal muscle results are shown in ***Figure 3-6***. We did not detect any significant differences in liver IGF-I content in any of the groups tested.

In male mice, there was no difference in skeletal muscle IGF-I content between any of the genotypes regardless of how data was expressed. Female MC3R KO mice had significantly lower skeletal muscle IGF-I levels compared to WT when the data was expressed in terms of grams of tissue. When expressed as a function of protein content, the skeletal muscle IGF-I content in female MC3R KO was not significantly reduced compared to WT, however a trend towards reduced levels is evident.

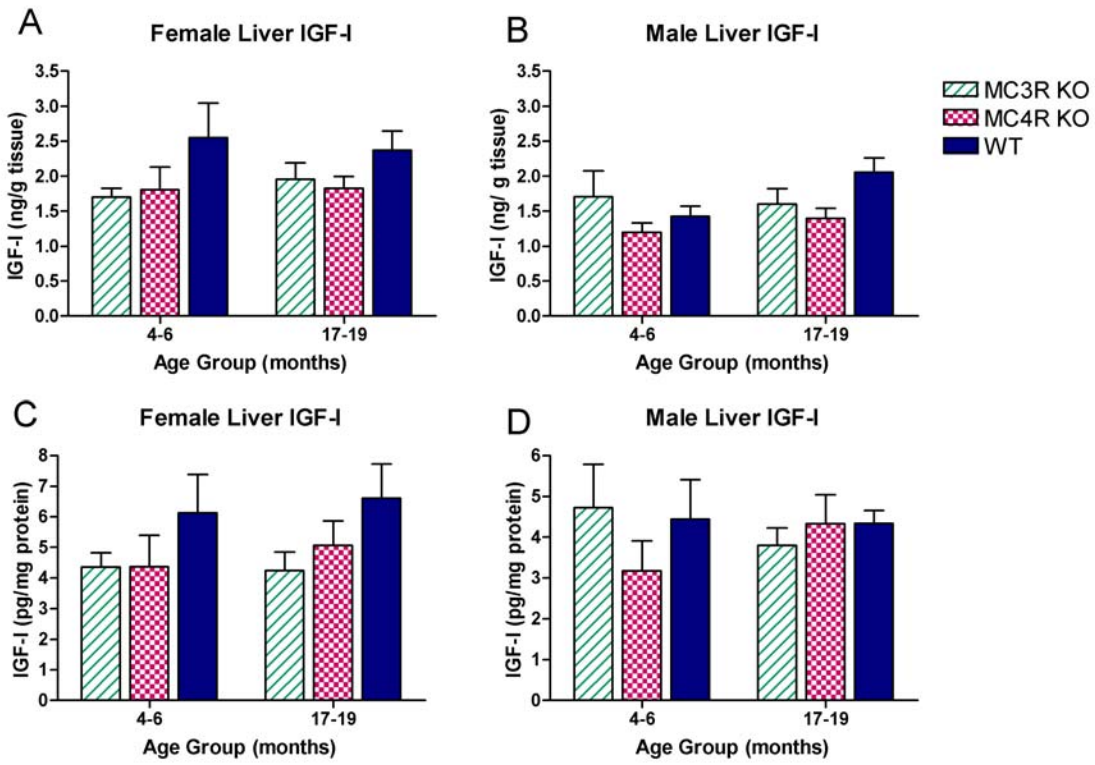

Figure 3-5. Liver IGF-I content. n= 5-9 per group except male 17-19 month WT where n=3. Graphs A and B show liver IGF-I content expressed as ng IGF-I/ g tissue. Graphs C and D show liver IGF-I content expressed as pg IGF-I/mg protein. Bars represent mean ± SEM

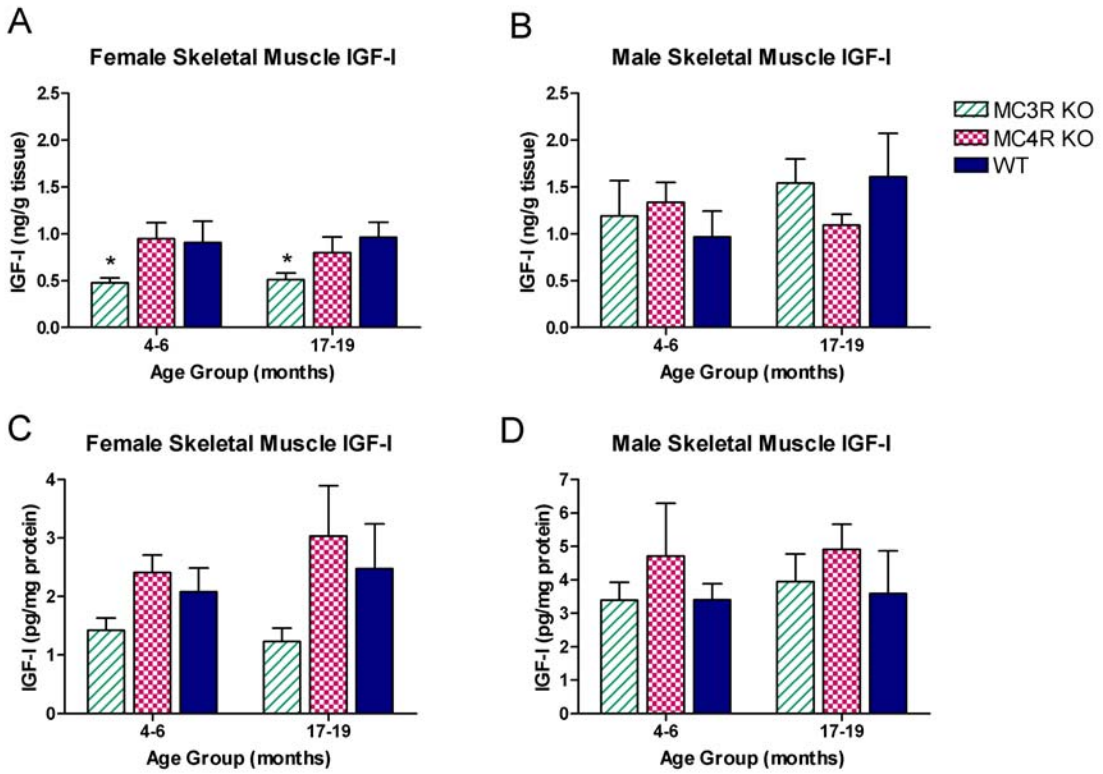

Figure 3-6. Skeletal muscle IGF-I content. n= 5-9 per group except male 17-19 month WT where n=3. Graphs A and B show IGF-I content expressed as ng IGF-I/ g tissue. Graphs C and D show IGF-I content expressed as pg IGF-I/mg protein. Bars represent mean ± SEM *p<0.05 vs WT.

Hypothalamic GHRH Content

The GHRH peptide content in the hypothalamus was measured in the youngest and oldest age groups, 4-6 months and 17-19 months (***Figure 3-7***). In female mice, ANOVA revealed a main effect of genotype ($F_{(2,35)} = 5.25$; $p=0.01$), which was due to a difference between MC3R KO and WT. There was no difference in GHRH content between female MC4R KO or WT animals. In male animals, there was no difference between MC3R KO or MC4R KO mice and WT mice. GHRH levels in male MC4R KO mice actually increased with age ($p=0.02$), which is opposite of that observed in male WT mice that show a trend towards reduced GHRH content with age (results not significant).

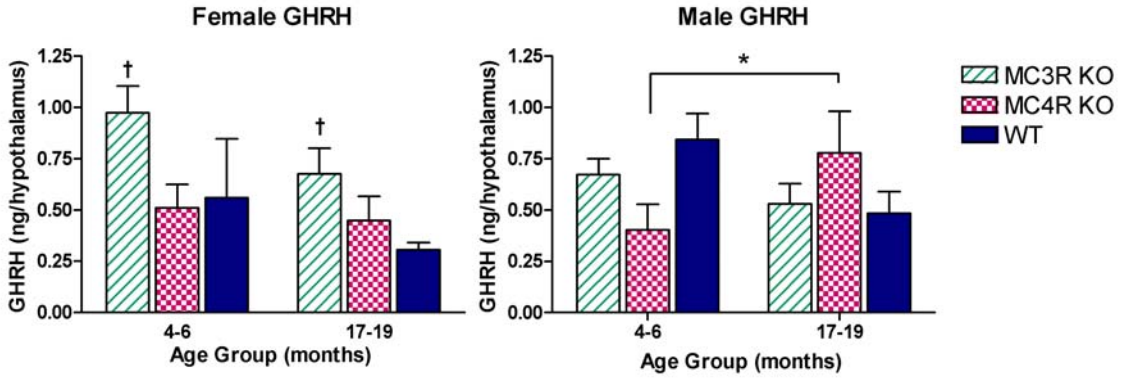

Figure 3-7. Hypothalamic GHRH content. n=4-9 per group. Bars represent mean ± SEM *p<0.05 between 4-6 month and 17-19 month male MC4R KO. †p<0.05 vs WT

Hypothalamic SRIH Content

The SRIH peptide content in the hypothalamus was measured only in the youngest and oldest age groups, 4-6 months and 17-19 months and the results are shown in ***Figure 3-8***. There was no significant difference in hypothalamic SRIH levels between WT and either MC3R KO or MC4R KO mice in both males and females. Only female MC3R KO showed a significant reduction in SRIH levels from 4-6 months to 17-19 months of age (p=0.01).

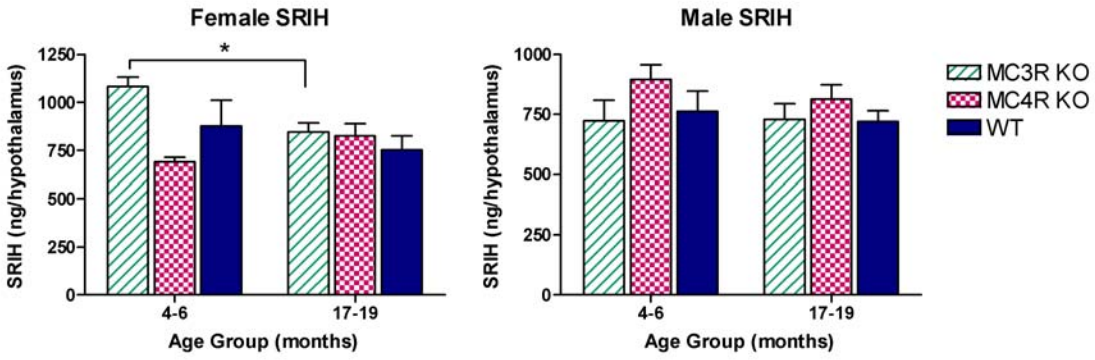

Figure 3-8. Hypothalamic SRIH content. n= 5-9 per group Bars represent mean ± SEM *p<0.05 between 4-6 month and 17-19 month female MC3R KO mice.

Discussion

This study was initiated to characterize the GH secretory axis in MC3R KO and MC4R KO male and female mice throughout their lifespan. Because both MC3R KO and MC4R KO mice exhibit age-onset obesity, we studied four age groups: 4-6 months, 8-9 months, 11-13 months, and 17-19 months old. We sought to identify any age-related changes that may occur in the GH-IGF-I axis that could contribute to the observed obesity and body length phenotypes of the knockout mice. The age groups of animals selected for this study were primarily based on availability of animals, however, it is important to correlate these age groups to various stages in the mouse lifespan. The average lifespan of a mouse is between 2 and 3 years depending on the strain [194]. Therefore, our oldest age group, 17-19 months, can be considered early or healthy aging. It has been suggested that observations in aging mice should be compared with mice aged 4-6 months rather than to mice 2-3 months of age, which are not yet mature [195]. Thus, the 4-6 month group was used as a comparison to the aged mice in our studies. Reproductive function in female mice begins around 1 month of age [196] and usually lasts until 9-13 months of age [197]. We therefore feel that our age groups cover both young and old animals as well as animals with declining reproductive function.

The body weights of male and female MC3R KO, MC4R KO, and WT mice at various age groups were measured before sacrificing the animals. As expected and previously reported in the literature [31], both male and female MC4R KO mice exhibit significantly higher body weight at 4-6, 8-9, and 11-13 months of age when compared to wild-type (WT) controls. Interestingly, by 17-19 months of age, the body weights of WT animals have increased while the body weights of MC4R KO animals have decreased such that there is no longer a difference between the groups.

Female MC3R KO mice have significantly higher body weight at 8-9 months of age but do not differ from their WT controls in any other age group. In addition, there was no difference in body weights between male MC3R KO mice and their age-matched WT controls. This agrees with previous reports of MC3R KO mice in that they do not differ greatly in body weight compared to WT mice. However, body composition analysis of MC3R KO mice reveals that they are obese in that they have a greater amount of fat mass and a decrease in lean body mass [32].

To assess basal GH status, we measured circulating GH and pituitary GH content in both male and female mice. Compared to wild-type controls, both male and female MC4R KO mice displayed reduced serum GH levels compared to WT while there was no difference between MC3R KO and WT mice. Pituitary GH content was reduced in young female MC4R KO and MC3R KO animals but was not different from WT controls in the oldest age group. In addition, GH pituitary content declines with age in female WT animals whereas this trend is not observed in either MC3R KO or MC4R KO. From this data, it can be argued that pituitary GH production in young and middle-aged female MC3R KO and MC4R KO mice is similar to that seen in aged WT mice and suggests that pituitary function may be altered in these animals much earlier than that seen in WT animals. Young and middle-aged male MC4R KO mice displayed a trend towards reduced pituitary GH content compared to WT controls and as observed in female mice, there was no difference between the groups at 17-19 months of age. Although not as marked as that seen in females, pituitary GH content declines somewhat with aging in male WT mice; whereas, pituitary GH content in male MC4R KO mice stays the same throughout the lifespan with levels similar to those of aged WT mice. In other words, the

pituitary content of a young male MC4R KO mouse resembles that of an aged WT mouse, similar to the trend observed in female mice. In addition, in contrast to female mice, male MC3R KO mice do not display reduced pituitary GH content in any age group compared to WT controls. A decline in the amount of pituitary GH has been observed in obese Zucker rats which is due to a decrease in somatotroph cell number rather than a decrease in the GH content per cell [198]. However, the reduction in pituitary GH content is not usually considered the primary cause of the diminished GH secretion seen in obesity and aging as the pituitary is still responsive to various GH-releasing compounds [97]. Although it is not considered a primary factor, reduced pituitary GH content is likely a contributor to the lower GH levels observed in obesity and aging and as well as in MC4R KO mice.

