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Investigating the Role of Melanocortin System in Regulating Linear Growth and Growth Hormone Secretion

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Abstract

Growth hormone (GH) secretion declines with increased adiposity, culminating in GH deficiency in obesity. Dysfunction of the melanocortin 4 receptor (MC4R) results in disrupted satiety signaling, resulting in hyperphagia, hyperinsulinemia, rapid weight gain, and the development of obesity. Clinically, obese hyperinsulinemic MC4R deficient adults demonstrated a partial recovery of GH secretion when compared to healthy body mass index (BMI)-matched individuals. Moreover, as with childhood onset obesity, these adults present with increased adult height. Consequently, defective MC4R signaling is thought to contribute to the recovery of GH release in obesity, and that GH hypersecretion contributes to rapid linear growth in hyperphagic MC4R deficient children. Second to this, it is thought that activation of MC4R contributes to the suppression of GH secretion during weight gain and in obesity.

Observations demonstrate a progressive shift in the patterning of GH secretion from puberty throughout early adulthood in mice, reflecting the maturation of the GH axis. This was characterized by the establishment of regular GH secretory events; reminisce of that observed in all adult mammals characterized to date. Relative to pubertal mice at 4 weeks of age, pulsatile GH secretion declined at 8 and 16 weeks of age, correlating with the progressive slowing in the rate of growth velocity. This change in GH secretion did not occur in response to altered somatotropin release inhibitory factor (*Srif*, the primary inhibitor of GH release) mRNA expression, or changes in *Srif* mRNA distribution throughout the periventricular /arcuate nucleus (PeVN/ARC) complex of the hypothalamus. When considered alongside published observations, these data suggest that the age-associated changes in pulsatile GH release in mice likely occur in response to a loss of hypothalamic growth hormone releasing hormone (GHRH) neurons and the synchronization of the somatotroph network.

Based on limited clinical observations, it was anticipated that rapid pubertal growth associated with MC4R deficiency occurs alongside hypersecretion of GH. However, observations herein confirmed that rapid linear growth in hyperphagic MC4R knockout (MC4RKO) mice occurred in the absence of GH hypersecretion. Rather, MC4RKO mice were GH deficient by 8 weeks of age. Moreover, the progressive decline in GH secretion with age in wild-type (WT) mice was greatly exaggerated in MC4RKO mice, confirming that the eventual slowing of growth rate in these mice did not coincide with the gradual withdrawal of GH release. In addition, circulating or local insulin-like growth factor 1 (IGF-1) remained unchanged throughout rapid linear growth. These observations confirmed that GH/IGF-1 hypersecretion does not account for the rapid linear growth in MC4RKO mice.

Consequently, altered GH release in MC4RKO mice declined relative with increased adiposity. As with rats, immunohistochemistry analysis confirmed that the MC4R does not directly contribute to GH release, and thus the loss of MC4R expression does not directly contribute to the recovery of GH release in obesity. Rather, changes in GH release occurred alongside metabolic alterations following hyperphagia-induced weight gain in MC4RKO mice.

Interestingly, the suppression of GH release and corresponding rapid linear growth in hyperphagic MC4RKO mice occurred alongside a progressive elevation in circulating levels of insulin. This preceded the development of insulin resistance in adult MC4RKO mice. In humans, the progressive rise in circulating insulin levels during hyperphagia is necessary to sustain circulating nonesterified free fatty acids (NEFAs) and glucose homeostasis, regardless of rapid weight gain and increased adiposity. Moreover, the suppression of GH secretion in response to hyperphagia is thought to maintain insulin-driven NEFAs and glucose clearance relative to calorie intake. To this extent, the suppression of GH release in hyperphagic hyperinsulinemic MC4RKO mice occurred alongside normal circulating NEFA and glucose levels. Consequently, observations in MC4RKO mice reflected metabolic adaptations that sustain NEFAs/glucose homeostasis, similar to that observed in humans. Given that insulin interacts with IGF-1 receptor (IGF-1R) to promote linear growth, it is likely that hyperinsulinemia contribute to sustained rapid pubertal growth in hyperphagic GH-deficient MC4RKO mice. Accordingly, rapid pubertal growth in MC4RKO mice occur secondary to metabolic alterations in response to hyperphagia.

To prevent hyperphagia-induced hyperinsulinemia, MC4RKO mice were pair fed (PF) to restrict food intake to that observed in age-matched WT littermates (WT LM). Observations confirmed that the prevention of hyperphagia-associated hyperinsulinemia results in the normalization of rate of linear growth, and a restoration of pulsatile GH secretion in PF MC4RKO mice. Thus, the consequential metabolic responses that occur in response to hyperphagia promote rapid pubertal growth. Of importance, current data infer that metabolic adaptations in response to hyperphagia that are essential to sustain insulin-driven fatty acid and glucose homeostasis likely mimic the effects of GH in promoting pubertal growth. While confirmed in MC4RKO mice, these mechanisms may be conserved in pubertal obese children, accounting for rapid pubertal linear growth in this population. Findings provide valuable insights underlying altered somatic growth in mouse models of pubertal hyperphagia, and set the precedent for future studies that will define mechanisms

of growth in the absence of GH/IGF-1, while addressing misconceptions of GH dependent growth in obese hyperinsulinemic MC4R deficient adolescence.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

First authored manuscripts:

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Contributor	Statement of contribution
Primary author, Hwee Yim Tan (Candidate)	Experimental design, data collection, interpretation of observations (70%) Preparation of the manuscript (80%)
Co-author, Lili Huang	Assistance in experiments (10%) Proof reading of manuscript
Co-author, David Simmons	Interpretation of observations (5%) Proof reading of manuscript
Co-author, Johannes D Veldhuis	Deconvolution analysis of pulsatile measures of GH secretion (5%)
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Corresponding Author, Chen Chen	Proof reading of manuscript

Contributions by others to the thesis

Professor Chen Chen (principal supervisor) and Dr Frederik Steyn (associate supervisor) contributed significantly to the design of the project, interpretation of results and revising of manuscripts. Professor Johannes Veldhuis contribute to the deconvolution analysis of pulsatile measures of GH secretion in this thesis.

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Keywords

Growth hormone, melanocortin 4 receptor, insulin, rapid pubertal linear growth, obesity, hyperphagia

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List of Abbreviations

AC	Adenylyl cyclase
ACTH	Adrenocorticotropic Hormone
AgRP	Agouti related Peptide
ALS	Acid labile subunit
AMPK	Adenosine monophosphate-activated protein kinase
ARC	Arcuate Nucleus
ATGL	Adipose triglyceride lipase
BBB	Blood Brain Barrier
bGH	Bovine Growth Hormone Transgenic mice
BMI	Body Mass Index
BSA	Bovine Serum Albumin
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and Amphetamine-regulated Transcript
CNS	Central Nervous System
DH	Dorsal Hypothalamus
DIO	Diet-induced obesity
EDTA	Ethylenediaminetetraacetic acid
ELs	Extracellular loops
FSH	Follicle-stimulating hormone
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
GH	Growth Hormone
GHD	Growth Hormone Deficiency
GHR	Growth Hormone Receptor
GHRH	Growth Hormone Releasing Hormone
GHRHR	Growth Hormone Releasing Hormone Receptor
GHRKO	Growth Hormone Receptor Knockout mice
GHRP	Growth Hormone Releasing Peptide
GHS	Growth Hormone Secretogogue
GHSR	Growth Hormone Secretogogue Receptor
GOAT	Ghrelin-O-acyltransferase
GSIS	Glucose stimulated insulin secretion
GPCR	G-protein-coupled receptor
HCL	Hydrochloric Acid

HOMA-IR	Homeostasis model assessment-estimated insulin resistance
HSL	Hormone-sensitive lipase
HPT	Hypothalamic-pituitary-thyroid
IGF-1	Insulin-like Growth Factor
IGFBPs	Insulin-like growth factor binding proteins
InsR	Insulin Receptor
ISH	*In situ* Hybridization
JAK-STAT	Janus kinase/signal transducers and activators of transcription
LepR	Leptin Receptor
LH	Lateral Hypothalamus
LH	Luteinizing Hormone
MC4R	Melanocortin 4 Receptor
MC4RKO	Melanocortin 4 Receptor knockout mice
ME	Median Eminence
MPP	Mass of GH secreted per pulse
MSH	Melanocyte Stimulating Hormone
MT II	Melanotan II
NDS	Normal donkey serum
NEFAs	Non-esterified Fatty Acids
NGS	Normal goat serum
NPY	Neuropeptide Y
OPD	O-phenylenediamine
PB	Phosphate buffer
PBST	Phosphate Buffered Saline with Tween 20
PeVN	Periventricular Nucleus
PF	Pair Feeding
PKA	Protein Kinase A
PLC/IP3	Phospholipase C/ Inositol 1,4,5-trisphosphate
POMC	Pro-opiomelanocortin
PRL	Prolactin
PVN	Paraventricular Nucleus
qPCR	Quantitative Real-time Polymerase Chain Reaction
rhGH	Recombinant human Growth Hormone
SIGFRKO	Somatotroph specific IGF-1 receptor knockout mice
SRIF	Somatostatin Release Inhibitory Hormone
SSTR	Somatostatin Receptor

T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
Tg	Transgenic
TG	Triglycerides
TMs	Transmembrane
TSH	Thyroid stimulating hormone
WT	Wild-Type
WT LM	Wild-Type Littermates

CHAPTER ONE: LITERATURE REVIEW, AIMS AND HYPOTHESIS

1. Introduction

Growth hormone (GH) promotes longitudinal bone and muscle growth during childhood [1]. GH deficiency in humans [2-5] and rodents [6-8] results in severe postnatal growth retardation, whereas early treatment with recombinant human GH (rhGH) in humans increased growth velocity [9, 10]. In this regard, GH is a principal determinant of somatic growth during pubertal development [11]. Assessment of GH secretion in hyperphagic melanocortin 4 receptor (MC4R) deficient adults demonstrate a partial increase in pulsatile GH secretion relative to BMI-matched obese adults. As with childhood obesity, obese MC4R deficient individuals present with increased adult height. Consequently, it is thought that accelerated linear growth in obese MC4R deficient adults is associated with pubertal growth hormone (GH) excess. Second to this, it is proposed that the activation of MC4R may, in part, contribute to the suppression of pulsatile GH secretion during obesity. Measures of pulsatile GH release during the pubertal growth spurt in MC4R deficient humans do not exist, and thus the premise of GH hypersecretion contributing to rapid linear growth in obese MC4R deficient adults remains unsubstantiated. Moreover, the premise that increased GH release contributes to an increase in linear growth in obese MC4R deficient children contradict the anabolic actions of GH and insulin in sustaining endogenous fatty acid levels during periods of excess dietary consumption. This thesis will review the role of GH and metabolic changes associated with hyperphagia, with regard to increased GH secretion in promoting linear growth during excess energy availability.

Obesity [12, 13] and hyperphagia [14] are associated with impaired GH secretion, resulting in GH deficiency. Similarly, pubertal childhood obesity is associated with GH deficiency, regardless of increased growth velocity [15]. This suggests that factors other than GH may facilitate growth at this time. Of interest, obesity [16-18] and hyperphagia [14] are associated with elevated insulin production. Insulin is a potent anabolic growth hormone [19], and thus may counter the effects of suppressed GH to promote growth in obese children. Furthermore, based on the premise that increased adiposity (and obesity) is negatively correlated with GH [12, 13], the recovery of GH secretion in obese MC4R deficient adults appears to contradict current pharmacological observations that exclude the role of the MC4R as a direct intermediate regulator of GH secretion [20-22].

Using a novel approach to assess GH secretion from serial samples collected in mice, this thesis assessed the role of the MC4R in regulating linear growth and GH release. *Specifically, this study addressed whether MC4R directly contributes to altered GH release during hyperphagia-induced weight gain. Second to this, this thesis investigated whether*

GH hypersecretion as a consequence of defective MC4R signaling contributes to enhanced pubertal linear growth. Finally, the cause for aberrant linear growth in MC4R deficient mice was addressed, predominantly focusing on hyperphagia-associated hyperinsulinemia. In support of these studies, I first characterized the pattern of GH release in mice throughout rapid linear growth, thereby establishing a model for the assessment of the role of GH relative to pubertal growth in wild-type (WT) mice. I validated changes in GH release throughout pubertal growth in mice. In doing so, I further assessed the role of GH in promoting rapid linear growth in MC4R knockout (MC4RKO) mice, while briefly addressing the potential mechanisms that may contribute to altered GH release during the transition between puberty and adulthood.

1.1 Hypothalamic Control of Energy Balance

The hypothalamic arcuate nucleus (ARC) regulates feeding and energy expenditure by modifying food intake and energy balance through the integration of peripheral factors that signal short- and long-term energy status [23]. Orexigenic and anorexigenic neuronal populations within the ARC responds to circulating hormones and/or peptides outside the blood brain barrier (BBB) [24]. Leptin and insulin change in proportion to the amount of energy consumed following food intake and body fat stores, suggesting that both leptin and insulin are essential short- and long-term regulators of feeding [25, 26]. In addition, peripheral peptides including GH, ghrelin, glucocorticoids, and non-esterified free fatty acids (NEFAs) may interact with hypothalamic feeding centers to influence energy balance and food intake [27]. Together with leptin and insulin, these peptides may act rapidly to modify food intake and thus regulate energy homeostasis. To this extent, the control of body composition and metabolism are tightly regulated by multiple, complex interactions of central and peripheral pathways. Two distinct leptin/insulin responsive neuronal populations within the ARC are critical for appetite regulation. Neurons expressing the neuropeptides pro-opiomelanocortin (POMC) and cocaine-and amphetamine-regulated transcript (CART) (stimulated by leptin/insulin) suppress food intake, whereas neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons (inhibited by leptin/insulin) stimulate food intake. Thus, the balance between these neuronal signals regulate energy homeostasis (Figure 1-1) [23, 28].

1.2 The Melanocortin System

The hypothalamic-melanocortin system is the key regulator of energy homeostasis and food intake. The melanocortin system is composed of two sets of leptin-responsive

neurons. These neurons are located within the ARC and express NPY/AgRP or POMC/CART [29]. The melanocortin system signals primarily via the melanocortin receptors to modulate diverse functions in the central nervous system (CNS), the adrenal gland and the skin [23, 29, 30]. These receptors respond to endogenous agonists from POMC-derived peptides including alpha-, beta-, gamma-melanocyte stimulating hormone (α-MSH, β-MSH, γ-MSH), adrenocorticotropic hormone (ACTH), or antagonist including agouti or AgRP. The melanocortin 1 receptor (MC1R) is the classical MSH receptor expressed primarily in the skin and in hair follicles where it regulates pigmentation [31, 32]. The melanocortin 2 receptor (MC2R) is an ACTH receptor expressed in the adrenal cortex where it mediates the effects of ACTH on steroidogenesis [31]. Melanocortin 3 receptor (MC3R) and MC4R are predominantly expressed in the CNS and are involved in the regulation of food intake and energy homeostasis. The melanocortin 5 receptor (MC5R) is expressed widely in the skin, skeletal muscle, adrenal gland, adipocytes, ovary and testis, and is only known to facilitate exocrine actions [33]. Amongst these, the MC4R is the predominant regulator of the melanocortin system, and facilitates energy homeostasis by integrating signals from α-MSH and AgRP [34]. MC4Rs are abundantly expressed in feeding centres of the hypothalamus, within the paraventricular (PVN), dorsal hypothalamus (DH) and lateral hypothalamus (LH) [35]. MC4R is a member of class A G-protein coupled receptor (rhodopsin-like GPCR) with seven transmembrane (TMs) connected by alternating extracellular loop (EL) and intracellular loops [34]. The highly conserved cysteine residues at the top of TM3 and EL2 in rhodopsin that forms the disulfide bond connecting to TM3 and EL2 is lacking in the MC4R, although an intraloop disulfide bond exist in EL3 [36]. Thus, MC4R is considered one of the shortest members in the GPCR superfamily.

Downstream activation of the melanocortin system through the MC4R occurs via upstream activation of leptin (Figure 1-1). Leptin is an adipocyte derived hormone which relays peripheral signals of adiposity and food intake to regulate energy homeostasis [37]. Following feeding, elevated circulating levels of leptin suppress appetite [38]. Conversely, during periods of calorie excess, leptin stimulates anorexigenic POMC neurons by binding to the leptin receptor (LepR), while inhibiting orexigenic NPY/AgRP neurons (Figure 1-1). Activated POMC neurons secrete α-MSH to bind to MC4R-expressing neurons, triggering a series of anorexigenic signals to reduce food intake and to increase energy expenditure through upregulated thermogenesis [39-41]. Accordingly, elevated leptin levels (hyperleptinemia) in obese humans or animal models fail to suppress food intake and reduce body weight, likely as a consequence of impaired leptin signaling/central leptin

resistance [42, 43]. It is however, unknown, whether increased leptin secretion or impaired leptin signaling contributes to obesity-related disorders [44, 45]. Conversely, during calorie restriction, leptin levels decreases, thus inactivating POMC neurons while stimulating NPY/AgRP neurons to increase appetite and conserving energy [46, 47]. In agreement with this, the sequential fall in circulating leptin is accommodated by an upregulation of *Npy/Agrp* mRNA expression following 9 hours of fasting, whereas the decline in POMC mRNA expression does not occur only until much later (18 hours of fasting) [48]. Observations are consistent with previous findings confirming that leptin directly inhibits NPY/AgRP neurons [49], and is required to maintain POMC expression [46]. It should be noted that although the melanocortin system is the predominant mediator of leptin signaling, independent and additive effects of anorexigenic/orexigenic systems have been reported [50, 51]. Furthermore, factors such as age, gender and diet may influence the interaction between these two systems [52].

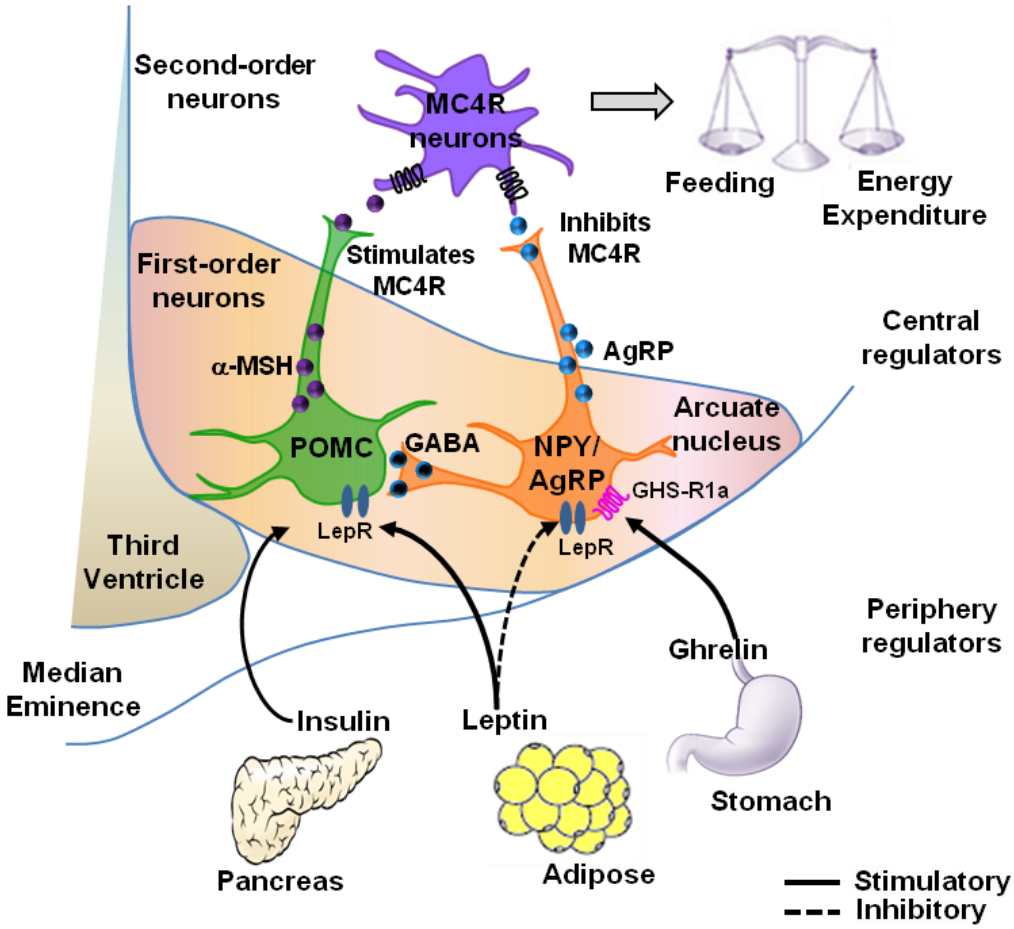

Figure 1-1: Central and peripheral regulators specific to food intake, mediated through the melanocortin system.