Because GH is secreted in a pulsatile fashion, the use of a single sample to assess circulating GH levels can be very misleading. Many of the actions of GH are mediated by its primary effector protein, IGF-I, which has a longer half-life than GH. IGF-I is also secreted more continuously than GH and is therefore considered a useful indicator of GH secretory status [199]. Despite the lowered circulating GH levels, serum IGF-I levels remained unchanged or were significantly elevated in MC4R KO mice, except for females 17-19 months of age in which IGF-I levels were reduced compared to wild-type controls. MC3R KO females 17-19 months of age had significantly reduced IGF-1 levels, similar to that observed in 17-19 month MC4R KO females while no differences in serum IGF-I levels were observed in any other MC3R KO groups compared to their WT controls.

The findings of increased IGF-1 levels and lower circulating GH levels in MC4R KO mice are similar to those observed in other cases of obesity. Children with idiopathic obesity [200] and obese Zucker rats [201] exhibit GH deficiency while maintaining normal or increased levels of IGF-I and linear growth rates. Thus, alterations in the GH-IGF-I axis may be responsible, in part, for the increased linear growth observed in the MC4R KO mice, specifically through actions of IGF-I. In humans, IGF-I levels usually correlate with body size and tall children have been shown to have elevated plasma IGF-I levels [66] supporting a role for IGF-I in regulating growth. The higher insulin levels observed in MC4R KO mice [31] may directly affect circulating IGF-I or IGF binding proteins contributing to normal or elevated levels of IGF-I in spite of lowered GH secretion. Insulin causes a decrease in IGF-I binding protein levels, which may result in higher levels of free, active IGF-I [201]. In addition, peripheral GH sensitivity may be increased in obesity due to an increase in GH receptor content and GH binding protein levels as reported in obese patients [202, 203], thus contributing to the normal or elevated IGF-I levels observed. It is also possible that the elevated IGF-I levels observed in MC4R KO mice may be directly related to the reduced pituitary GH content, as well as the lower circulating GH levels observed in these mice. This is due to that fact that IGF-I acts as a negative feedback signal to the pituitary and hypothalamus causing an inhibition of GH secretion [64, 82].

In liver specific IGF-I gene knockout mice, serum IGF-I levels are significantly reduced, but these animals do not show a decrease in linear growth suggesting that circulating IGF-I levels may not be a major regulator of growth [192]. It has been suggested that paracrine or autocrine derived IGF-I may be important for regulating

growth. Female MC3R KO mice do in fact demonstrate reduced skeletal muscle IGF-I content, which may contribute to their observed decrease in lean body mass. However, reduced skeletal muscle IGF-I levels were not observed in male MC3R KO mice, therefore a direct connection between IGF-I content in peripheral tissues and the phenotypes of MC3R KO mice cannot be made. The obesity syndrome of MC4R KO mice is characterized, in part, by elevated fat mass and normal levels of lean body mass [31]. The normal levels of skeletal muscle IGF-I content in these animals may contribute to the maintenance of normal lean body mass in spite of elevated fat mass and reduced GH secretion. Bone IGF-I levels were not measured in this work; however, this would be important for future studies and would help determine if changes in linear bone growth in these animals is due to altered bone IGF-I content.

Impaired function of hypothalamic neurons producing GHRH has been suggested to be a major factor in the reduced GH secretion observed in obese male Zucker rats [204, 205]. In addition, pituitary responsiveness to GHRH is decreased in obese rats, both *in vivo* and *in vitro* [198, 206]. Therefore, we evaluated whether hypothalamic GHRH content was altered in obese MC3R KO or MC4R KO mice. In female MC3R KO mice hypothalamic GHRH content was greater than WT mice, which may be important in preserving the normal levels of GH observed in these animals. There was also an interesting finding in male mice in regards to the patterns of change in GHRH content with age in MC4R and WT mice. Male WT mice show an age-associated decrease in GHRH content although this effect is not significant. This agrees with previous findings in aged male animals, which demonstrate that hypothalamic GHRH content and gene expression are reduced, compared to young animals [115, 207]. In

contrast, male MC4R KO mice actually show an increase in GHRH content with age. In the younger age group (4-6 months), male MC4R KO mice have reduced GHRH content compared to age-matched WT mice, and this observation is almost significant ($p=0.06$). Previous studies in rats have shown that GHRH gene expression is reduced in obese male rats compared to lean but is not altered in obese female rats [204]. When we consider the body weight profiles of these animals, MC4R KO mice lose weight as they age and WT mice gain weight as they age. In our oldest age group, 17-19 months, the body weights of all three genotypes are similar. Based on our observations and those of other groups, it is likely that changes in GHRH content with age are directly influenced by body weight, at least in male animals.

It has been suggested that an increase in central SRIH tone may contribute to the hyposecretion of GH in obesity. Administration of drugs such as pyridostigmine and arginine, which lower hypothalamic SRIH release, improves the GH response to GHRH in obese patients [96]. In our studies, there was no difference in hypothalamic SRIH content between WT and MC3R KO or MC4R KO mice in either males or females. Female MC3R KO mice did show an age-related reduction in SRIH content but this was not observed in any of the other groups. This agrees with previous findings of normal SRIH levels in obese rats and mice with reduced pituitary GH content [205, 207] and suggests that hypothalamic SRIH content is not related to GH function in these animals.

It is also important to note that our measurements of hypothalamic GHRH and SRIH only reflect the levels of the protein within the tissue. We did not address the possibility of altered release and this may be a more important factor than the just the tissue content of the peptide. In aging, it has been demonstrated that there is a shift in the

primary type of SRIH released from the hypothalamus. SRIH-28, which is more potent that SRIH-14, is preferentially released from aged hypothalami suggesting that this form of SRIH may be important for inhibiting GH function in aged animals [114]. Thus, studies addressing the release profiles of both GHRH and SRIH would further our analysis of hypothalamic influences on GH secretion in these animals.

This data suggests that alterations in the GH-IGF-I axis may contribute to and/or result from the obesity syndromes observed in MC3R and MC4R KO mice. Our data also shows the existence of sexual dimorphism in the GH axis in these models of obesity and is in line with similar observations in obese male and female Zucker rats [204]. Studies in humans suggest that the pulsatile aspect of GH secretion is more important for mediating many of its actions on peripheral tissues. Discrete GH peaks are able to stimulate linear growth and influence IGF-I gene expression in skeletal muscle better than basal GH exposure [208]. Therefore, assessment of GH pulsatility in these animals would be beneficial to better relate changes in the GH axis with the phenotypes of the knockout mice. However, serial blood sampling over a long enough time period to accurately assess pulsatility was not feasible in mice due to their small size and blood volume.

It would also be interesting to further assess the GH axis in these animals by measuring levels of IGF-BPs as well as IGF-I content in other tissues such as bone and pituitary. Alterations in bone IGF-I levels may be important for mediating the changes in linear growth observed in these animals. In addition, analysis of peripheral receptor levels for GH and IGF-I as well as GHRH and SRIH receptors in the pituitary would help to determine if altered sensitivity and/or signaling mechanisms could contribute to the

phenotypes of these animals. Extension of our studies would help to further elucidate the potential interactions of the GH secretory axis and the melanocortin system in the control of body weight and energy homeostasis and promote a better understanding of the changes that occur in the GH axis with both obesity and aging.

CHAPTER 4
GHRELIN-INDUCED FOOD INTAKE IS REDUCED IN MC3R KO AND MC4R KO MICE

Introduction

Ghrelin, the endogenous ligand for the (GHS-R), potently stimulates GH secretion [79] and affects energy regulation by stimulating food intake and adiposity in rodents [151, 173, 176]. Circulating ghrelin levels and stomach ghrelin mRNA levels are modified by short-term changes in energy status as they increase during fasting [144, 146] and before meals [147] and decrease following meals [144, 148]. Ghrelin levels are also influenced by chronic states of altered energy balance as they are increased in anorexic patients [43] and decreased in human obesity [119]. In the arcuate nucleus (ARC), GHS-R mRNA is co-localized with neurons containing GH-releasing hormone (GHRH) and somatostatin (SRIH) and 94% of neuropeptide Y (NPY)/agouti-related peptide (AGRP) neurons contain GHS-R mRNA [136]. The orexigenic actions of ghrelin are thought to be mediated primarily by activation of these leptin responsive NPY/AGRP neurons in the ARC. Central and peripheral administration of ghrelin induces *c-fos* in these neurons [173, 180] and increases hypothalamic NPY and AGRP mRNA expression [131]. In addition, administration of antibodies and antagonists of NPY and AGRP [173] and genetic deletion of both NPY and AGRP [57] abolishes the feeding response to ghrelin. This data are suggestive of a critical role for both NPY and AGRP in mediating the orexigenic effects of ghrelin.

The ability of AGRP to stimulate food intake involves its antagonistic actions on melanocortin receptors, including the melanocortin 3 and 4 receptors (MC3R, MC4R) [41]. Studies utilizing mice deficient in the MC3R and MC4R have revealed distinct functions of the two receptors that act to modulate overall body composition [56]. MC4R knockout (MC4R KO) mice exhibit maturity-onset obesity characterized by excess food intake, elevated fat mass and normal levels of lean body mass as compared to their wild-type (WT) littermates [31, 55]. MC3R knockout (MC3R KO) mice also develop obesity, which is characterized by increased fat mass and reduced lean body mass and is thought to result from changes in metabolism, such as increased feed efficiency [32], rather than hyperphagia as in MC4R KO mice**.** In the present study, we sought to further investigate the role of AGRP/melanocortin signaling in ghrelin-induced food intake by utilizing both the MC3R KO and MC4R KO mouse models. We hypothesized that deletion of either of the central melanocortin system receptors, MC3 or MC4, would result in a diminished feeding response to peripherally administered ghrelin. We were also interested in determining if sensitivity to the feeding effects of ghrelin is altered with age. Finally, we measured circulating ghrelin levels in these animals to determine if their obesity syndromes are accompanied by a reduction in ghrelin levels as seen in human obesity and other animal models of obesity.

Methods

Animals

Male and female MC3R KO, MC4R KO, and wild-type (WT) mice aged 3-5 and 14-15 months were used for these studies. The MC3R KO mice were originally provided by Merck [32] and the MC4R KO mice were provided by Millennium Pharmaceuticals [31] but our mice were bred in the laboratory of Dr. Carrie Haskell-Luevano. Animals

were group-housed (3-4 per cage) and received food and water *ad libitum* until the start of the feeding studies. Each genotype was divided into 2 groups: a control group receiving an intraperitoneal (i.p.) injection of sterile saline or a treatment group receiving an i.p. injection of ghrelin at a dose of 400 μg/kg. This ghrelin dose was based on previous studies reported in the literature utilizing mice and peripheral injections of ghrelin to measure both food intake and growth hormone secretion [174, 189-191]. For the measurement of serum ghrelin levels, WT, MC3R KO and MC4R KO mice aged 11-13 months were sacrificed by decapitation and trunk blood was collected, centrifuged and serum stored at -20°C until day of assay. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Florida and were conducted in accordance with animal welfare guidelines. Genotypes of animals were verified upon sacrifice by PCR as described in the ***General methods section***.

Peptide Preparation

Rat ghrelin (BACHEM; Torrance, CA) was dissolved in 1 ml sterile saline and diluted to a concentration of 80 μg/ml. The diluted peptide was separated into 1.5 ml aliquots and stored at -80 °C. Immediately before the start of the experiments, one aliquot of the peptide solution was thawed and kept on ice for the duration of the experiments.

Feeding Studies

Three days (14-15 month old mice) or 5-7 days (3-5 month old mice) prior to the start of feeding studies, animals were housed individually and allowed to acclimate. The light phase studies were begun in the morning (0900-1100h) and the dark-phase studies were begun at the onset of the dark period (around 1800h). Non-fasted animals (n= 3-9

per group) were injected with either rat ghrelin (400μg/kg, i.p.) or saline and returned their cages containing a preweighed amount of food. Food intake was measured at 30 min, 1, 2, 4 and 24 hours post injection by weighing the amount of food remaining in the hopper. After the 24-hour measurement, animals were returned to group housing and allowed several weeks before the start of the GH studies (described in ***Chapter 5***).

Radioimmunoassay (RIA)

Serum ghrelin levels were analyzed (n= 3-6 animals per group) using a total ghrelin RIA kit (Linco Research, Inc., St. Charles, MO), which measures both the octonylated and des-octonylated forms of ghrelin in human, rat, mouse, and canine samples. The assay sensitivity was 100 pg/ml and the range of detection was from 100 pg/ml to 10,000 pg/ml when using a 100 μl sample size. Due to higher levels of ghrelin in mouse serum, a sample size of 25 μl was used for unknowns and the volume was made up to 100 μl using supplied assay buffer. Again, all samples were assayed together to minimize inter-assay variability. The intra-assay coefficient of variation was 3.2%.

Real-Time RT-PCR for Hypothalamic GHS-R mRNA levels

Total RNA was isolated from the hypothalamus of the young mice collected after sacrifice. RNA was isolated using TRIreagent (Sigma, St. Louis, MO) and was subsequently treated to remove any contaminating DNA using a DNase treatment kit (Sigma, St. Louis, MO). RNA was then reverse-transcribed using the Reverse Transcription reagents kit (Applied Biosystems, Foster City, CA) and cDNA was stored at -80°C until PCR was performed.