Following food intake, increased circulating levels of adipose derived leptin activates first-order POMC neurons by binding to LepR expressed within the ARC. In turn, activated

POMC neurons secrete α-MSH to bind to second order MC4R-expressing neurons to inhibit food intake. Following feeding, reduced circulating leptin results in the loss of inhibition of NPY/AgRP neurons and the consequential inhibition of POMC neurons via inhibitory γ-aminobutyric acid (GABA) inputs from NPY/AgRP neurons. During excess calorie intake, elevated pancreatic insulin levels may indirectly regulate blood glucose levels by activating POMC neurons in the ARC, thereby inhibiting food intake. Ghrelin is an orexigenic hormone secreted from the stomach which activates the NPY/AgRP neurons via GHSR-1a, thereby stimulating food intake (the role of ghrelin will be reviewed in Chapter 1.8.1). Abbreviations; neuropeptide Y; NPY, agouti-related peptide; AgRP, proopiomelanocortin; POMC, melanocortin 4 receptor; MC4R, alpha-melanocyte stimulating hormone; α-MSH, gamma-aminobutyric acid; GABA, leptin receptor; LepR, insulin receptor; InsR, growth hormone secretagogue receptor-1a; GHS-R1a.

1.3 Melanocortin 4 receptor knockout (MC4RKO) mice

Defects in melanocortin signaling specifically in the POMC, agouti or AgRP gene, MC3R and MC4R result in hyperphagia and obesity [53-60]. These observations confirmed that hypothalamic melanocortinergic neurons exert a tonic inhibition on appetite, and disruption of this functional role accounts for an impairment in energy homeostasis. Of particular importance, development of the MC4RKO mouse model confirmed that the MC4R acts as the dominant regulator of melanocortin actions on energy homeostasis [58].

Targeted deletion of MC4R results in maturity-onset obesity, characterized by hyperphagia, hyperinsulinemia and hyperleptinemia [58]. Heterozygous MC4RKO mice show an intermediate elevation in body weight compared to wild-type littermates (WT LM) and homozygous mice, suggesting that the severity of the obese phenotype of the MC4RKO mice is dependent on the degree of loss of MC4R function [60]. Central and peripheral administration of leptin decreases food intake and increase energy expenditure in rodents [61-64]. In contrast to non-obese WT mice, the inhibitory effects of leptin on feeding are not observed in MC4RKO mice [65, 66]. This suggests that the MC4R plays a significant role in mediating the inhibitory effects of leptin on food intake. This is consistent with the finding that *in vivo* leptin administration directly activates action-potential firing activity of MC4R neurons within the ARC-PVN to regulate energy homeostasis [67]. It should be noted, however, that obesity is associated with multiple endocrine impairments, and thus hyperleptinemia (seen in MC4RKO mice) associated with obesity may desensitize leptin signaling in obese MC4RKO mice. Consequently, the failure of leptin to reduce food intake in obese MC4RKO mice may occur in response to obesity-associated leptin insensitivity and not solely due to the deletion of the MC4R. Irrespective of this, both central and peripheral administration of the MC4R selective α-MSH-like agonist, melanotan II (MT II) had no effect on food intake and energy expenditure in MC4RKO mice

relative to WT LM mice. This suggests that the acute effects of α-MSH on energy homeostasis are mediated primarily through the MC4R [39, 66]. Thus, irrespective of obesity-associated leptin resistance, failure to activate MC4R results in the loss of satiety and a decline in metabolic function.

1.3.1 MC4R as a regulator of GH secretion

Alongside the severe metabolic phenotype as a consequence of MC4R deficiency, the obese phenotype of MC4RKO mice is associated with accelerated linear growth, suggesting that the melanocortin system may modulate growth by altering GH release. This may possibly occur through MC4R-expressing neurons interacting along the GH axis to modulate GH release. Accordingly, impaired MC4R signaling in humans results in a partially recovery of pulsatile GH release in MC4R deficient patients compared to BMI-matched controls. This suggests that the increase in adult height in obese MC4R deficient individuals may occur in response to pubertal GH hypersecretion, and thus enhanced GH-mediated pubertal growth [68]. The hypersecretion of GH in these individuals is disproportionate to the degree of obesity observed in both rodents and humans [58, 68, 69]. It should be noted, however, that BMI may not be an accurate representative indicator of adiposity, and thus may not account for variations in lean mass between the obese and MC4R deficient populations [70]. Given that MC4R deficient adults have increased lean mass [68] which may compromise the accuracy of BMI in determining fat mass, and that GH secretion declines with increased adiposity [12, 13], comparison of pulsatile GH secretion between MC4R deficient individuals to obese individuals with similar BMI may be misleading. Moreover, based on the premise that obesity is associated with the suppression of GH secretion [12, 13], accelerated linear growth in obese MC4R deficient adults as a consequence of GH hypersecretion may contradict the anticipated anabolic actions of GH and insulin in sustaining fatty acids flux during energy excess and in obesity (this will be reviewed in the following sub-chapters).

Recent measures demonstrate that impaired pulsatile GH secretion and increased insulin secretion in humans precede hyperphagia-induced weight gain [14, 71], and that the suppression of GH secretion with increased food consumption improves meal tolerance [71]. This suggests that prolonged overeating may influence key endocrine profiles to initiate metabolic adaptations that favours energy surplus. Given the opposing metabolic actions of GH and insulin in promoting dietary fat storage, impaired GH secretion relative to increased adiposity is thought to be a physiological adaptation that facilitates the homeostatic balance of circulating fatty acids. Given this, one may assume

an inverse relationship between circulating GH and insulin secretion during periods of energy consumption. *In this regard, the assumption of GH hypersecretion to promote rapid linear growth in obese MC4R deficiency individuals may not be valid. This thesis will address the hypothesis whether increased GH secretion contributes to rapid linear growth associated with MC4R dysfunction.* Measures of pulsatile GH release during pubertal growth in humans with MC4R deficiency currently do not exist. Considering that GH is the key hormone regulator of linear growth in childhood, the incorporation of MC4RKO mouse model will address the role of MC4R in regulation of GH secretion, specific to pubertal linear growth. Specifically, pulsatile GH secretion will be assessed in pubertal MC4RKO mice throughout peak periods of rapid linear growth. This is further discussed in Chapter 5.

1.4 The Hypothalamo-Pituitary Axis

The hypothalamo-pituitary axis regulates pituitary function by producing stimulatory and inhibitory hormones to release or inhibit pituitary hormone production [72]. Classically, the pituitary gland is composed of two lobes, the posterior pituitary (known as neurohypophysis) and the anterior pituitary (or adenohypophysis). The region lies distance between the anterior and posterior lobes of the pituitary is known as the intermediate lobe. The intermediate lobe differentiates from the same embryological origin as the anterior lobe, and synthesizes POMC peptides including MSH [72]. Despite it close proximity to the anterior pituitary lobe, the posterior lobe originates from a downgrowth (infundibulum) of the neuroectoderm of the development brain, and secretes anti-diuretic hormone (ADH) and oxytocin, which functions independent of the anterior pituitary [72]. In contrast, majority of pituitary endocrine cells synthesized in embryonic cells derived from the Rathke's pouch in the anterior lobe of the pituitary gland. These endocrine cells secretes specific hormones and are released directly into the blood circulation to promote essential homeostatic functions such as reproduction, lactation, stress, growth and metabolism [72]. Under the influence of the hypothalamic trophic hormones, the specific cell types and their respective pituitary hormones include gonadotrophs (secreting luteinizing hormone (LH)) and follicle-stimulating hormone (FSH)), lactotrophs (secreting prolactin (PRL)), corticotrophs (secreting ACTH), thyrotrophs (secreting thyroid stimulating hormone (TSH)) and somatotrophs (secreting GH) [73]. Amongst these, the somatotrophs are the major endocrine cells of the anterior pituitary lobe, constituting approximately 50% of the entire cell population [74]. Recent exploration in rodent studies reinforced the concept of the two-dimensional plasticity of pituitary organization. Advanced high-resolution imaging of cellular structures in transgenic mice expressing green fluorescent protein (GFP) in

somatotrophs revealed that the functional integration of the anterior pituitary gland is highly organized in a three-dimensional network [73, 75]. The dynamic integrity of the anterior pituitary lobe adjusts accordingly at varying stages in response to physiological stimuli [76]. To this extent, increased clusters of the somatotroph cells correspond to a profound increase in GH secretion in pubertal mice [75], suggesting that altered integrity of the somatotroph network facilitates coordinated pulsatile GH secretion to encourage pubertal growth (further discussed in Chapter 1.5.2). Of importance, the presence of growth hormone releasing hormone (GHRH) markedly enhances the assembly of somatotroph cells [75, 77], indicating that synchrony of the somatotroph network appears to be dependent on hypothalamic GHRH. This is in consistent with previous observations, whereby transgenic ablation of hypothalamic GHRH neurons in mice results in severe derangement of the somatotroph cluster organization within the pituitary [78]. Thus, observations emphasize the importance of synchronized network architecture within the anterior pituitary in modulating pituitary GH system (this is further discussed in Chapter 3.4).