Real-time RT-PCR reactions were conducted using primers and reagents from Applied Biosystems. Taqman Gene Expression Assays containing predesigned primers

and probes based on the mouse GHS-R sequence (Genbank accession #NM177330 and AK049671) and eukaryotic 18s ribosomal RNA were used. The total reaction volume was 25 ul and contained Taqman universal PCR master mix, Taqman gene expression assay mix containing primers and probes and cDNA. Expression levels of GHS-R in the hypothalamus were calculated using the $2^{-\Delta\Delta Ct}$ method [193] using 18s RNA as an internal control. To perform this calculation, the difference in cycle threshold (ΔCt) between the GHS-R and the corresponding 18s threshold for each sample was calculated. Mean ΔCts were then calculated for each genotype and tissue type. The ΔΔCt was then calculated by subtracting the mean ΔCt for WT mice from that of each genotype, and this was done separately for each sex and tissue type. Numbers were then normalized to the WT group within sex by calculating the fold change in expression ($2^{-\Delta\Delta Ct}$).

Statistics

All data shown are mean ± standard error of the mean (SEM). Statistical analysis was performed using SigmaStat 3.0 software. Differences in serum ghrelin levels were analyzed by two-way ANOVA for the factors of genotype and sex with post-hoc comparisons using the Tukey test. Cumulative food intake at each time point post-injection was analyzed using two-way repeated measures ANOVA for the factors of genotype and treatment with post-hoc comparisons using the Tukey test. Differences in GHS-R mRNA levels were analyzed by one-way ANOVA using the delta Ct data for analysis. Statistical significance was set at $p<0.05$.

Results

Serum Ghrelin Levels

Although the feeding studies and GH release studies (***Chapter 5***) were done using young (3-6 months) and old (14-17 months) mice, we first assessed circulating ghrelin levels in 11-13 month old mice in the fed state. This age group was selected based on availability of animals and allowed an initial assessment of serum ghrelin levels in WT, and obese MC3R KO and MC4R KO mice. Ghrelin levels were similar in both male and female MC3R KO and WT animals, whereas MC4R KO mice had reduced ghrelin levels compared to WT controls (***Figure 4-1***), with significance observed in female MC4R KO mice. Although ghrelin levels appear to be reduced in male MC4R KO mice compared to WT, this result was not significant. This is likely due to insufficient power in this analysis. In addition, ghrelin levels in females were significantly higher than males.

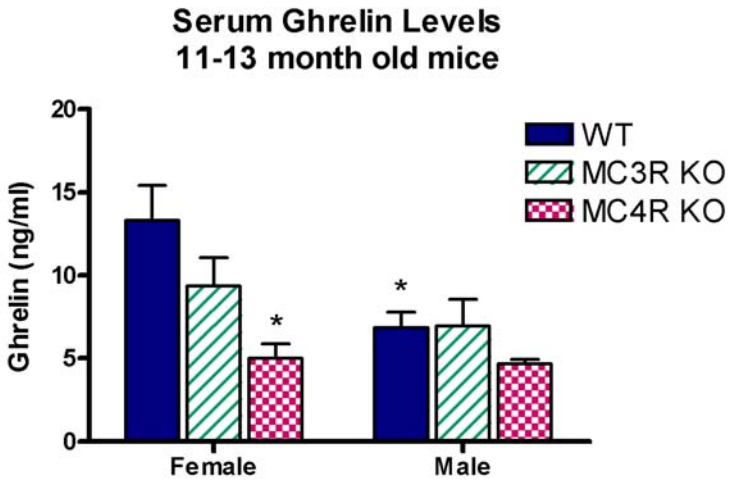

Figure 4-1. Serum ghrelin levels. Values are mean ± SEM. n=5-6 animals per group except MC4R KO males where n=3. * $p<0.05$ vs. female WT; two-way ANOVA, Tukey test for comparisons.

Ghrelin-Induced Food Intake in Young Mice

To determine whether signaling through the MC3R or MC4R was necessary for ghrelin-induced food intake, we measured the feeding response to ghrelin in young male and female MC3R KO, MC4R KO, and WT mice during the light phase. ***Figure 4-2*** shows that the amount of food consumed in response to ghrelin in young female WT mice were significantly greater than that observed after a saline injection. This effect was significant up to 4 hours post injection. Young female MC3R KO mice also demonstrated an increase in food intake after a peripheral injection of ghrelin with significance observed at 1 and 2 hours post injection compared to their saline treated controls. Ghrelin treatment in female MC4R KO mice did not cause an increase in food intake compared to their saline-treated controls. Although only significant at 30 minutes post injection, the magnitude of the feeding response to ghrelin was higher in WT animals compared to either MC3R KO or MC4R KO mice. The 1 and 2 hour time points in this analysis were nearly significant.

The feeding response to a peripheral injection of ghrelin in young male mice is shown in ***Figure 4-3***. Only male MC3R KO mice exhibited a significant increase in food intake after ghrelin administration compared to saline-treated controls and this effect was only significant at 30 minutes post injection. There was no difference in food intake between ghrelin-treated WT or MC4R KO mice compared to saline treatment.

Ghrelin-Induced Food Intake in Aged Mice

To determine whether the feeding response to ghrelin is altered in aged MC3R KO and MC4R KO mice, we injected ghrelin peripherally and measured food intake. Both female (***Figure 4-4***) and male (***Figure 4-5***) WT mice had a robust increase in food intake after ghrelin administration at 30 minutes and 1-hour post injection compared to

their saline-treated controls. This effect was also observed up to 4 hours in WT male mice. However, the acute effects of ghrelin were not significantly different from saline-treated controls in both male and female MC3R KO and MC4R KO mice except at the 1-hour time point in male MC4R KO mice. In addition, wild-type animals exhibited a significantly greater response to the orexigenic effects of ghrelin compared to MC3R KO and MC4R KO mice.

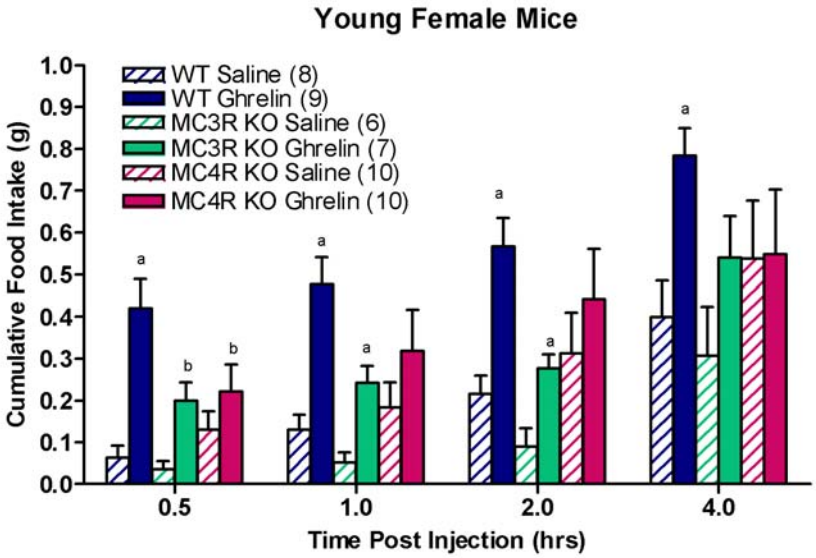

Figure 4-2. Ghrelin-induced food intake in young female mice during the light phase. Group numbers are indicated in figure legends and values are mean ± SEM. a= $p<0.05$ vs. saline- injected control within genotype; b= $p<0.05$ vs. ghrelin-injected WT

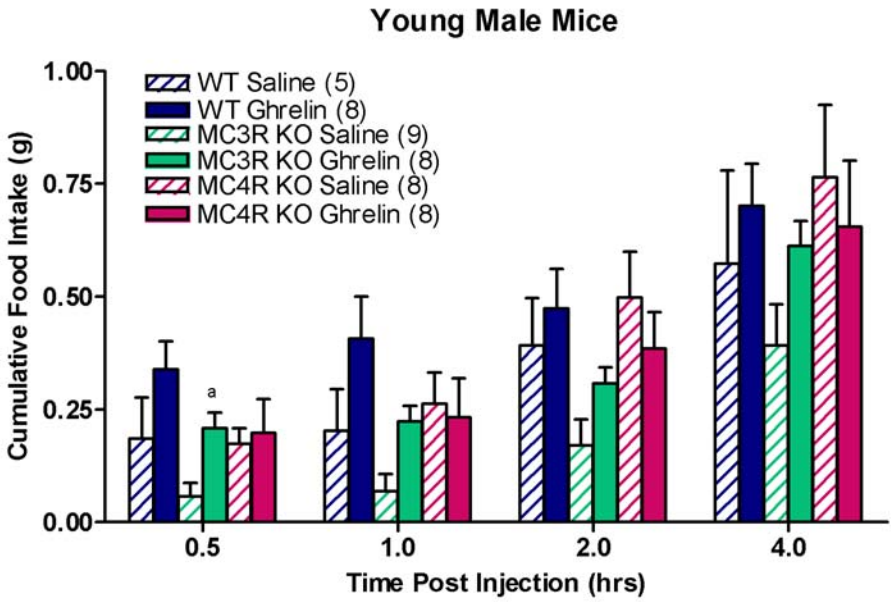

Figure 4-3. Ghrelin-induced food intake in young male mice during the light phase. Group numbers are indicated in figure legends and values are mean ± SEM. a= $p<0.05$ vs. saline- injected control within genotype

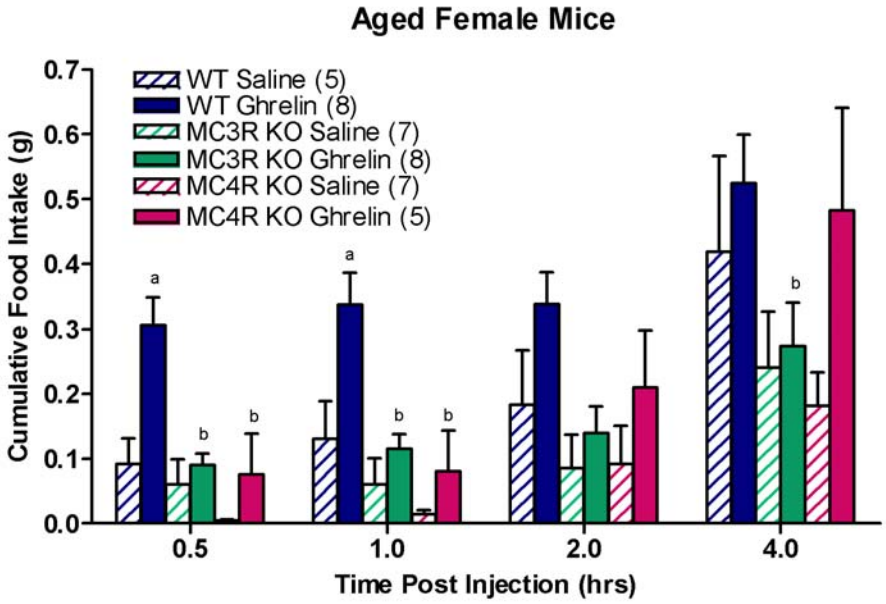

Figure 4-4. Ghrelin-induced food intake in aged female mice during the light phase. Group numbers are indicated in figure legends and values are mean ± SEM. a= $p<0.05$ vs. saline- injected control within genotype; b= $p<0.05$ vs. ghrelin-injected WT

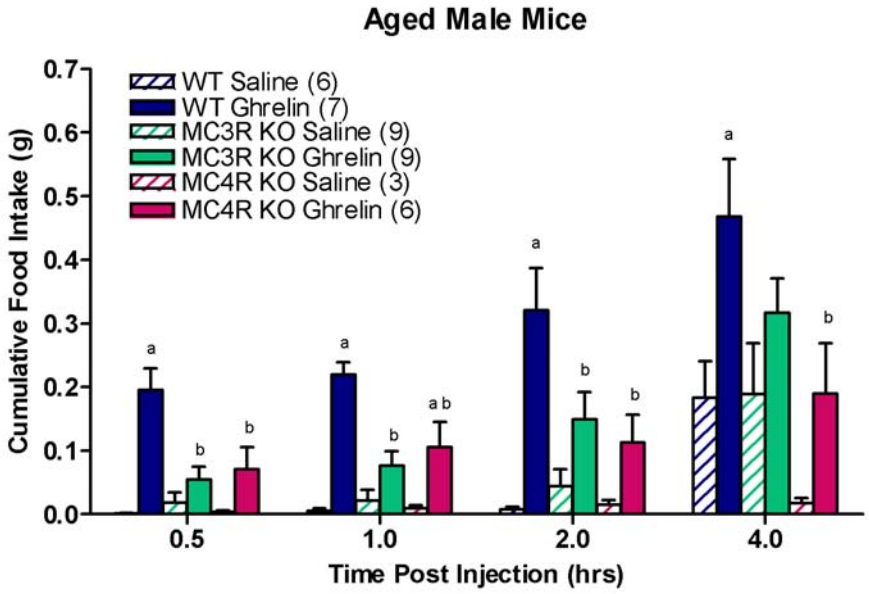

Figure 4-5. Ghrelin-induced food intake in aged male mice during the light phase. Group numbers are indicated in figure legends and values are mean ± SEM. a= $p<0.05$ vs. saline- injected control within genotype; b= $p<0.05$ vs. ghrelin-injected WT

Twenty-Four Hour Food Intake

As expected, ghrelin did not affect 24-hour food intake in any of the groups tested as shown in ***Figure 4-6*** (female data) and ***Figure 4-7*** (male data). In some groups, it originally appeared as if there was a difference in 24-hour food intake between ghrelin and saline injected animals. However, when we compared the amount of food ingested in the 24 hours before the injection (the day before the experiment) to the amount of food ingested in the 24 hours after the injection, no differences were detected.

Ghrelin-Induced Food Intake During Dark-Phase Feeding

Because rodents are nocturnal and do the majority of their eating during the dark phase, we wanted to evaluate the effects of peripheral ghrelin on food intake during this period. Animals were injected with ghrelin at the beginning of the dark phase (just after lights out) and only young animals (4-5 months of age) were used for these experiments. There were no significant differences in food intake in response to the same dose of

ghrelin given in the light-phase studies in any of the groups tested compared to their saline-treated controls (***Figure 4-8***). Only MC3R KO mice showed a slight increase in food intake at the early time points compared to saline but these results were not significant.

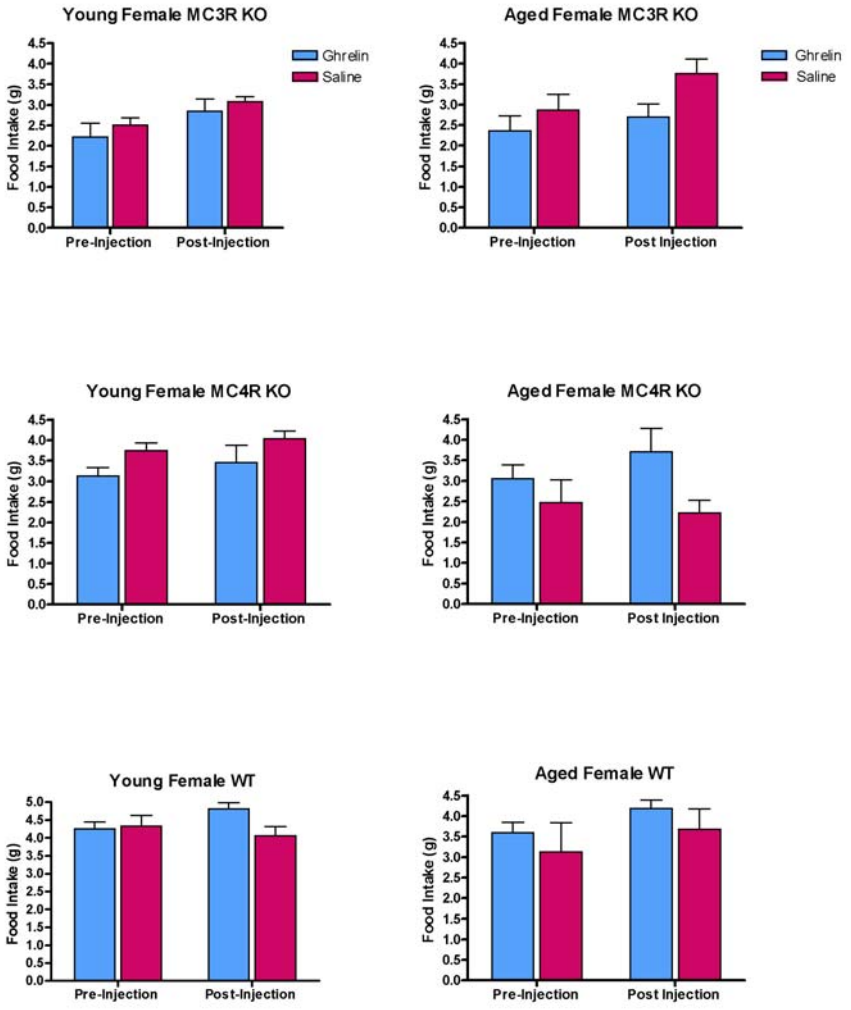

Figure 4-6. Twenty-four hour food intake in young and old female mice during the light phase. Pre-injection refers to the 24 hours prior to the start of the studies and post-injection is the 24 hours after the injection.

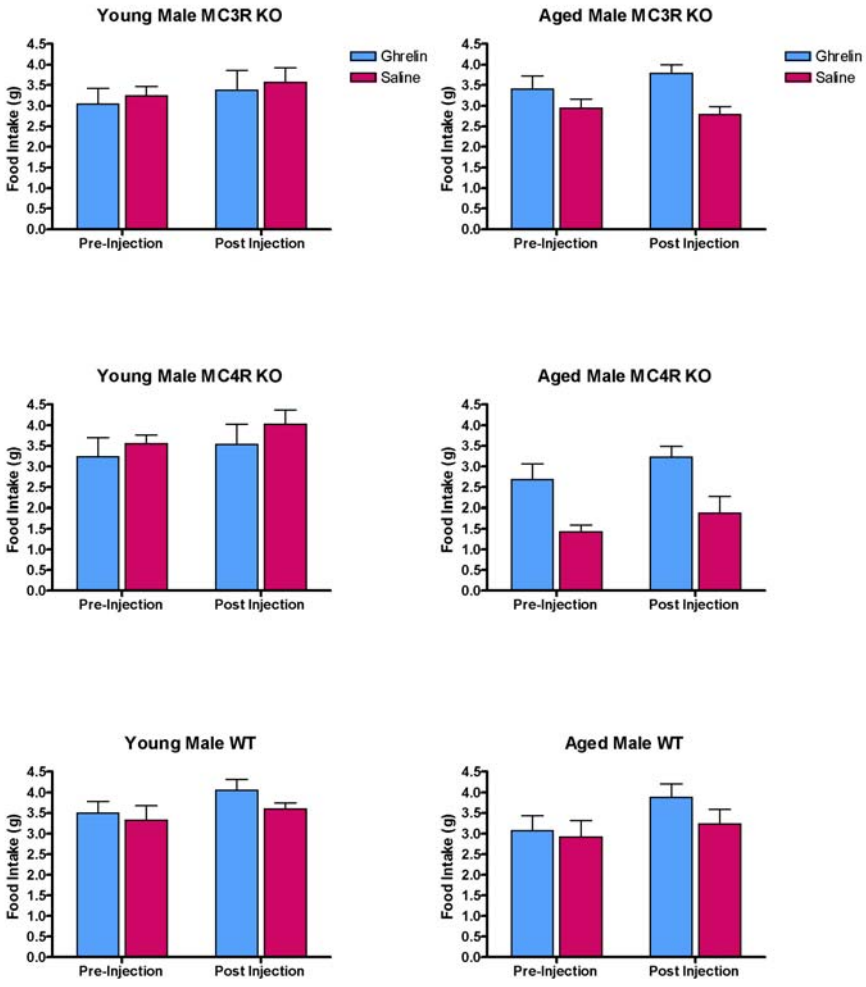

Figure 4-7. Twenty-four hour food intake in young and old male mice during the light phase. Pre-injection refers to the 24 hours prior to the start of the studies and post-injection is the 24 hours after the injection.

GHS-R Levels in the Hypothalamus

The primary tissues expressing GHS-R mRNA are the hypothalamus and the pituitary [77]. Arcuate nucleus NPY/AGRP neurons co-express GHS-R mRNA and it is thought that the orexigenic actions are mediated through activation of this receptor on these feeding neurons. We therefore characterized the mRNA levels of the GHS-R in the hypothalamus of MC4R KO, MC3R KO and WT male and female mice (see ***Figure 4-9***).

We did not have the sufficient tissues from aged animals to conduct this analysis; therefore, these measurements were only done in the young animals.

In male MC3R KO mice, there was approximately a 2-fold increase in hypothalamic GHS-R mRNA levels compared to male WT controls. We did not detect any difference in male MC4R KO mice compared to WT. In female mice, there was no difference in hypothalamic GHS-R mRNA levels between any of the groups.

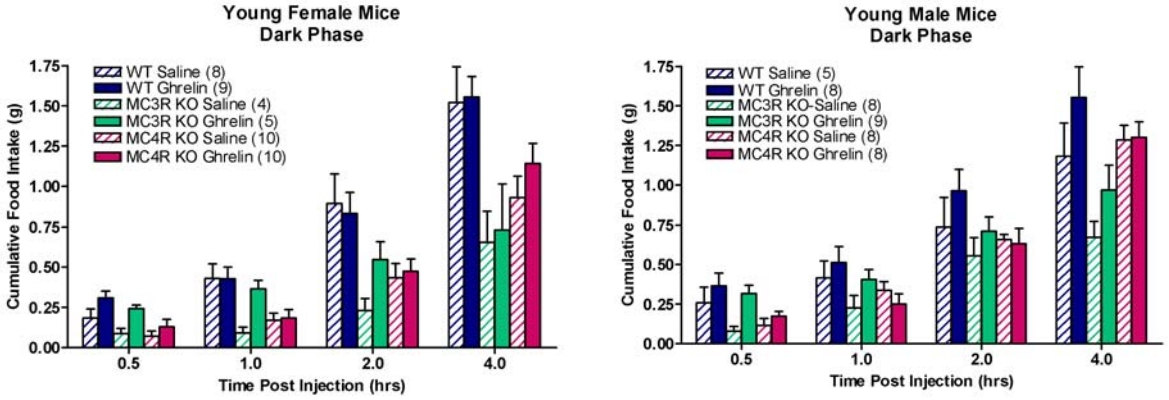

Figure 4-8. Ghrelin-induced food intake in young female and male mice during the dark phase. Group numbers are indicated in figure legends and values are mean ± SEM.

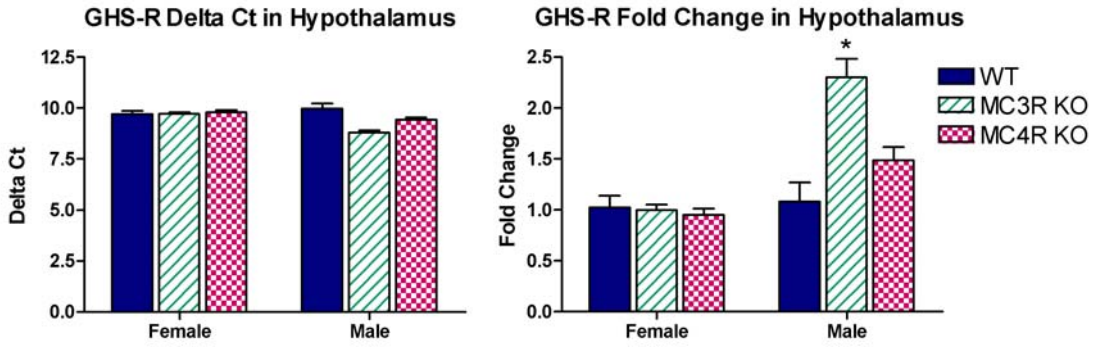

Figure 4-9. Hypothalamic GHS-R mRNA levels. The left graph shows data expressed as the delta Ct and the right graph shows data expressed as fold-change compared to sex-matched WT control. n = 5-6 animals per group. *$p<0.05$ vs sex-matched WT control.

Discussion

The regulation of energy balance and body composition is a complex process that involves the interactions of many central and peripheral systems. The stomach hormone ghrelin is a potent stimulator of both GH secretion and food intake and its actions are mediated by the GHS-R in the hypothalamus and pituitary. The involvement of the melanocortin antagonist, AGRP, in ghrelin-induced adiposity and food intake and the modulatory effects of ghrelin on melanocortin neurons suggests that the central melanocortin system is important for mediating the effects of ghrelin on energy homeostasis. Thus, this work was aimed at studying the effects of peripherally administered ghrelin on food intake in MC3R KO and MC4R KO mice.

Our results show that serum ghrelin levels are reduced in obese MC4R KO mice with a more marked reduction in female mice. These results are consistent with previous studies in obese humans [119, 209] and mice (*ob/ob, db/db* and *agouti)* [210, 211], which demonstrate that circulating ghrelin levels are reduced in obesity and/or conditions of hyperleptinemia. MC4R KO mice are hyperleptinemic [31] and this may contribute to the reduction in ghrelin levels seen in these animals, since previous studies have shown that ghrelin is negatively regulated by leptin [119, 133, 212]. However, MC3R KO mice also have high circulating leptin levels [32] yet they have normal or only slightly reduced serum ghrelin levels compared to WT animals. This suggests that other factors besides leptin may contribute to the regulation of serum ghrelin concentrations in obesity or at least in the MC3R KO animal model of obesity. Both male and female MC4R KO mice are hyperinsulinemic [31], whereas male MC3R KO mice exhibit only mild hyperinsulinemia and female MC3R KO mice have normal insulin levels [32]. Therefore, alterations in insulin status may also influence circulating ghrelin levels in

MC3R KO and MC4R KO mice. Some studies have indeed shown a reduction in circulating ghrelin levels in response to insulin infusion in humans [154, 213] or in conditions of hyperinsulinemia [214, 215]. While both MC3R KO and MC4R KO mice are characterized by an increase in fat mass and a reduction in lean body mass, their body weight profiles are different in that MC4R KOs are significantly heavier. Previous studies have indicated that there is an inverse relationship between body weight and ghrelin levels [209, 216, 217] therefore, the higher body weights observed in MC4R KO mice may contribute to the reduction in ghrelin levels. Thus, the alterations in ghrelin levels seen in obesity seem to result from a complex interaction involving body composition, leptin and insulin, and presumably other metabolic factors as well.

Peripheral administration of ghrelin to rats and mice causes an acute increase in food intake [163, 174, 176], which is thought to occur through its modulation of NPY/AGRP neurons in the ARC [173, 180]. To further characterize the role of the central melanocortin system in mediating the orexigenic effects of ghrelin, we compared the feeding response to ghrelin in MC3R KO and MC4R KO mice to that of wild-type mice. In addition, we utilized both young and old animals to determine if sensitivity to the feeding effects of ghrelin is altered with age. Our results indicate that both young and old male and female MC3R KO and MC4R KO mice are less sensitive to the orexigenic effects of ghrelin in comparison to WT mice. Although there is some food consumption in MC3R KO and MC4R KO mice in response to ghrelin, the amount of food consumed by WT mice in response to the same treatment is significantly greater. In addition, the ghrelin dose used in our study is similar to that recently reported by Sun *et al* [145] which was shown to produce serum ghrelin levels in the range seen in fasting mice and is

therefore likely to be physiologically relevant. Our data suggests that melanocortin signaling is important for ghrelin-induced food intake and supports previous studies demonstrating a role for AGRP action in mediating the effects of ghrelin. The obesity syndrome and phenotypic changes observed in MC4R KO mice strongly resemble those observed in the *agouti* mouse model [31] and both normal weight and obese *agouti* mice were shown to be resistant to the orexigenic effects of peripheral ghrelin [211]. In addition, MC3R/MC4R double knockout mice do not show an increase in 4-hour food intake in response to a peripheral injection of ghrelin [57]. Taken together, our results and those in previous studies support the hypothesis that suppression of melanocortin tone via NPY and AGRP is essential for mediating the orexigenic effects of ghrelin. Although, the MC4R is thought to be the primary mediator of central melanocortin signaling, the MC3R has also been shown to play an important role in energy balance [32, 56]. The fact that ghrelin-induced food intake is suppressed in MC3R KO mice to a similar extent as MC4R KO mice supports the idea that both of these receptors are important for mediating the effects of ghrelin on food intake.