1.4.1 Growth hormone (GH)

Growth hormone (GH), also known as somatotropin, is synthesized and secreted by somatotrophs located within the anterior pituitary gland [79]. The regulation of episodic GH secretion from the pituitary gland is predominantly driven by the reciprocal actions of hypothalamic hormones, GHRH and somatotropin release inhibitory factor (SRIF or somatostatin) [80]. GHRH determines the amplitude of GH secretion, whereas SRIF determines trough levels of GH secretion by inhibiting GHRH release and GHRH-induced GH secretion [81, 82]. GH exerts a wide range of physiological functions and affects substrate metabolism via direct and indirect mechanisms. GH acts by binding to the GH receptor (GHR), expressed predominantly in the liver and induces intracellular signaling by activating the Janus kinase-signaling transducer and activator of transcription (JAK-STAT) pathway [83]. GH stimulates the production of hepatic IGF-1 via GHR, a potent growth and differentiation peptide that mediates many growth-promoting actions of GH [84] (discussed in Chapter 1.7.3).

1.4.2 Pulsatile mode of GH secretion

The secretion of GH occurs in a pulsatile manner, characterized by low basal GH levels that are dispersed by several dominant pulses that occur at periodic intervals [85, 86]. This mode of GH release is sexually dimorphic, and the pulsatile pattern of GH release is

conserved across all mammalian species characterized to date [79]. Schematic reproduction in Figure 1-2 illustrates the pulsatile pattern of GH secretion in healthy humans (Figure 1-2A), rats (Figure 1-2C) and mice (Figure1-2E). During periods of energy excess, increased adiposity and weight gain results in a marked suppression of GH release in obese humans (Figure 1-2B), in rats (Figure 1-2D) and in mice (Figure 1-2F). Pulsatile GH secretion events in healthy men occur approximately every 2 to 3 hours, and whilst women tend to have significantly greater GH levels (higher pulse amplitude and baseline GH secretion), the pulse frequency is similar to that seen in men [86-88]. In male rats, GH secretion occurs in distinct pulses with relatively low interpeak levels at approximately 3 to 3.3-hour intervals. By comparison, female rats exhibit lower irregular pulses with higher interpeak levels [85, 89, 90]. The regulation of GH secretion between males and females differ significantly due to the endogenous estrus cycle associated with ovulation [91, 92]. Consequently, differences in gonadal steroid profiles between males and females may significantly alter or impair GH release and function, respectively. In this regard, an elevation in estrogen in females enhanced GH secretory dynamics [92]. Consequently, GH secretion in females is believed to be elevated [92]. To this extent, the metabolic role of GH between males and females may differ significantly.

Observations of pulsatile GH release from mice are limited [93, 94]. Due to their small size, prior observations of pulsatile GH secretion in mice were limited to one-off measures [93, 94]. This does not accurately reflect overall GH secretion, and thus interpretation of the actions of GH based on single measures may be misleading. The recent development of methodology to allow the detection of pulsatile GH release in mice provided a renewed opportunity to incorporate transgenic mouse lines in research aimed at deciphering GH release [95]. This methodology revealed that, as with humans and rodents [85, 89, 93, 96], mice display a regular periodicity in GH secretion, characterized by peak secretion periods and interpulse stable baseline secretion periods [48, 95, 97-99]. *Measures of GH secretion in this thesis are assessed based on pulsatile GH secretion profiles, and limited to male physiology.*

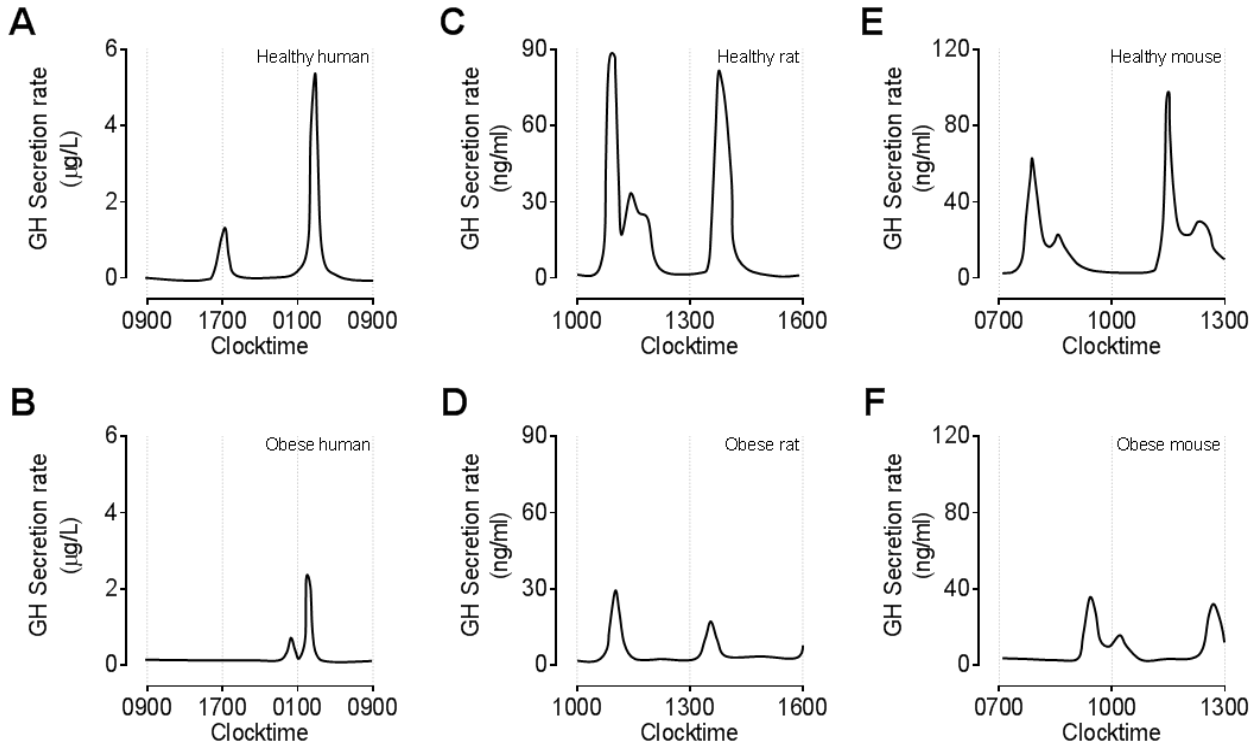

Figure 1-2: Schematic examples illustrating the pulsatile profiles of GH secretion in normal and obese humans (24-hour profile) and rodents (6-hour profile). Pulsatile GH secretions of male profiles were reproduced from [13, 88, 97, 100].

1.5 Physiological aspects of GH

GH exerts its effect directly via the GHR. During pubertal development, peaks in GH secretion activate the GH/IGF-1 axis [1, 11]. The rise in GH levels is necessary to stimulate longitudinal bone and skeletal muscle growth during childhood, allowing children to grow to a normal adult height [101]. Consequently, GH deficiency (GHD) as a consequence of pituitary disease during childhood results in severe growth retardation. In GH deficient dwarf rats, treatment with GH promoted femur bone growth [102]. Similarly, exogenous infused of GH at the site of epiphyseal growth plate of hypophysectomized rats stimulated unilateral growth and restored longitudinal bone growth [103]. Accordingly, elevated IGF-1 mRNA expression or local IGF-1 peptide in the epiphyseal growth plate of the long bone parallels the administration of GH in hypophysectomized rats [104-106]. To this extent, observations support the concept of stimulatory effects of GH in the clonal expansion of epiphyseal chondrocytes for bone growth. Subsequent measures demonstrated that the infusion of GH in combination with IGF-1 antiserum abolished the stimulatory effects of bone growth in response to GH, indicating that the stimulatory effects of longitudinal bone growth is dependent on the local effects of autocrine/paracrine actions of IGF-1 [107, 108]. Moreover, the loss of liver-derived circulating IGF-1 does not impair

postnatal growth following liver-specific deletion of IGF-1 in mice [109, 110]. Thus, circulating IGF-1 is thought to modulate long-term changes in GH release through negative feedback [111]. Given that the effects of pulsatile GH infusion in stimulating bone and/or muscle IGF-1 mRNA expression in hypophysectomized male rats [112] or GH-deficient dwarf rats [102] were more pronounced compared to continuous GH administration, the autocrine/paracrine mode of IGF-1 actions appears to be dependent on the rhythmic release of GH [112]. This suggests that the secretory pattern of GH delivery is a major determinant of GH action for optimal linear growth.