An alternative explanation for our findings in MC3R KO and MC4R KO mice is that ghrelin is simply ineffective due to the obesity of the animal. A recent study by Perreault *et al,* [218] demonstrated that mice made obese through the feeding of a high fat diet were less sensitive to the orexigenic effects of ghrelin compared to lean mice fed a normal or low fat diet. In these animals, sensitivity to ghrelin improved with weight loss indicating a link between obesity and resistance to the effects of ghrelin. It is possible that the reduction in the effectiveness of ghrelin in MC3R KO and MC4R KO mice is due to both the absence of the receptors and the obesity of these animals.

As expected, there was no increase in food intake at 24 hours in response to peripheral ghrelin in any of the groups tested, which supports previous work demonstrating that peripheral ghrelin acts as an acute stimulator of food intake. In some of the data, especially in the aged animals, it appears that MC4R KO mice are actually hypophagic compared to WT mice. This is in contrast to reports of marked hyperphagia observed in the MC4R KO mouse [31]. Many labs working with these animals have suggested that MC4R KO animals are more sensitive to stress than WT animals, although these findings have not been published. Therefore, we feel that the normal to decreased food intake observed in our studies in MC4R KO mice may be due to stress caused by handling or the injection of the animal, or to inadequate time for the animal to adjust to individual housing. As described in Chapter 2, the feeding experiments were first conducted in the aged animals and these animals were only allowed 3 days to acclimate to new housing. When we repeated the studies in the young animals, we allowed the animals more time to acclimate and the feeding responses were more in line with what has been previously reported in MC4R KO mice. Therefore, the stress response may play a role in the hypophagia observed in some groups of MC4R KO mice both acutely and at 24 hours.

We also measured levels of hypothalamic GHS-R mRNA to determine if the diminished responses to ghrelin in the knockout animals are simply due to a reduction in levels of the receptor for ghrelin. To our knowledge, there is no commercially available antibody for the GHS-R; therefore, protein levels of the receptor were not quantified. We observed an increase in GHS-R mRNA levels in the male MC3R KO mice compared to WT but no differences were seen between male or female MC4R KO or female MC3R

KO mice and their WT controls. This suggests that mechanisms other than GHS-R expression may affect the orexigenic effects of ghrelin.

It is also interesting to look at the effects of aging on the orexigenic effects of ghrelin in the WT animals only. Our results demonstrate that both young and old female WT mice consume a similar amount of food after a peripheral injection of ghrelin, which is significantly greater than the food intake seen after a saline injection. In contrast, in WT male mice, there is an age-related difference in the feeding response to ghrelin. The amount of food consumed in young male WT mice in response to ghrelin is not significantly greater than that seen after an injection saline. However, in the aged male WT mouse, ghrelin potently stimulates food intake compared to saline. Therefore, there appears to be sexually dimorphic effects in the feeding response to ghrelin, which is related to the age of the animal. It is important to point out that we did not perform a dose response curve for the concentration of ghrelin to use in these studies, but based it on previous reports in the literature. Thus, young male WT mice may simply not be as responsive to this dose of ghrelin but may respond to a higher ghrelin dose with effects similar to that observed in females and aged males. Although we did not have the tissues to analyze GHS-R levels in the aged animals, it would be interesting to look at this in future studies. This would help to determine if the age-related changes in male mice are simply due to altered GHS-R expression or if other factors are involved.

Because rodents are nocturnal and consume the majority of their food during the dark phase, we wanted to evaluate the effects of ghrelin on food intake in these animals during the dark phase. The dose of ghrelin used in these studies was unable to elicit an increase in food intake during the dark phase in male and female WT and MC4R KO

mice. There was a small increase in food intake in response to ghrelin in male and female MC3R KO mice, which was similar to that observed in light phase feeding but this effect, was not significant. The inability of ghrelin to stimulate dark phase food intake in WT animals may be because ghrelin levels in the circulation rise prior to the consumption of food [147]. Therefore, the onset of the feeding period in WT mice may be accompanied by an increase in ghrelin levels, resulting in resistance to further ghrelin administration at least in young animals. We did not conduct dark phase feeding studies in aged mice but it would be interesting to determine if the effects in aged mice are similar to those of young mice.

In conclusion, we have shown that MC4R KO mice have reduced serum ghrelin levels and that both MC3R KO and MC4R KO mice are less sensitive to the orexigenic effects of ghrelin compared to WT mice. This supports a role for melanocortin signaling involving both the MC3R and MC4R in mediating the effects of ghrelin on food intake. Thus, altered responses to the orexigenic actions of ghrelin occur in both MC3R KO and MC4R KO mice, providing further information regarding the mechanism of action of ghrelin and its effect in this animal model of obesity.

CHAPTER 5
THE GH RESPONSE TO GH-RELEASING STIMULI IS REDUCED IN THE OBESITY SYNDROMES OF MC3R KO AND MC4R KO MICE

Introduction

Pulsatile GH secretion is controlled by the interactions of the stimulatory hypothalamic peptide, GHRH, and the inhibitory hypothalamic peptide SRIH, which act via their respective receptors in the anterior pituitary [219]. In addition, synthetic peptidyl and non-peptidyl growth hormone secretagogues (GHS) can potently stimulate GH secretion both directly via its receptor, the GHS-R, found in the pituitary and indirectly via its interactions with GHRH and SRIH [220]. The discovery of the endogenous ligand for the GHS-R, ghrelin, in 1999 [79] verified a physiological role for GHS-R ligands in regulating GH secretion and supported a model of tripeptidyl modulation of GH secretion [221].

In obesity, circulating levels of GH are extremely low, its half-life is reduced, and the amplitude of GH pulses can be nearly undetectable [96]. Furthermore, obese patients show an impaired responsiveness to the GH-releasing effects of GHRH alone, but do show, interestingly, an increase in GH secretion in response to various GHS moieties such as GH-releasing peptide-6 (GHRP-6) and hexarelin [96]. However, the overall GH response to various secretagogues is less than that of lean patients. Recently, ghrelin was shown to potently stimulate GH secretion when administered alone or in combination with GHRH to obese patients [222]. The fact that administration of GH to obese rats causes a decrease in body weight [80] and a normalization of GH secretion after weight

loss in humans [103] supports a role for GH in obesity. We therefore wanted to determine whether the obesity syndromes associated with the deletion of the MC3R or MC4R are accompanied by decreased sensitivity to the GH-stimulating effects of ghrelin as well as GHRH.

As described in previous chapters, ghrelin also stimulates food intake and adiposity, which is thought to occur primarily through activation of NPY/AGRP neurons in the ARC [164, 182]. AGRP stimulates food intake by antagonizing the actions of α-MSH at the central melanocortin receptors, MC3R and MC4R [223]. Therefore, ghrelin is indirectly modifying the central melanocortin system to stimulate food intake. In understanding the dual actions of ghrelin it is necessary to determine whether the GH-releasing and orexigenic effects are mediated through similar pathways or if these effects occur independently through different mechanisms. We therefore investigated the effects of ghrelin on GH secretion in MC3R KO and MC4R KO mice to determine if deletion of these receptors alters the ability of ghrelin to stimulate GH secretion.

Materials and Methods

Animals

Male and female MC3R knockout, MC4R knockout, and wild-type (WT) mice 5-6 and 15-17 months of age were used for these studies. The MC3R KO mice were originally provided by Merck [32] and the MC4R KO mice were provided by Millennium Pharmaceuticals [31]. Our animals were bred in the laboratory of Dr. Carrie Haskell-Luevano. Following the completion of the feeding studies, the same animals were allowed at least 1 week for drug washout and were then used for these GH release experiments. Animals were group-housed (3-4 per cage) and received food and water *ad libitum*. Each genotype was randomly divided into 2 groups: a control group receiving an

intraperitoneal (i.p.) injection of sterile saline or a treatment group receiving an i.p. injection of ghrelin at a dose of 400 μg/kg. This ghrelin dose was based on previous studies reported in the literature utilizing mice and peripheral injections of ghrelin to measure both food intake and GH-secretion [174, 189-191]. After the completion of the ghrelin injections and blood sampling for the measurement of GH levels, the mice were allowed a 2-3 week recovery period based on the animal welfare requirements for repeated blood sampling in mice without replacement. Each genotype was again randomly divided into 2 groups: a control group receiving an i.p. injection of sterile saline or a treatment group receiving an i.p. injection of human GH-releasing hormone (hGHRH) at a dose of 50 μg/kg which is based on previous studies in mice and rats [191, 192]. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Florida and were conducted in accordance with animal welfare guidelines. Genotypes of animals were verified upon sacrifice by PCR as described in the ***General methods***.

Peptide Preparation

Rat ghrelin (BACHEM; Torrance, CA) was dissolved in 1 ml sterile saline and diluted to a concentration of 80 μg/ml. The diluted peptide was separated into 1.5 ml aliquots and stored at -80°C. Immediately before the start of the experiments, one aliquot of the peptide solution was thawed and kept on ice for the duration of the experiments. Human GHRH (Bachem; Torrance, CA) was dissolved in 1 ml sterile water and diluted to a concentration of 0.5 mg/ml. The diluted peptide was separated into 50 μl aliquots and stored at -80 °C. Just prior to the start of the experiments, one aliquot was thawed

and further diluted with 2.45 mls of sterile saline to a working concentration of 10 μg/ml. The peptide was kept on ice throughout the duration of the experiments.

GH Release Studies

These experiments were conducted between 0900-1200 to minimize diurnal variations in GH levels between groups of animals. Mice (n= 3-9 mice per group) were anesthetized using a ketamine (100 mg/kg), xylazine (15 mg/kg) mixture administered i.p before the first blood sample was taken. Immediately following the first blood sample, the animals were injected with either rat ghrelin (400 μg/kg, i.p.) or saline in the first set of experiments or either hGHRH (50 μg/kg) or saline in the second set of experiments. Blood samples were collected at 15, 30, and 60 minutes post injection and all blood samples were collected by saphenous venipuncture. We tried to limit each blood sample to around 100 μl maximum in order to avoid removing too much blood volume from the animals. The University of Florida IACUC approved this procedure in accordance with animal welfare guidelines. The blood was centrifuged and serum was collected and frozen at –20°C until measurement of GH by RIA. Following the 60-minute blood sample, animals were allowed to recover from anesthesia. While the animals were under anesthesia, naso-anal body length was measured. We used this measurement to calculate the Obesity Index of Lee [224], which was used as a measure of adiposity in the animals. The equation for calculating the obesity index is:

$$\text{Obesity Index} = \frac{\sqrt[3]{\text{weight (g)}}}{\text{naso-anal length (mm)}} \times 10^4$$

Radioimmunoassay

Serum GH concentrations were determined by radioimmunoassay (RIA) using reagents supplied by the NIDDK's National Hormone and Peptide Program and Dr. A.F.

Parlow. The mouse GH RIA had a sensitivity of 2.5 ng/ml and a range of detection from 2.5 ng/ml to 200 ng/ml. Intra-assay coefficient of variation was less than 5% and inter-assay coefficient of variation was 4.5%**.**

Real-Time RT-PCR for the GHS-R

Total RNA was isolated from the pituitary of the young mice collected after sacrifice. RNA was isolated using TRIreagent (Sigma, St. Louis, MO) and was subsequently treated to remove any contaminating DNA using a DNase treatment kit (Sigma, St. Louis, MO). RNA was then reverse-transcribed using the Reverse Transcription reagents kit (Applied Biosystems, Foster City, CA) and cDNA was stored at -80°C until PCR was performed.

Real-time RT-PCR reactions were conducted using primers and reagents from Applied Biosystems. Taqman Gene Expression Assays containing predesigned primers and probes based on the mouse GHS-R sequence (Genbank accession #NM177330 and AK049671) and eukaryotic 18s ribosomal RNA were used. The total reaction volume was 25 µl and contained Taqman universal PCR master mix, Taqman gene expression assay mix containing primers and probes and cDNA. Expression levels of GHS-R in the pituitary were calculated using the $2^{-\Delta\Delta Ct}$ method [193] using 18s RNA as an internal control. To perform this calculation, the difference in cycle threshold (ΔCt) between the GHS-R and the corresponding 18s threshold for each sample was calculated. Mean ΔCts were then calculated for each genotype and tissue type. The ΔΔCt was then calculated by subtracting the mean ΔCt for WT mice from that of each genotype, and this was done separately for each sex and tissue type. Numbers were then normalized to the WT group within sex by calculating the fold change in expression ($2^{-\Delta\Delta Ct}$).

Statistics

GH data were transformed using a natural log transformation to allow for parametric analysis. After transformation, differences in GH levels in response to injection of ghrelin were analyzed by two-way ANOVA for the factors of genotype and treatment at each time point with post-hoc comparisons using the Tukey test. GH area under the curve (AUC) was calculated by trapezoidal integration and analyzed by Kruskal-Wallis one-way ANOVA on Ranks with post-hoc testing using Dunn's method. Differences in body weight and obesity indices were analyzed by two-way ANOVA and post-hoc testing with the Tukey test. Differences in pituitary GHS-R mRNA levels were determined by one-way ANOVA using the delta Ct values for the analysis. Data is expressed as delta Ct and fold change compared to sex-matched WT controls. No comparisons were made between sex for GHS-R levels.