1.5.1 Metabolic actions of GH

The physiological actions of GH change dramatically following the attainment of peak linear height following pubertal maturation. In adulthood, GH primarily maintains body composition and metabolism [113, 114]. Treatment with rhGH in GH deficient adults increased lean muscle mass and muscle strength resulting in improved physical activity [115, 116] and decreased abdominal fat mass [117-119]. In contrast, termination of GH treatment in GH-deficient adults on long term rhGH replacement therapy results in an increase in subcutaneous and visceral fat mass and total waist circumference [120], whereas muscle mass and resulting physical activity output were compromised in adults with childhood onset GH deficiency [121]. Collectively, observations confirm that continuous GH therapy is essential to maintain the lipolytic and therapeutic effects of GH on body composition and skeletal muscle profile. As an anabolic hormone, GH may enhance fat utilization as the preferential energy substrate to retain lean muscle mass. Prior observations demonstrate that GH increases whole-body lipolytic rate in humans [122-124], resulting in the augmentation of NEFAs during periods of calorie restriction. This suggests that endogenous GH may be the predominant regulator of lipolysis during short-term fasting. The elevation of circulating NEFAs represent a physiological adaption in response periods of calorie restriction (or metabolic stress), where mobilized NEFAs act as the primary source of energy fuel to preserve lean body mass [124, 125]. Unlike humans, the fasting-induced release of NEFAs in mice does not occur in response to an elevation in GH [48]. While this suggests that the release of NEFAs in response to fasting may differ between mammalian systems, current observations do not dismiss the role of GH in mediating NEFA release in mice in general. Given that GH is a major regulator of lipolysis, long-term changes in endogenous GH are more likely to significantly alter body composition. Accordingly, GH excess is associated with decreased body fat mass in bovine GH (bGH) transgenic mice, while loss of GH signaling results in the accumulation

of subcutaneous fat mass in GHR knockout (GHRKO) mice [126, 127]. Thus, regardless of the differential role of GH mediated fasting-induced changes in NEFAs flux in mice, GH is a predominant factor that regulates adiposity, and presumably long-term fatty acid flux in mice.

1.5.2 Physiological role of GH in regulating Linear Growth

GH is a major determinant of postnatal longitudinal growth during childhood. In humans, the pubertal growth spurt is associated with increased GH secretion [101, 128]. The rise in GH is critical in the remodeling of bone formation, and this occurs directly through interactions with GHR, or indirectly through autocrine/paracrine effects of IGF-1 to promote linear growth [129]. Accordingly, idiopathic childhood onset GHD as a consequence of pituitary dysfunction results in decreased bone density and impaired growth height [2, 3]. This is restored following early rhGH treatment, allowing these children to achieve adult height [9], confirming that GH is critical in promoting optimal bone health and linear growth. Defects in the GHR signaling in humans result in an overall short stature [4, 5]. Similarly, severe postnatal growth retardation is observed in mouse models with disrupted GHR signaling [6-8, 130]. For example, the rate of growth determined by the body length in pubertal GHRKO mice was reduced between 2 and 6 weeks of age, and this occurred as a consequence of reduced chondrocyte proliferation and cortical bone growth [6]. Thus, GH acting via the GHR is important in bone formation and pubertal growth at this time. In line with this, the administration of GH to GH deficient hypophysectomized adult [103] or dwarf rats [102] restored longitudinal bone growth. Moreover, the correction of bone growth mediated by pulsatile GH infusion was more effective than continuous GH therapy [102, 112], suggesting that pulsatile GH release is critical for optimal linear growth during puberty. In this regard, pubertal growth is dependent on the normal pulsatile GH secretion.

1.5.3 Secretion of GH from Puberty throughout Ageing

In humans, GH production begins early in fetal life [131], rising steadily throughout puberty before declining progressively thereafter [101, 132]. During puberty, maximal GH secretion increases up to 3-fold [101], and the mass of GH secreted per burst increases between 2- to 10-fold [101, 133]. In humans, the augmentation of GH release during puberty is attributed to increased GH pulse amplitude as a consequence of an increase in the amount of GH secreted per secretory event [101, 128]. Given that GHRH determines the amplitude of GH secretion, an elevation in circulating GHRH activity is thought to account for peak GH secretion during puberty. In agreement with this, the administration of

neonatal monosodium glutamate to mimic GHRH deficiency abolished the pubertal increase in *Gh* mRNA, pituitary GH content and severely impaired growth rate in male and females rats [134]. Thus, while not assessed alongside pulsatile GH secretion, observations suggest that GHRH may contribute to enhanced pubertal GH secretion at this time.

Following the cessation of pubertal growth, GH secretion declines with age by approximately 14% per decade in humans [12]. This is characterized by a reduction in the amplitude of GH secretory burst [79]. Furthermore, this age-associated decline in GH secretion correlates with decreased gonadal hormone concentrations in men and women [135, 136], suggesting that testosterone and estradiol may be important regulators of GH secretion. In ageing individuals, the pituitary gland remains responsive to GHRH stimuli, regardless of its decreased sensitivity [82, 137]. While this suggest that the GH-releasing effect of somatotrophs in response to GHRH may be preserved during ageing, the attenuation of GHRH-induced GH secretion in elderly individuals [82, 138] suggest that age-associated changes in GH may occur in response to decreased hypothalamic GHRH release or diminished somatotroph function. In agreement with human observations, the age-associated decline GH secretion in male and female rats is accompanied by a decrease in pituitary GH content [139, 140]. Moreover, a reduction in *Ghrh* mRNA resulting in decreased GHRH synthesis was impaired in the hypothalamus of senescent male rats [141]. Consequently, alterations in hypothalamic GHRH-induced GH secretion are likely one of the primary mechanisms responsible for the age-associated loss of GH release.

Prior observations in humans suggested that that the age-associated decline in GH secretion may occur in response to somatostatinergic hyperactivity. Co-administration of arginine enhances the stimulatory effect of GHRH on GH responses in elderly adults by suppressing the SRIF release [142]. Similarly, arginine in combination with GH releasing peptide (GHRP) hexarelin, a GH secretagogue (GHS), virtually restores stimulated GH secretion to levels seen in young adults [143]. This suggests that SRIF excess in hyposomatotropism with age contributes to reduced GH release. To this extent, pretreatment of pyridostigmine (a cholinergic agonist) to reduce SRIF release and to stimulate GHRH release normalized blunted GH secretion and plasma IGF-1 concentrations in healthy aged men [144]. Collectively, these studies support the notion that age-associated changes in GH input may occur in response to a reduction in GHRH release, and, not excluding to the contribution of enhanced SRIF input. The role of SRIF in modulating age-associated decline in GH secretion from puberty into adulthood, however, is less defined. While increased somatostatinergic tone is thought to contribute to the

progressive reduction in GH secretion with age, observations of structural changes in SRIF expression from ageing rats are conflicting. For instance, the loss in SRIF immunoreactivity in aged rats [81, 145] is contradicted by observations showing sustained SRIF immunoreactivity regardless of age [81]. Moreover, measures directly assessing the role of SRIF in modulating GH secretion alongside pulsatile GH secretion do not exist. Consequently, the potential role of SRIF in accounting for age-associated changes in pulsatile GH secretion from puberty throughout adulthood remains unsubstantiated. This will be addressed in Chapter 4.

1.5.4 Secretion of GH in Obesity

Obesity reflects the consequence of chronic long-standing positive energy balance when energy intake (overeating) exceeds energy expenditure (sedentary physical activity). Such a state in humans results in excess calories being stored as body fat, resulting in the progressive weight gain culminating in obesity. Thus, prolonged excess calorie intake is thought to be the underlying cause for obesity and obesity-associated metabolic disorders. In this context, the secretion of GH is directly proportional to the amount of energy consumption. In humans, the reduction in GH release is often observed during periods of excess calorie intake, including hyperphagia and obesity [12-14]. The degree of GH attenuation correlates with the amount of total and visceral adipose mass [12, 13, 146]. In support of this, reductions in basal and stimulated GH concentrations are observed in obese individuals. This is characterized by a reduction in GH half-life, and reduced peak amplitude and frequency of GH secretory pulses [12, 13, 147, 148]. As seen in humans, genetically obese rodent models of obesity (including Zucker rats, ventromedial hypothalamus-lesioned rats) and obese adult rhesus monkeys exhibit GH deficiency [149-152]. The underlying mechanisms responsible for the suppression of GH in obesity are not completely understood. In humans, the restoration of GH release was observed following normalization of body weight [153-155]. Thus, observations suggest that metabolic alterations associated with increased adiposity alongside weight gain are major determinants of impaired GH secretion, and that these changes are reversible.

Recent clinical observations demonstrate that impaired pulsatile GH secretion and consequently GH deficiency in humans following sustained calorie excess (hyperphagia) precedes dietary induced weight gain [14]. Moreover, the suppression of GH secretion relative to hyperphagia is thought to ameliorate insulin resistance, thereby improving meal tolerance to prevent hyperlipidemia [71]. This suggests that impaired GH release in obese individuals occurs prior to the onset of obesity, and while facilitating the removal of lipids

from circulation, may predispose hyperphagic adults to the development of obesity. These observations present a renewed perspective on the role of GH relative to excess calorie consumption. In agreement with this, circulating GH concentrations were suppressed in obesity-susceptible rats when compared to obesity-resistant rats [156]. Importantly, measures of GH levels in obese-prone rats revealed a deficit in GH secretion that occurs prior to weight gain and the onset of obesity [157]. Of interest, the suppression of GH release prior to dietary induced weight gain in hyperphagic adults was accompanied by a rise in postprandial levels of insulin (hyperinsulinemia) [14]. While not directly assessed, hyperphagia-associated hyperinsulinemia may contribute to the progressive decline in GH secretion following dietary induced weight gain. The role of insulin in regulating GH release relative to food intake and weight gain will be addressed in Chapter 5.

1.6 Regulation of GH release

The secretion of GH from the anterior pituitary gland is controlled by the stimulatory actions of hypothalamic GHRH neurons, and the inhibitory influence of hypothalamic SRIF neurons. GHRH and SRIF neurons, released through the hypophyseal portal vessels act in concert to regulate the release of GH secretion from the somatotrophs (Figure 1-3). Released GH acts upon the liver to stimulate the production of IGF-1, which inhibits GH secretion through a negative feedback loop [80]. Circulating IGF-1 inhibits GH secretion through negative feedback at the level of anterior pituitary gland and hypothalamus. GH also regulates its own secretion via short-loop feedback circuit to inhibit hypothalamic GHRH and stimulate SRIF release into the hypophyseal portal circulation (Figure 1-3). Furthermore, the hypothalamic regulation of GH release occurs via an ultra-short loop feedback exerted by both GHRH and SRIF on their own secretion [79]. The functional interactions between principal regulatory neurohormones are supplemented by a host of neurotransmitters, neuropeptides and peripheral metabolic parameters (including insulin, leptin, ghrelin, NEFAs and glucose) that couple nutritional status with GH secretion [13, 79]. In this thesis, I will discuss the pathophysiological aspects of these parameters in modulating GH secretion relative to energy homeostasis.

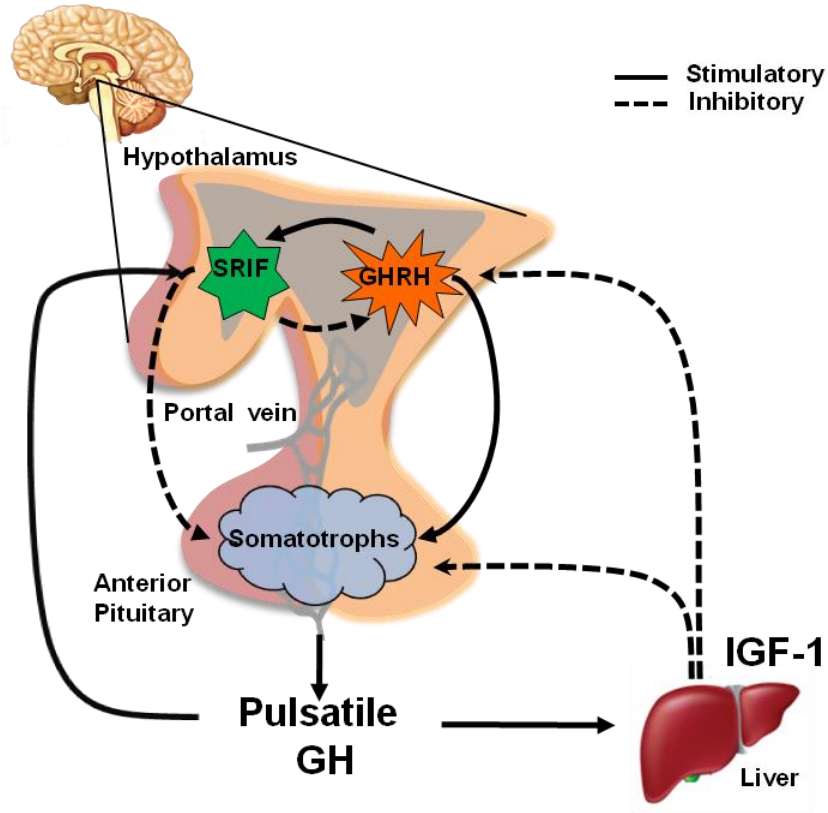

Figure 1-3: Feedback regulation of the hypothalamic-pituitary-growth hormone (GH/IGF-1) axis.

The secretion and release of GH from the anterior pituitary gland is stimulated by GHRH and inhibited by SRIF neurons. Released GH limits its own secretion by exerting short-loop negative feedback to increase SRIF activity in the periventricular nucleus. GH stimulates the production of insulin like growth factor 1 (IGF-1) from liver. Peripheral IGF-1 inhibits GH secretion through negative feedback at the level of anterior pituitary gland and hypothalamus. Abbreviations; growth hormone releasing hormone; GHRH, somatostatin releasing inhibitory factor; SRIF, growth hormone; GH, insulin-like growth factor-1; IGF-1.

1.7 The GH/IGF-1 axis – Primary (Central) Regulators of GH secretion

1.7.1 Growth hormone releasing hormone (GHRH)

GHRH is synthesized and produced by GHRH neurons, located within the ARC of the hypothalamus (Figure 1-3). The release of GHRH neurons projects into the hypophyseal artery at the base of the median eminence ME to initiate the release and synthesis of GH from the somatotrophs within the anterior pituitary gland [158]. GHRH stimulates GH secretion by binding to GHRH receptors (GHRH-R) expressed on pituitary somatotrophs [159, 160]. The binding of GHRH to GHRH-R (class B (secretin receptor) GPCR superfamily), activates the heterotrimeric stimulatory G protein (Gs), composed of a Gα-, and the tightly associated Gβ- and Gγ-subunits. This triggers the dissociation of the Gα subunit from the Gβ and Gγ complex, leading to the activation of adenylyl cyclase (AC), and thus the production of cyclic adenosine monophosphate (cAMP) (Figure 1-4). The

release of GH from somatotrophs is entirely dependent on an increase in intracellular Ca^{2+} following the influx of Ca^{2+} via voltage-dependent Ca^{2+} channels [161]. Increased levels of cAMP acts as second messenger which activates the protein kinase A (PKA) signaling pathway [162]. In addition, the binding of GHRH to GHRH-R also activates the phospholipase C/inositol phosphate 3 (PLC/IP3) [161] pathway, leading to the release of intracellular Ca^{2+} from the endoplasmic reticulum to further facilitate GHRH-induced GH release from the somatotrophs.

The hypothalamic control of GH secretion is governed by the release of GHRH. In humans, administration of competitive GHRH antagonists severely impairs spontaneous pulsatile GH secretion [163] and blunts the response to GH-releasing stimuli [164, 165]. Thus, observations suggest that endogenous GHRH is likely the predominant stimulatory regulator of GH secretion. During periods of energy excess, the secretion of GH is impaired in response to all pharmacological stimulus acting at the level of hypothalamus [166]. In overweight individuals, exogenous pulsatile or continuous GHRH treatment to stimulate GH-releasing somatotrophs fails to evoke a rise in GH secretion [167, 168]. In line with this, decreased circulating levels of GH coincide with a reduction in hypothalamic *Ghrh* mRNA expression and GHRH protein content in obese Zucker rats, [151, 169]. Subsequent findings reported that the blunted GH secretion observed in obese leptin-deficient mice (Ob/Ob), and dietary induced obese (DIO) mice is accompanied by a reduction pituitary *Ghrh-r* and *Gh* mRNA expression and GH content [170]. This reduction in pituitary GH content is consistent with the GH deficiency observed in obese humans [16, 155, 171]. Regardless of alterations at the pituitary level, hypothalamic *Ghrh* mRNA expression remains unchanged in DIO mice compared to that seen in lean mice [170]. Similar observations demonstrating that obese rats placed on a cafeteria-style diet display a reduction in GH secretion alongside normal hypothalamic GHRH expressions, and were unresponsive to exogenous GHRH treatment irrespective of the extent of obesity [172]. While this suggests that obesity-associated GH suppression may be due to a primary pituitary defect initiated by systemic factors, the release of GHRH may possibly be altered independent of changes in gene expression. Moreover, current observations reflecting alterations in pulsatile GH release alongside measures of hypothalamic GHRH output do not exist. Regardless, observations suggest that altered GHRH neuronal output may contribute to the suppression of GH in response to energy excess. Thus, impaired GH secretion in obesity may be due, at least in part, to an overall reduction in pituitary GH expression as well as defects in the capacity of somatotrophs to respond to GHRH stimuli. This awaits further investigation.

1.7.2 Somatotrophin releasing inhibitory factor (SRIF)

SRIF is abundantly distributed throughout the CNS, as well as peripheral tissues [173], SRIF-producing neurons involved in the regulation of GH secretion are predominantly found within the periventricular nucleus (PeVN) of the hypothalamus. SRIF neurons within the PeVN projects to the ME and release SRIF from neurosecretory terminal, after which is transported by the hypophyseal portal system to the anterior pituitary gland to inhibit GH release, thereby modulating the amplitude of GH pulses (Figure 1-3) [158]. Although SRIF receptors (SSTRs) are widely expressed in the anterior pituitary gland [174], the antagonistic actions of SRIF on GHRH-induced GH release from the somatotrophs in rodents are primarily mediated through SSTR2 and SSTR5 [174]. SRIF inhibits GH secretion either directly by inhibiting somatotroph activity via SSTRs, or indirectly by inhibiting GHRH release from the ARC (Figure 1-4). SRIF inhibits GH release from the somatotrophs by limiting adenylyl cyclase (AC) activity and reduces cAMP accumulation, while opening of the K^+ channels. This leads to the hyperpolarization of membrane potential and consequently, inhibiting Ca^{2+} influx through voltage-dependent calcium channels (Figure 1-4). This decreases intracellular Ca^{2+} levels, thereby maintaining basal GH release from the pituitary somatotrophs at low levels [175, 176].

The mechanism of decreased GH secretion in obese state is thought to be attributed to the hypersecretion of hypothalamic SRIF, given that the administration of exogenous pyridostigmine or arginine (to inhibit hypothalamic SRIF release) in combination with GHRH or GHRP-6 restored reduced GH secretion in obesity [142, 177, 178]. However, while treatments in obese adults evoked a GH response, the magnitude of GH release following pyridostigmine or arginine treatment in obese adults was not completely reversed to that seen in normal weight individuals. Thus, observations suggest that SRIF may not solely contribute to the decline in GH secretion in obesity. In this context, the decline in GH secretion in either obese Zucker rats [100, 172] or DIO mice [170] may not occur solely in response to alterations in hypothalamic *Srif* mRNA or protein content. Moreover, passive immunization with specific SRIF antiserum fails to restore the amplitude of GH release in obese rats [100]. Collectively, observations suggest that central SRIF actions may not solely contribute to obesity-associated GH deficiency.