Results

Body Weight and Obesity Index

The body weights and obesity indices of the animals used in these studies are shown in ***Table 5-1***. In female mice, the body weight and obesity index of MC4R KO mice was greater than that of WT mice and there was no difference between MC3R KO and WT mice. In addition, there was an overall difference in the obesity index and body weight between young and aged female mice, which did not depend on the genotype of the animal. In male mice, the obesity index of young and old MC4R KO mice differed from that of their age-matched WT controls but body weight was only different between young MC4R KO and WT mice. There was also a significant interaction of genotype and age in male mice. The obesity indices and body weights of male MC3R KO and WT

increased with age whereas there was no age-related increase in obesity in male MC4R KO mice.

Table 5-1. Body weights and obesity indices of animals used in GH release studies.

		Body Weight (g)		Obesity Index	
	Genotype	Young	Aged	Young	Aged
Female	WT	24.4 ± 0.5†	31.5 ± 2.4	327.3 ± 1.9†	342.0 ± 3.0
	MC3R KO	22.8 ± 0.6†	36.0 ± 1.3	329.1 ± 2.4†	356.9 ± 4.2
	MC4R KO	40.3 ± 1.8*†	48.6 ± 3.7*†	365.1 ± 4.5*†	384.3 ± 6.6*
Male	WT	28.4 ± 0.5^{a}	46.3 ± 2.9	336.4 ± 1.9^{a}	360.4 ± 4.6
	MC3R KO	31.3 ± 1.0^{a}	44.4 ± 2.2	345.2 ± 2.5^{a}	367.7 ± 3.8
	MC4R KO	48.1 ± 2.0*	51.3 ± 2.8	380.0 ± 3.8*	383.0 ± 6.6*

Values are mean ± SEM. *$p<0.05$ vs. sex matched WT control within age group; $^{\dagger}p<0.05$ vs. sex matched aged mice (overall difference not dependent on genotype), $^{a}p<0.05$ vs. sex matched aged mice within genotype.

Effects of Ghrelin on GH Secretion in Young WT, MC3R KO and MC4R KO Mice

In young male and female WT and MC3R KO mice, ghrelin caused a significant rise in GH levels at 15 and 30 minutes post injection compared to their saline treated controls (***Figure 5-1 and 5-2***) and this effect was also seen at 60 minutes post injection in male MC3R KO mice. There was no increase in GH levels in response to ghrelin in male and female MC4R KO mice compared to their saline treated controls and this response was significantly different from WT mice. In addition, the total AUC for the GH response to ghrelin in female MC4R KO mice was significantly different from the WT response. This trend was also seen in male MC4R KO mice, however these results were not significant.

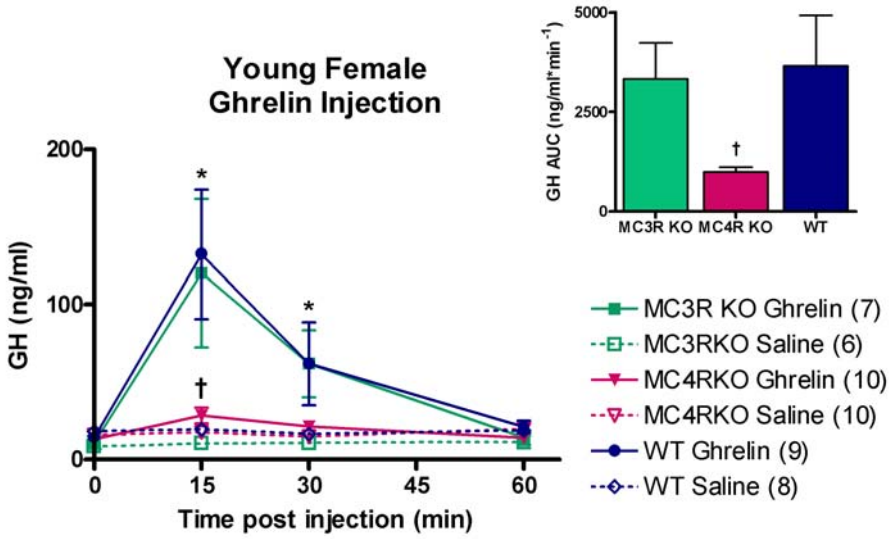

Figure 5-1. Ghrelin-induced GH secretion in 4-5 month old female mice. The inset graph shows area under the curve that was calculated by the trapezoidal method. Group numbers are indicated in figure legends and values are mean ± SEM. *p<0.05 vs. saline-treated control within genotype; † p<0.05 vs. ghrelin-treated WT.

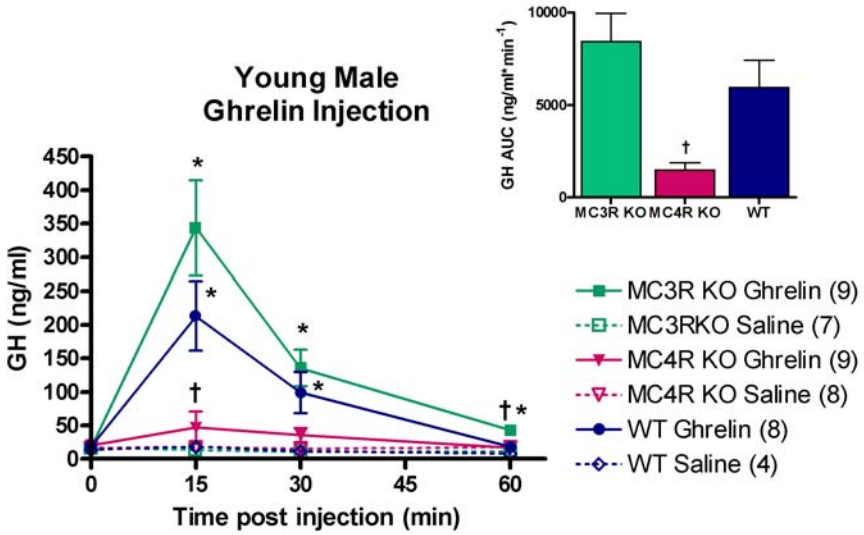

Figure 5-2. Ghrelin-induced GH secretion in 4-5 month old male mice. The inset graph shows area under the curve that was calculated by the trapezoidal method. Group numbers are indicated in figure legends and values are mean ± SEM. *p<0.05 vs. saline-treated control within genotype; † p<0.05 vs. ghrelin-treated WT.

Effects of Ghrelin on GH Secretion in Aged WT, MC3R KO and MC4R KO Mice

Ghrelin significantly stimulated GH secretion at 15 minutes post injection in WT, MC3R KO, and MC4R KO females as compared to their saline-treated controls (***Figure 5-3***). The response observed in WT females was greater than that of both MC3R KOs and MC4R KOs with significance being observed at 30 minutes post injection in WT vs MC3R KO. Although, there was not significance observed at 15 minutes post injection in MC3R KO and MC4R KO and at 30 minutes in MC4R KO, the AUC was significantly lower in both female genotypes compared to WT. In addition, there was one female WT mouse whose GH response was delayed and did not peak until the 30-minute sample, which contributed to the high variability observed in the female WT animals. Thus, a diminished responsiveness to the GH-releasing effects of ghrelin was observed in aged female mice deficient in the melanocortin receptors. Male mice were not as sensitive as female mice to the GH stimulating effects of ghrelin. Male MC3R KOs exhibited a significantly greater GH response to ghrelin at 15 and 30 minutes post injection while the response to ghrelin in male MC4R KOs was increased at 30 minutes post injection compared their respective WT controls (***Figure 5-4***). A comparison of the GH responses to ghrelin in both young and old male and female mice are shown in ***Figure 5-5***.

GHS-R mRNA Levels in the Pituitary

Because ghrelin and GHS can affect GH secretion by indirect actions at the hypothalamus and direct actions on the pituitary we wanted to assess whether alterations in GHS-R levels in either the pituitary or hypothalamus could contribute to the altered ghrelin-induced GH secretion in these studies. We reported the hypothalamus GHS-R data in ***Chapter 4***; therefore we will only present the pituitary data in this chapter. As with the hypothalamus, we did not have sufficient tissues from aged animals to conduct

this analysis; therefore, these measurements were only done in the young animals. In female mice, there was a decrease in hypothalamic GHS-R mRNA levels in MC3R KO mice compared to WT controls, but no difference between MC4R KO and WT. In male mice, MC4R KO mice had significantly reduced GHS-R mRNA levels compared to WT controls. Male MC3R KO mice had a reduction in GHS-R levels as well, however, these results were not significant.

Effects of GHRH on GH Secretion in Young Mice

GHRH significantly stimulated GH secretion at 5 minutes post-injection in male and female MC3R KO mice compared to their saline-treated controls (***see Figures 5-7 and 5-8***). This effect remained significant at 15 minutes post-injection in male MC3R KO mice. GHRH was able to significantly stimulate GH secretion in male WT at 5 minutes post injection and a small increase in GH in female WT mice was also observed although this effect was not significant. GHRH did not stimulate GH secretion in either male or female MC4R KO mice at any time point post injection.

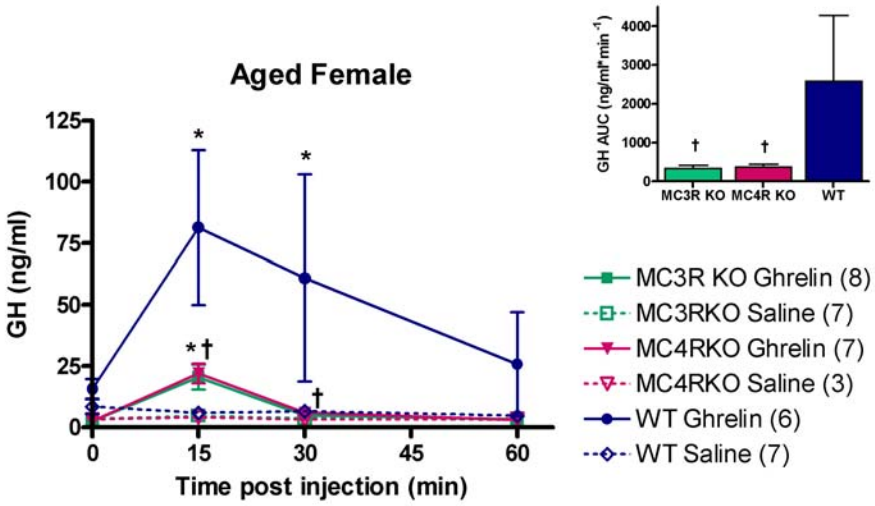

Figure 5-3. Ghrelin-induced GH secretion in 16-17 month old female mice. The inset graph shows area under the curve. Group numbers are indicated in figure legends and values are mean ± SEM. *p<0.05 vs. saline-treated control within genotype; † p<0.05 vs. ghrelin-treated WT.

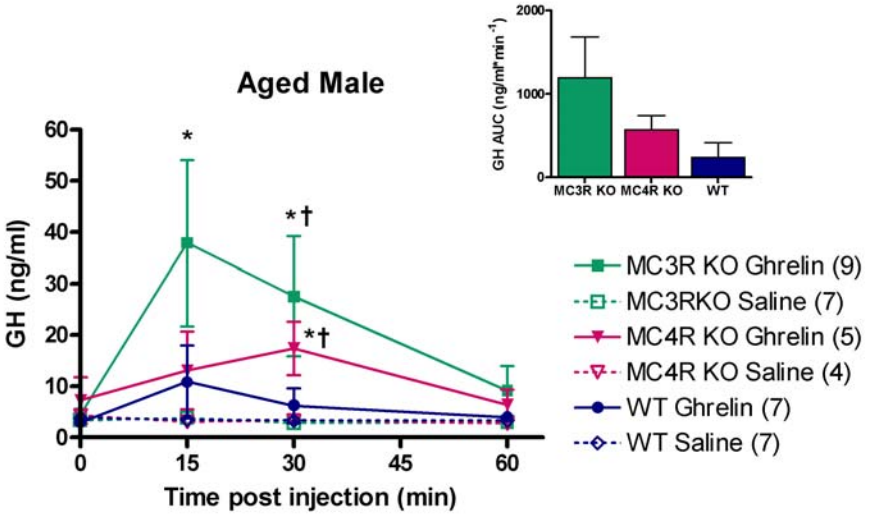

Figure 5-4. Ghrelin-induced GH secretion in 16-17 month old male mice. The inset graph shows area under the curve. Group numbers are indicated in figure legends and values are mean ± SEM. *p<0.05 vs. saline-treated control within genotype; † p<0.05 vs. ghrelin-treated WT.

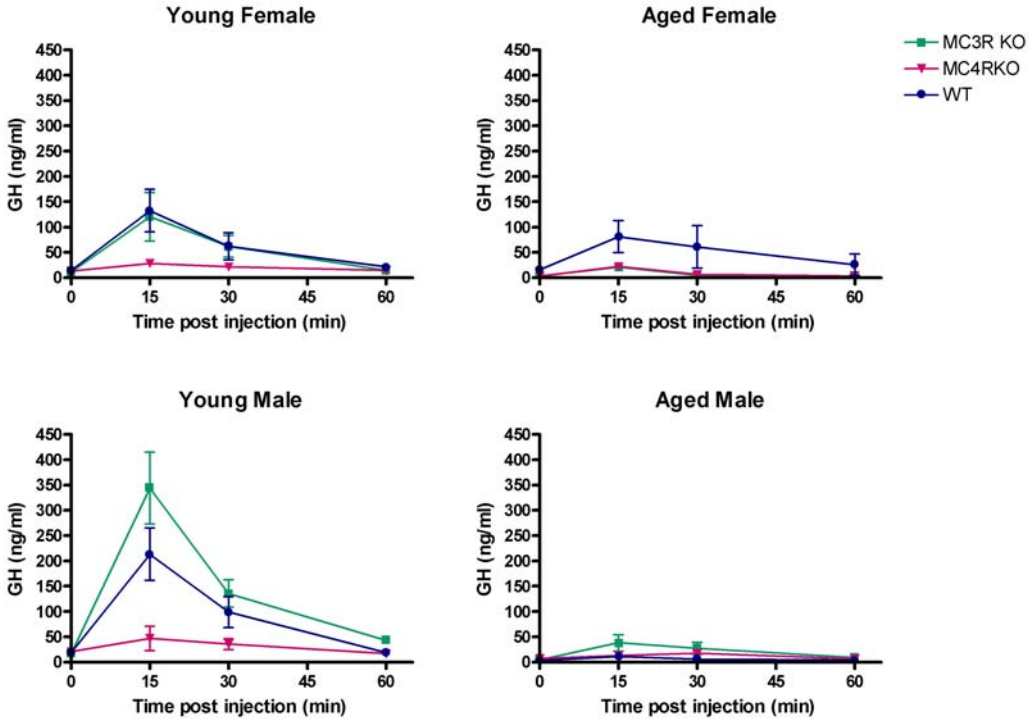

Figure 5-5. Comparison of ghrelin-induced GH response in young and old male and female mice. Graphs display only ghrelin treated groups; saline treated groups were omitted for the sake of clarity.

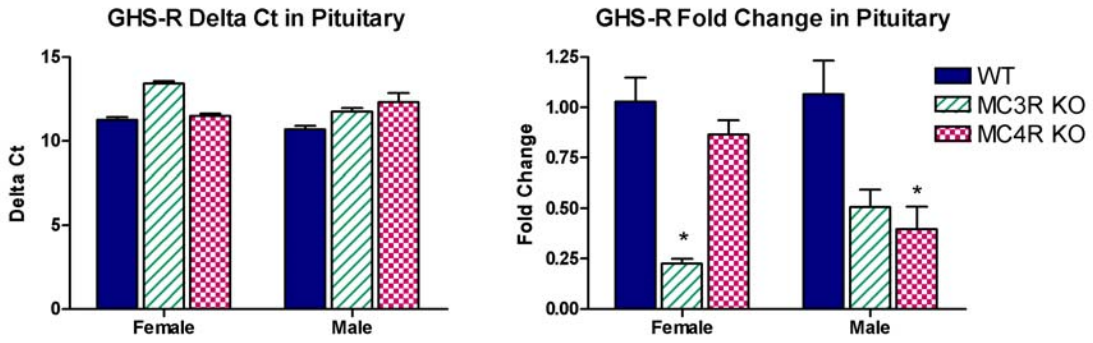

Figure 5-6. Pituitary GHS-R mRNA levels. The left graph shows data expressed as the delta Ct and the right graph shows data expressed as fold change compared to sex-matched WT control. n = 5-6 animals per group. *$p<0.05$ vs sex-matched WT control.

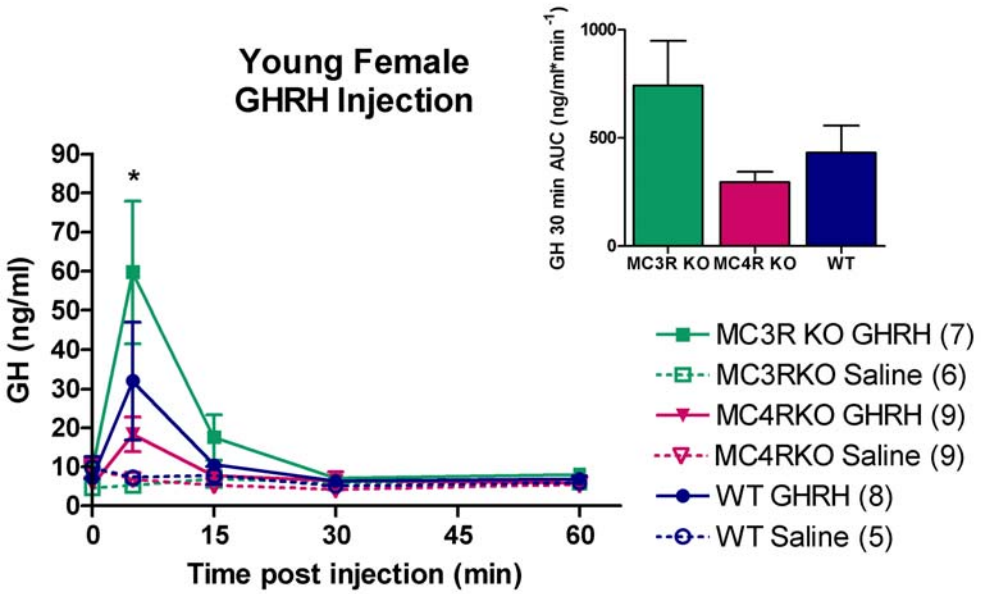

Figure 5-7. GHRH-induced GH secretion in 5-6 month old female mice. The inset graph shows 30 minute area under the curve. Group numbers are indicated in figure legends and values are mean ± SEM. *p<0.05 vs. saline-treated control within genotype.

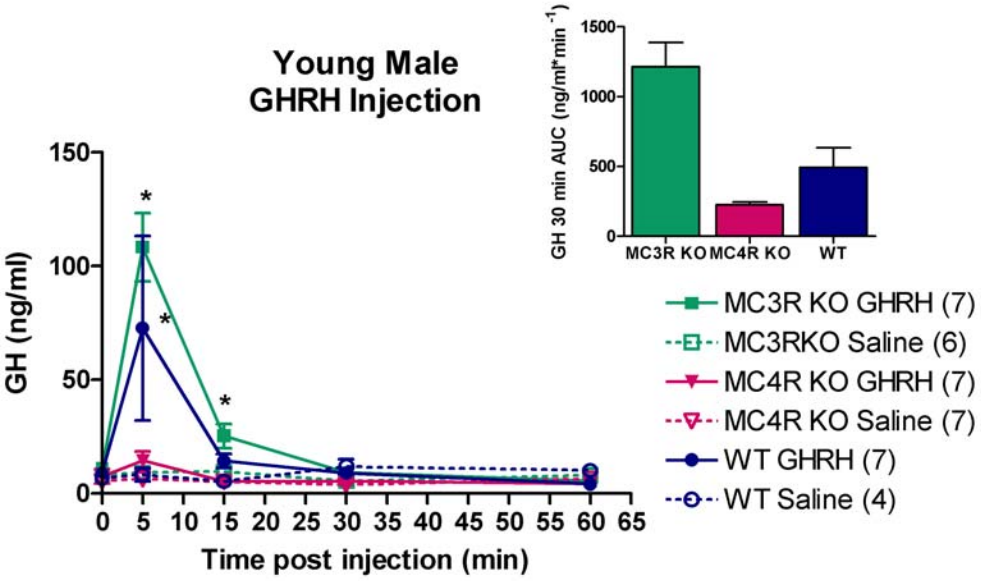

Figure 5-8. GHRH-induced GH secretion in 5-6 month old male mice. The inset graph shows 30 minute area under the curve. Group numbers are indicated in figure legends and values are mean ± SEM. *p<0.05 vs. saline-treated control within genotype.

Discussion

The different phenotypes of MC3R KO and MC4R KO mice support the hypothesis that these two receptors have independent and non-redundant functions in regulating energy homeostasis. The obesity syndrome of MC3R KO mice is characterized by increased fat mass and a reduction in lean body mass without excess food intake or weight gain. Thus, the obesity observed in these mice is thought to result from changes in metabolism. In addition, these animals have a shorter body length than WT controls [32]. MC4R KO mice also exhibit maturity-onset obesity with increased fat mass and reduced body mass, however these animals are hyperphagic and weigh significantly more than WT controls. It is thought that obesity in these mice results primarily from excess food intake. In contrast to the MC3R KO mouse, MC4R KOs exhibit increased linear growth compared to WT controls [31]. Because ghrelin is a potent GH secretagogue and GH secretion affects both body composition and linear growth, we sought to determine whether altered responsiveness to the GH-releasing affects of ghrelin could contribute to the phenotypes of MC3R KO and MC4R KO mice. In addition, reductions in basal as well as stimulated GH secretion have been widely demonstrated in obesity [96, 225]. We therefore wanted to determine if the obesity syndromes observed in MC3R KO and MC4R KO mice are also characterized by a decreased sensitivity to ghrelin. These experiments were also designed to help determine if the GH-releasing effects of ghrelin are mediated by the same pathways as the orexigenic effects of ghrelin, specifically through modulation of the central melanocortin system.

Our results show that in response to a single peripheral injection of ghrelin, GH levels are dramatically increased in both young and old female WT mice. Circulating

levels of GH are also increased in young female MC3R KO after ghrelin administration and this effect is identical in magnitude to that seen in WT controls. However, the ability of ghrelin to stimulate GH secretion in aged female MC3R KO mice is significantly smaller than the response seen in aged female WT mice. Both young and old female MC4R KO mice exhibit only a minimal increase in GH secretion after ghrelin administration; and the magnitude of the response is much smaller than that seen in WT controls. The obesity syndrome of MC4R KO mice is more severe than that of the MC3R KO mouse and the diminished GH response in these animals in both age groups is likely related to the extent of obesity in these animals. Young MC3R KO mice are less obese than older MC3R KO mice and the GH response to ghrelin appears to correlate with the extent of obesity in these animals as well. These results concur with previous work showing that a decrease in the somatotrope responsiveness to ghrelin occurs in obese women compared to lean women [96, 226] and suggest that the obesity syndromes of MC4R KO and to a lesser extent, MC3R KO mice are accompanied by a reduced sensitivity to the GH-releasing effects of ghrelin.

Young male MC3R KO and WT mice display a large increase in GH secretion in response to peripheral ghrelin administration, similar to the results observed in female mice. Also, as in young female MC4 R KO mice, young male MC4R KO mice exhibit only a minimal increase in GH release after ghrelin injection. In the aged male group, all three genotypes show reduced responsiveness to ghrelin compared to the effects seen in young animals. Therefore, as in female mice, the ability of ghrelin to stimulate GH secretion in both MC3R KO and MC4R KO animals seems to depend on the extent of obesity in the animal. MC4R KO mice do not respond to ghrelin in either age group

whereas young MC3R KO mice respond to ghrelin and aged MC3R KO do not. The obesity index of WT mice increases with age to a similar extent as MC3R KO mice. Therefore, the diminished responsiveness to ghrelin in aged male WT mice may also be due to an increase in adiposity. It cannot be ruled out, however, that other factors besides the extent of obesity may contribute to the reduction in ghrelin sensitivity that occurs in MC3R KO mice with age.

We also measured levels of pituitary GHS-R mRNA to determine if the diminished responses to ghrelin in MC4R KO animals are simply due to a reduction in levels of the receptor for ghrelin. We observed a decrease in pituitary GHS-R mRNA levels in male and female MC3R KO and male MC4R KO mice compared to WT but no differences were seen between female MC4R KO mice and WT controls. It is possible that the reduced GHS-R mRNA levels in male MC4R KO mice may contribute to the small GH response to ghrelin observed in these animals. However, male and female MC3R KO mice also have reduced pituitary GHS-R levels yet exhibit a normal GH response to ghrelin. In ***Chapter 4***, we reported an increase in hypothalamic GHS-R in male MC3R KO mice. This may contribute to the intact GH responsiveness to ghrelin in these animals through indirect actions of ghrelin on GH secretion. However, we do not feel that there is any clear-cut relationship between the levels of GHS-R expression in either the hypothalamus or pituitary and the GH response to ghrelin. This suggests that mechanisms other than GHS-R expression may influence the GH-releasing effects of ghrelin.

In is also interesting to look at the ghrelin responses in male and female WT mice independently. From ***Figure 5-5*** it is evident that the GH response to ghrelin remains

intact in normal WT female mice as they age, whereas the ghrelin response in aged WT male mice is no longer intact. Therefore, there appears to be sexually dimorphic effects related to the GH-releasing ability of ghrelin that occur with aging. In fact, obese Zucker rats and high fat diet-induced obese rats show an impairment of the GH axis with female rats being better preserved than male rats [107]. This suggests that the alterations of the GH axis in obesity and aging may be gender-related and possibly influenced by gonadal function. However, in humans, the GH response to ghrelin was higher in young subjects compared to elderly subjects, and there were no gender-related differences observed [227].

In order to determine if the reduced sensitivity to the GH–releasing effects of ghrelin observed in MC4R KO mice and the varied responses in MC3R KO mice were specific to ghrelin and directly related to deletion of the central melanocortin receptors, we evaluated the GH response in these mice using another GH secretagogue, GHRH. We completed the ghrelin experiments in the aged animals and based on the results of these experiments we wanted to test the effects of GHRH in knockout animals. We did not have further aged animals available for this study; therefore, GHRH experiments were only conducted in the young animals following the completion of the ghrelin injections. In addition, based on previous studies in the literature, we added a 5-minute time point to the GHRH experiments to better assess the GH release induced by this peptide. We therefore cannot make direct comparisons as to the actions of GHRH and ghrelin in these animals and we are simply testing the ability of the pituitary to release GH in response to another GH secretagogue, which acts through a different mechanism than ghrelin. In both male and female MC3R KO and male WT mice, GHRH causes a significant

increase in serum GH levels. Female WT mice show a small increase in GH levels, but this result was not significant. As seen with ghrelin, both male and female MC4R KO mice do not show an increase in GH secretion in response to GHRH. Taken together, these results suggest that in MC4R KO mice, the diminished responsiveness to ghrelin and GHRH is likely due to the obesity syndrome of these animals and is not specific for effects mediated through the MC4R. These findings agree with previous studies in Zucker rats, which demonstrate that pituitary responsiveness to GHRH is reduced in both male and female obese rats compared to lean rats. This effect is observed both *in vivo* and *in vitro* using cultured anterior pituitary cells [198, 206]. In young male and female MC3R KO mice, both ghrelin and GHRH are able to elicit a strong release of GH from the pituitary. Therefore, it appears that the pituitary is still highly responsive to GH secretagogues in young MC3R KO mice. The mechanism behind the diminished GH secretion in response to ghrelin in aged MC3R KO mice is less clear however, as we do not have data from GHRH injections in these animals. We hypothesize that the effects of GHRH in aged MC3R KO mice would mimic those of ghrelin in that responsiveness would be reduced with age. This would give further support to an obesity-associated mechanism responsible for decline of responsiveness to GH secretagogues and the overall decline of GH in obesity and aging.