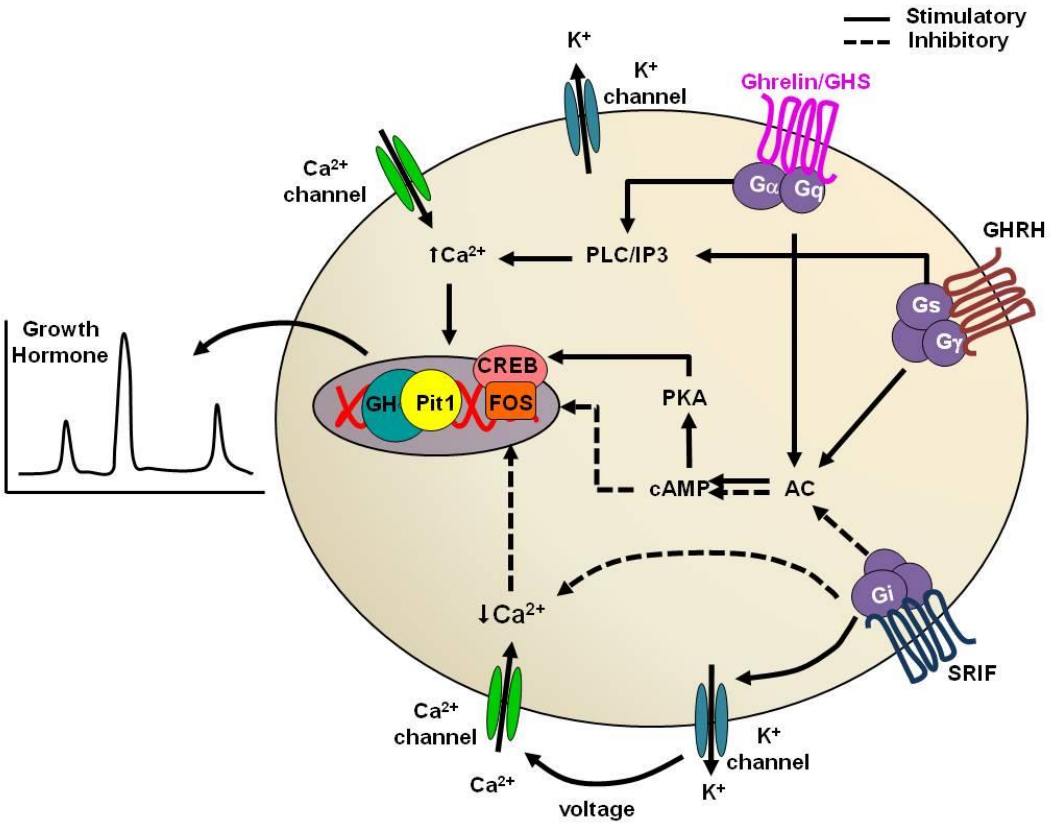

Figure 1-4: Schematic regulatory pathways of GHRH and SRIF in pituitary somatotrophs.

The binding of GHRH to GHRH receptor dissociates γ-subunit from β- and γ-subunits of stimulatory Gs. This leads to the activation of AC to generate cyclic AMP (cAMP) from ATP. cAMP activates PKA which phosphorylates and activates nuclear localization of CREB. CREB activates transcription factor FOS, and together with activated Pit1, activates transcription of GH. To inhibit GH release, SRIF activates inhibitory Gi protein which inhibits AC and reduces intracellular calcium levels. Ghrelin or GHS stimulate GH release by activating multiple interdependent signal transductions involving PKA, PLC/IP3 and intracellular calcium systems. Abbreviations: G protein; Gs, adenylyl cyclase; AC, cyclic adenosine monophosphate; cAMP, ghrelin secretagogues; GHS, protein kinase A; PKA, phospholipase C; PLC, Inositol 1,4,5-trisphosphate; IP3.

1.7.3 Insulin-like growth factor-1 (IGF-1)

IGF-1, otherwise known as somatomedin-C, is a member of the IGF-1 family. This family is comprised of ligands (IGF-I, IGF-II and insulin), cell surface receptors for IGF-I, IGF-II and insulin and IGF-1 binding proteins (IGFBP1-6) [179]. In circulation, the majority of IGF-1 binds to high affinity IGFBPs to regulate the actions of IGF-1 on target tissues, thereby influencing the secretion of GH. Approximately 90% of IGF-1 in circulation is bound to the ternary 150kDa IGFBP complex consisting of IGF-1, IGFBP-3 and an acid-labile subunit (ALS, synthesized within the liver), which prolongs the plasma half life of IGF-1, and modulates the metabolic actions of IGF-1 [180]. Since IGF-1 and IGFBP-3 are dependent on GH release, they are indicators of changes in GH bioavailability and action [111].

IGF-1 is best known as a GH-dependent peptide that mediates the biological effects of GH (Figure 1-5). Circulating IGF-1 is primarily produced within the liver in response to GH secretion. The classical theory of IGF-1 regulation originates in the liver where the growth promoting actions of GH are enhanced by stimulation of hepatic IGF-1 [111]. This notion is supported by observations of stimulated body growth following the systemic administration of IGF-1 to humans or rodents displaying GH deficiency or dysfunction of the GH receptor [181-184]. In line with these observations, systemic deletion or disruption of IGF-1 or IGF-1 receptor signaling in mice and humans results in severe growth retardation as a consequence of postnatal growth failure [185-188]. Taken together, these findings propose that IGF-1 may be essential for normal growth. Importantly, while resulting in a 75% reduction in circulating IGF-1 levels, liver-specific deletion of IGF-1 in mice does not impair postnatal growth [109, 110, 189]. Thus, while confirming that liver is the primary source of circulating IGF-1, these observations suggest that liver-derived IGF-1 is not critically involved in promoting linear growth. Of importance, liver-specific deletion of IGF-1 in mice results in an elevation of plasma GH levels (based on single GH measures), whereas free IGF-1 levels remain unchanged. Thus, sustained postnatal growth in liver-specific deletion of IGF-1 mice may occur in response to sustained actions of endogenous GH, or via autocrine/paracrine effects of locally expressed IGF-1.

IGF regulates GH secretion via long loop negative feedback at the anterior pituitary gland by directly inhibiting GH secretion, and indirectly by stimulating the release of SRIF while inhibiting the activity of GHRH neurons (Figure 1-5). Accordingly, a reduction in circulating IGF-1 occurs alongside an increase in circulating *Gh*, *Ghrh* and *Ghs-r* mRNA expression in the pituitary gland following liver-specific deletion of IGF-1 in mice [109]. In somatotroph specific IGF-1 receptor KO (SIGFRKO) mice, *Gh* mRNA expression and GH proteins are increased, regardless of elevated circulating IGF-1 levels [190]. This occurs alongside a decrease in hypothalamic *Ghrh* and *Srif* mRNA expression presumably in response to the loss of pituitary IGF-1 feedback [190]. While GH is an important regulator of linear growth, the modest elevation in circulating GH did not contribute to enhanced linear growth in SIGFRKO mice when compared to WT controls. This is presumably as pubertal growth may have ceased in these adult SIGFRKO mice [190]. Rather, the metabolic effects of elevated GH levels are more pronounced maintaining body fat mass, since total body fat mass was decreased in SIGFRKO mice compared to WT controls [190]. Although measures of GH in these studies were based on one-off measures that may not accurately reflect the pulsatile nature of GH secretion [109, 190], observations

confirmed the role for liver-derived IGF-1 in the negative feedback regulation of GH secretion at the level of pituitary gland and the hypothalamus.

In obesity, spontaneous GH secretion is diminished [166]. Thus, circulating levels of IGF-1 should be subnormal. However, measurements of total IGF-1 in obese subjects are reported to be normal [191-193] or elevated [194-196]. In contrast, measurements of free IGF-1 levels, which are thought to be the metabolically active form of IGF-1, are elevated in obese patients [191, 192, 197]. Thus, it is proposed that sustained bioavailability of free IGF-1 in obesity contributes to the suppression of GH secretion, although a precise mechanistic to clarify this has yet to be determined. In contrast, calorie restriction restores blunted GH secretion in obese patients and this coincides with a reduction in total and free IGF-1 levels [191, 198]. Collectively, observations demonstrate the reciprocal role of total and free IGF-1 in the negative feedback loop of GH secretion.

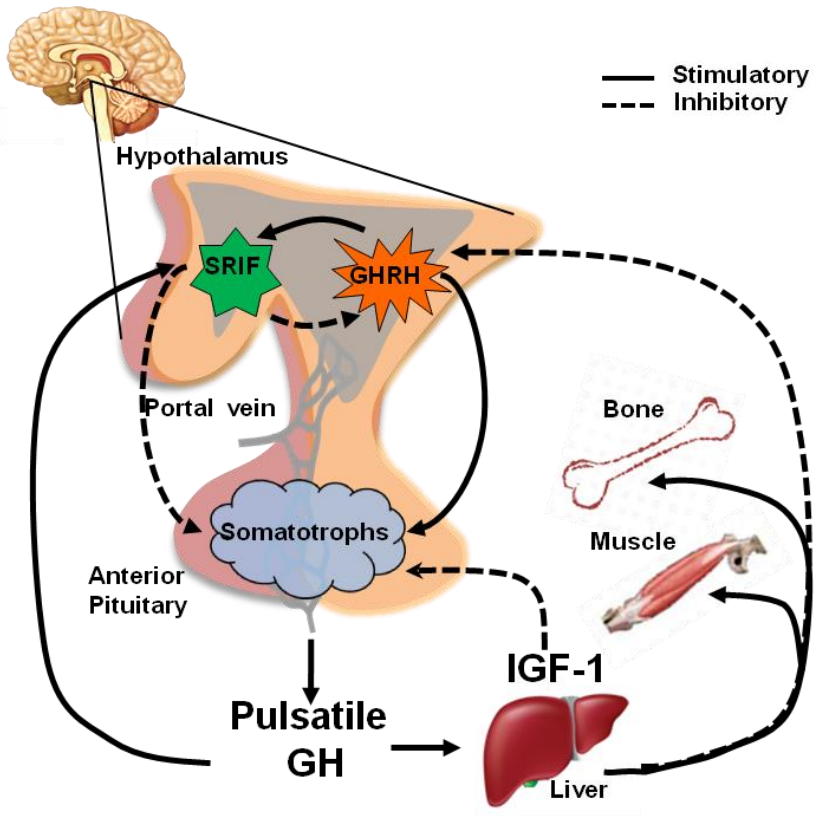

Figure 1-5: GH and liver-derived IGF-1 secretion in the hypothalamic-pituitary feedback system.