In summary, ghrelin was unable to elicit GH secretion in either young or old male or female mice MC4R KO mice. There was an age-related reduction in ghrelin responsiveness in MC3R KO mice, which is likely due to the extent of obesity in these animals. Sexually dimorphic effects were observed with age in WT mice which suggests that ghrelin may play a role in the altered GH secretion of aging and regulation by sex

steroids may be important in this response. Finally, the diminished GH response to ghrelin in these animal models of obesity does not appear to be directly related to deletion of the melanocortin receptors and gives further support for independent pathways of ghrelin in mediating food intake and GH secretion.

CHAPTER 6
GENERAL DISCUSSION

The regulation of energy balance and body composition is a complex process that involves the interactions of many central and peripheral systems. GH is important in building lean body mass and breaking down fat mass and its secretion is markedly suppressed in both obesity and aging. The synthesis and release of GH from the anterior pituitary is mediated by GHRH and SRIH, which stimulate and inhibit GH, respectively. The recent discovery of ghrelin, which stimulates GH through activation of the GHS-R, gives support to a tripeptidyl hypothesis of GH regulation in which GHRH, SRIH and ghrelin/GHS-R ligands are the primary mediators.

In addition to its effects on GH secretion, ghrelin has been shown to potently induce food intake, and this occurs at least in part through activation of ARC neurons containing the melanocortin receptor antagonist, AGRP. The central melanocortin system is important in regulating food intake and metabolism and animals deficient in the central melanocortin receptors, the MC3R and MC4R, exhibit interesting alterations in energy homeostasis. MC3R KO mice are obese and are characterized by increased fat mass, reduced lean body mass and reduced body length compared to WT littermates. Obesity is also a major characteristic of the MC4R KO mouse and these mice have increased fat mass and increased body length compared to WT littermates. Because of the important effects of GH on growth and body composition, we sought to investigate potential interactions of the central melanocortin system and the GH axis. The major objectives of this work were to examine the role of AGRP/melanocortin signaling in

mediating the effects of ghrelin on food intake, to evaluate the role of the MC3R and MC4R in ghrelin-induced growth hormone secretion in young and old animals and to characterize the GH secretory axis in MC3R KO and MC4R KO mice throughout their lifespan. This will help to better understand the mechanisms by which ghrelin affects metabolism, energy balance and GH secretion and further characterization of MC3R KO and MC4R KO mice will help to elucidate the role of the melanocortin system in regulating energy homeostasis and body composition.

One of the hypotheses of this dissertation was that alterations in the GH axis contribute to the obesity and body length phenotypes observed in MC3R KO and MC4R KO mice. In ***Chapter 3***, we presented work characterizing various components of the GH axis in these animals. To assess basal GH status, we measured circulating GH and pituitary GH content in both male and female mice. Although our results were not always significant, we detected a trend towards lower serum GH levels in both male and female MC4R KO mice throughout their lifespan compared to WT. We did not see any difference in serum GH levels between MC3R KO mice and WT mice. Pituitary GH content was reduced in young female MC4R KO and MC3R KO animals but was not different from WT controls in the oldest age group. In addition, GH pituitary content declines with age in female WT animals whereas this trend is not observed in either MC3R KO or MC4R KO. Therefore, it appears that pituitary GH production in young and middle-aged female MC3R KO and MC4R KO mice is similar to that seen in aged WT mice and suggests that pituitary function may be altered in these animals much earlier than that seen in WT animals. Male MC4R KO mice also showed a trend towards reduced pituitary GH content compared to WT controls but this was not observed in all

age groups. Although not as marked as that seen in females, pituitary GH content declines somewhat with aging in male WT mice whereas pituitary GH content in MC4R KO mice stays the same throughout the lifespan and mimics that seen in a normal aged mouse. Also in contrast to female mice, male MC3R KO mice do not display reduced pituitary GH content in any age group compared to WT controls. Although it is not considered a primary factor, reduced pituitary GH content is likely a contributor to the lower GH levels observed in obesity and aging and as well as in MC4R KO mice.

We also measured levels of the GH effector protein, IGF-I, in these animals. Despite the findings of lowered circulating GH levels, serum IGF-I levels remained unchanged or were significantly elevated in MC4R KO mice, except for aged females in which IGF-I levels were reduced compared to wild-type controls. Aged MC3R KO females also displayed reduced IGF-1 levels, similar to that observed in aged MC4R KO females while no differences in serum IGF-I levels were observed in any other MC3R KO groups compared to their WT controls. Thus, the presence of elevated IGF-I levels in MC4R KO mice may contribute to the longer body length and reduced GH secretion may influence the elevated fat mass observed in these animals. We did not find a significant pattern of GH-IGF-I axis alteration in MC3R KO mice, therefore it is not clear if this system influences the phenotypes of these animals.

It has been suggested that an increase in central SRIH tone may contribute to the hyposecretion of GH in obesity. In our studies, there was no difference in hypothalamic SRIH content between WT and MC3R KO or MC4R KO mice in either males or females. Female MC3R KO mice did show an age-related reduction in SRIH content but this was not observed in any of the other groups. Therefore, alterations in SRIH content do not

appear to mediate the reduced GH levels in MC4R KO mice. In cannot be ruled out, however, that alterations in downstream signaling mechanisms of SRIH, rather than SRIH content itself, could influence the GH axis in MC4R KO mice. Additional studies are needed to further elucidate the potential interactions of the GH secretory axis and the melanocortin system in the control of body weight and energy homeostasis.

In ***Chapters 4 and 5***, we sought to investigate the mechanisms involved in ghrelin-induced food intake and GH secretion. More specifically, we wanted to determine whether deletion of the MC3R and/or the MC4R in mice would alter the ability to of ghrelin to stimulate food intake and/or GH secretion. Our results show that serum ghrelin levels are reduced in obese MC4R KO mice with a more marked reduction in female mice. When ghrelin was injected peripherally, young and aged female WT and only aged male WT mice show an acute increase in food intake. Male and female MC3R KO and MC4R KO mice are less sensitive to the orexigenic effects of ghrelin in comparison to WT mice regardless of age. Our data suggests that melanocortin receptor signaling is important for ghrelin-induced food intake and supports previous studies demonstrating a role for AGRP action in mediating the effects of ghrelin. Taken together, our results and those in previous studies support the hypothesis that suppression of melanocortin tone via NPY and AGRP is essential for mediating the orexigenic effects of ghrelin.

GH secretion and the response to various GH secretagogues are markedly suppressed in obesity and aging. We therefore wanted to characterize the GH response to both ghrelin and GHRH to determine if suppression of their effects would be observed in obese MC3R KO and MC4R KO mice. Our results show that in response to a single

peripheral injection of ghrelin, GH levels are dramatically increased in both young and old female WT mice and young female MC3R KO but not in MC4R KO mice. The ability of ghrelin to stimulate GH secretion in aged female MC3R KO mice is reduced and mimics the minimal response seen in aged female MC4R KO mice. These responses are significantly smaller than the response seen in aged female WT mice. Young male MC3R KO and WT mice display a large increase in GH secretion in response to peripheral ghrelin administration, similar to the results in female mice. Also, as in young female MC4R KO mice, young male MC4R KO mice exhibit only a minimal increase in GH release after ghrelin injection. In the aged male group, all three genotypes show reduced responsiveness to ghrelin compared to the effects seen in young male WT and MC3R KO animals. Therefore, as in female mice, the ability of ghrelin to stimulate GH secretion in both MC3R KO and MC4R KO animals seems to depend on the extent of obesity in the animal. In both male and female MC3R KO and male WT mice, GHRH causes a significant increase in GH levels in the circulation. Female WT mice show a small increase in GH levels, but this result was not significant. As seen with ghrelin, neither male nor female MC4R KO mice show an increase in GH secretion after an injection of GHRH. Taken together, these results suggest that in MC4R KO mice, the diminished responsiveness to ghrelin and GHRH is likely due to the obesity syndrome of these animals and is not specific for effects mediated through the MC4R. In young male and female MC3R KO mice, both ghrelin and GHRH are able to elicit a strong release of GH from the pituitary, but the effects of ghrelin are diminished with age, as the obesity of these animals increases. It is also possible that the inability of ghrelin or GHRH to elicit GH release in MC4R KO animals is due to pituitary dysfunction as we demonstrated that

pituitary GH content was reduced in these animals throughout the lifespan. Therefore, the minimal GH release in response to GH releasing agents in MC4R KO mice may be due to reduced pituitary content available for release in these animals.

It is interesting to compare the overall trends in ghrelin-mediated GH secretion and food intake in all the groups tested and this is summarized in ***Table 6-1***. The responses to ghrelin in females were similar in that WT females increased their food intake and GH secretion while the responses seen in MC3R KO and MC4R KO animals were significantly diminished. In contrast, the feeding response to ghrelin in males was similar to that of females, whereas the GH response was different. Aged male WT mice did not exhibit a ghrelin-induced increase in GH levels at the dose given while the magnitude of responses in both knockouts were similar to that of aged females. In addition, in female WT mice the feeding and GH response to ghrelin remain intact as the animal ages, in that the overall responses are similar between young and aged WT mice. However, in male WT mice the feeding response to ghrelin is higher in the aged group, whereas the GH response is more robust in the young group. In fact, ghrelin was unable to stimulate GH secretion in the aged male WT mouse, whereas the ability to stimulate food intake was still present. This suggests that there may be a shift in the sensitivity to ghrelin in older males in regards to GH secretion but not to food intake. Previous studies demonstrated that obese Zucker rats and high fat diet-induced obese rats have an impairment of the GH axis with a more pronounced impairment observed in male rats. In these studies, male rats were less sensitive to the GH-releasing effects of GHRH and this was not dependent on the extent of obesity in the animal. In contrast, only female rats with extreme obesity showed a diminished responsiveness to GHRH-stimulated GH secretion [107]. This

suggests that the alterations of the GH axis in obesity may be gender-related and possibly influenced by gonadal function.

Table 6-1. Summary of the effects of ghrelin on food intake and GH secretion.

			Ghrelin-induced Feeding		Ghrelin-induced GH
			Light Phase	Dark Phase	
Female	Young	WT	↑↑	↔	↑↑
		MC3R KO	↑	↑	↑↑
		MC4R KO	↔	↔	↑
	Aged	WT	↑↑	N/A	↑↑
		MC3R KO	↔	N/A	↑
		MC4R KO	↔	N/A	↑
Male	Young	WT	↔	↔	↑↑
		MC3R KO	↑	↑	↑↑↑
		MC4R KO	↔	↔	↑
	Aged	WT	↑↑	N/A	↔
		MC3R KO	↔	N/A	↔
		MC4R KO	↑	N/A	↔

The number of ↑ in each cell indicates the magnitude of the increase mediated by ghrelin. No response to ghrelin is indicated by ↔ .

To further assess the element of obesity in the feeding and GH responses to ghrelin future experiments should be conducted. Various manipulations to reduce the obesity of both MC3R KO and MC4R KO mice, such as placing the animals in running wheel cages, pair-feeding the animals with their WT controls, or feeding the animals low fat or low calorie diets could be tested. If the GH responses to ghrelin were restored upon weight loss, it would support the hypothesis that obesity is the primary cause of reduced responsiveness to ghrelin. If not, it may be possible that the melanocortin system plays a role in ghrelin-induced GH secretion which would suggest that the feeding effects and the GH effects of ghrelin are mediated through similar pathways. Injections of GHRH in the

aged group of animals would also help to clarify the age related changes seen in the GH effects of ghrelin.

Ghrelin is an important hormone involved in the regulation of food intake and GH secretion. The actions of ghrelin on food intake involve signaling through the central melanocortin system and deletion of melanocortin receptors in mice results in profound metabolic alterations. We have shown that MC4R KO mice have reduced serum ghrelin levels and that both MC3R KO and MC4R KO mice are less sensitive to the orexigenic effects of ghrelin compared to WT mice. This finding supports a role for melanocortin signaling involving both the MC3R and MC4R in mediating the effects of ghrelin on food intake. Alterations in the ability of ghrelin to stimulate GH secretion have been observed in these animals and many of these effects were age- and sex-dependent. In addition, various components of the GH axis were disturbed in the knockout animals suggesting that GH may play a role in the phenotypes of MC3R KO and MC4R KO animals. Our results have provided further information regarding the mechanism of action of ghrelin and its effect in this animal model of obesity. With further studies, these results may help to explain the complex regulation of body composition, food intake and overall energy homeostasis and how these factors are altered in both obesity and aging. Better understanding of these interacting pathways could potentially lead to the development of therapeutic compounds for the treatment of obesity as well as for the negative metabolic changes that occur with aging.

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BIOGRAPHICAL SKETCH

Amanda Marie Shaw was born in Jacksonville, Florida, on February 23, 1977 to Robert and Rita Crews. She has a younger sister, Erin Crews, and is happily married to Jason S. Shaw. Amanda grew up in Jacksonville, attended Stanton College Preparatory School, and then went on to attend the University of North Florida where she graduated with a Bachelor of Science in biology. From there, Amanda began graduate school at the University of Florida in the Department of Pharmacodynamics in pursuit of a Ph.D. As the completion of this phase of her life draws near, Amanda is looking forward to the next step in her career and a rewarding, productive future in science.