The secretion of GH from somatotrophs is stimulated by GHRH and inhibited by SRIF. GH limits its own secretion through a short loop negative feedback to increase SRIF. Local IGF-1 is thought to mediate the anabolic effects of GH to promote bone and muscle growth. Circulating IGF-1 acts to inhibit GH release through long-loop negative feedback at the level of pituitary and hypothalamus. Abbreviations; growth hormone releasing hormone; GHRH, somatostatin releasing inhibitory factor; SRIF, growth hormone; GH, insulin-like growth factor-1; IGF-1.

1.8 Peripheral Regulators of GH secretion

Calorie restriction or weight loss in obese subjects restores spontaneous and stimulated GH release [166]. This suggests that impaired GH secretion in obesity may be associated with elevated peripheral factors that occur alongside weight gain. Of importance, increased circulating levels of adipose-derived hormones including leptin and insulin are typically associated with obesity [23, 26]. Likewise, endogenous ghrelin from the stomach promotes food intake, weight gain and adiposity, and is a potent GHS that augments GH release [199]. Alterations in these appetite related hormones relative to modest changes in food intake are thought to relay signals to the brain to modulate energy homeostasis. While contributing to altered metabolic output, these factors may also alter GH release. In this regard, metabolic alterations are tightly coupled to the regulation of energy balance, involving a complex coordination between peripheral hormones and metabolites. Given that the release of GH changes relative to energy balance, altered output of these metabolic factors associated with energy balance may contribute to altered GH secretion. The interactions between these factors with GH secretion will be discussed below.

1.8.1 Metabolic Hormones as regulators of GH release

1.8.1.1 Leptin

Leptin is a 16kDa adipocyte-secreted hormone that circulates in proportion to fat mass [200, 201]. Leptin maintains energy homeostasis by increasing energy expenditure and by decreasing energy consumption [27]. As discussed above, leptin inhibits appetite and promotes energy utilization by regulating hypothalamic POMC and NPY/AgRP neuron activity (Figure 1-1) [202-204]. Accordingly, leptin plays a vital role in controlling body fat mass through hypothalamic pathways that coordinate appetite and modulate efferent signals to the periphery, regulating intermediary metabolism and energy expenditure. These effects are mediated via interactions between orexigenic and anorexigenic neuropeptides (Figure 1-1) [205]. Central and peripheral administration of leptin decreased food intake and increase energy expenditure [206, 207]. While circulating leptin increases in proportion to increased adiposity [208], it should be noted, however that, leptin in obese individuals does not suppress food intake, suggesting that obesity itself is associated with central leptin resistance [209, 210].

The role of leptin in the regulation of GH/IGF-1 axis is well documented. Several studies showed that exogenous leptin stimulates GH release from pituitary explants, thus demonstrating that GH secretion is directly regulated by leptin [211-213]. Of importance, intracerebroventricular leptin infusion in adult rats augments both spontaneous pulsatile

GH and GHRH-induced GH release (Figure 1-6) [214-216]. Given that the release of GH secretion is facilitated by the episodic release of stimulatory GHRH, and inhibitory SRIF during GH trough periods, observations suggest that leptin serves as an important hormonal regulator of the GH axis. This notion is supported by the fact that hypothalamic GHRH neurons express LepR [217]. Moreover, SRIF is thought to inhibit its own secretion, and that of GHRH *in vitro* [218]. Thus, the facilitatory actions of leptin on GH secretion are likely mediated, at least, in part by modulating both GHRH and/or SRIF. Recent studies demonstrate that obese mice exhibiting leptin or LepR deficiency showed reduced proliferation of somatotrophs [219, 220]. Furthermore, deletion of somatotroph-specific LepR in mice results in a reduction in GH immunopositive cells and the consequential development of GH deficiency and obesity [221]. These findings suggest that leptin may directly regulate GH release and maintain somatotroph activity. In view of this, one may anticipate sustained GH release associated with hyperleptinemia during enhanced calorie intake. However, decreased GH secretion coexists with elevated leptin levels in obesity. This contradicts current opinions regarding the stimulatory effect of leptin on GH release, and may be attributed to impaired leptin signaling, and/or the development of central leptin resistance [44, 222]. Given that GH replacement therapy reduced adiposity and normalized hyperleptinemia in GHD adults [223, 224], presumably as a consequence of the lipolytic actions of GH, chronic hyperleptinemia in obesity may impair metabolic actions of leptin on GH and adiposity, thus leading to leptin resistance. In contrast, periods of calorie restriction in rodents result in a significant reduction in peak GH [48, 95, 225] and leptin secretion [95, 226]. Leptin infusion reverses the inhibitory effects exerted by fasting on *in vivo* pulsatile GH release [216, 227], suggesting that leptin acts as a metabolic signal to maintain GH secretion. Since the melanocortin system (via activation of POMC neurons) is a downstream target of leptin, the possible GH stimulatory actions of leptin may be indirectly mediated via interactions with the melanocortin system. Given that obesity is associated with altered endocrine profile, altered peripheral factors in modulating GH release may override the actions of leptin on GH secretion in obesity. Consequently, changes in leptin signaling may not solely account for altered GH release in obesity. These factors will be discussed in the following sub-chapters.

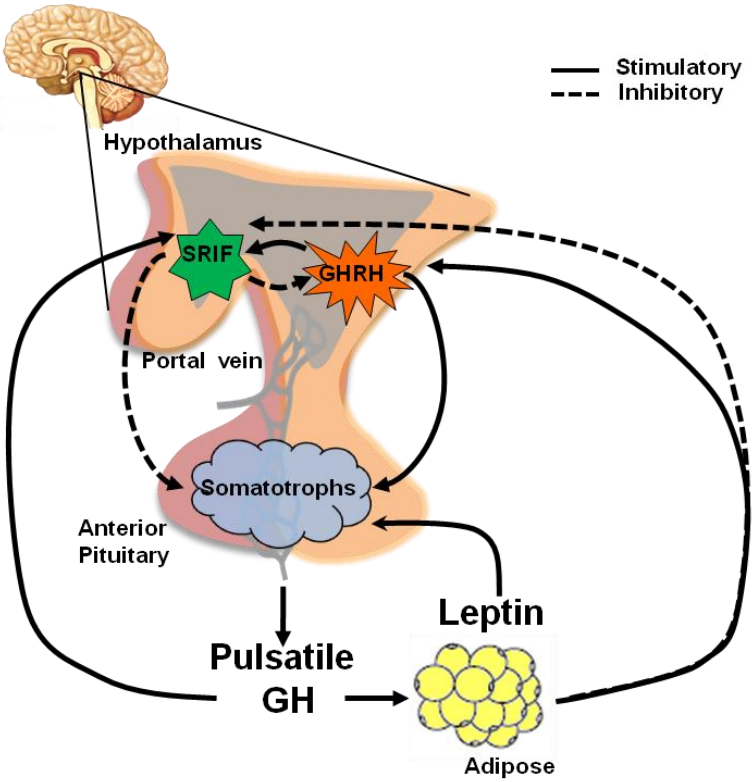

Figure 1-6: Schematic diagram illustrating the role of leptin in mediating GH secretion. Leptin may stimulate GH secretion by acting directly on pituitary somatotrophs and hypothalamic GHRH and SRIF neurons via leptin receptors expressed on these neurons. Abbreviations; growth hormone releasing hormone; GHRH, somatostatin releasing inhibitory factor; SRIF, growth hormone; GH.

1.8.1.2 Ghrelin

Ghrelin is a 28-amino acid peptide predominantly secreted by the oxyntic glands within the stomach, and regulate pituitary GH secretion, where ghrelin acts in a paracrine fashion to stimulate the release of GH from the anterior pituitary gland [228, 229]. Ghrelin circulates within the body in two forms: des-acyl and acyl ghrelin. Des-acyl ghrelin undergoes acylation mediated by ghrelin o-acyl transferase (GOAT) at the serine-3 residue of the peptide to produce active biological effects [230]. Circulating ghrelin levels are influenced by short-term alterations in energy status. Whereas the rise in ghrelin is observed prior to food intake [231, 232] and during periods of calorie restriction [233, 234], ghrelin levels are reduced after meals. Moreover, the reciprocity of enhanced ghrelin release before meals correlates with low leptin levels, whereas a reduction in ghrelin corresponds to elevated leptin levels after food consumption [235, 236]. Observations indicate a role for ghrelin (and leptin) in the physiological response to meal initiation and satiety. Additionally, ghrelin possess strong GH-releasing activity [237, 238]. Given the effects of ghrelin in driving

appetite and GH release, ghrelin is an important hormonal signal that regulates energy balance by modulating GH release relative to nutritional status.

Compelling evidence indicates that the effects of ghrelin-induced feeding are mediated through the ghrelin receptor, GHSR-la (the primary receptor mediating biological effects of ghrelin), expressed on NPY/AgRP expressing neurons [239]. Here, the binding of ghrelin to GHSR-1a activates orexigenic hypothalamic NPY/AgRP neurons (Figure 1-1) [239-242]. Moreover, the detection of GHSR-1a receptors on vagal afferent neurons [243] suggests that the orexigenic effects of ghrelin on hypothalamic NPY are transmitted via the vagus nerve (Figure 1-7). This is confirmed by previous studies wherein the loss of orexigenic effects of ghrelin was observed in rodents following ligation of the vagus nerve (vagotomy) [243, 244]. Intravenous administered ghrelin enhanced food take in humans [245]. In line with this, central and peripheral administration of GHRPs induced c-fos expression (neuronal marker) selectively in hypothalamic NPY/AgRP neurons in rodents [246-248]. Observations are also supported by electrophysiological recordings of *ex vivo* hypothalamic slices in which ghrelin directly increase the spontaneous activity of NPY/AgRP neurons, and indirectly hyperpolarized (to inhibit) POMC neurons by facilitating the pre-synaptic release of γ-aminobutyric acid (GABA) [249]. Considering that AgRP acts as a natural antagonist to MC4R, reduced food intake in response to ghrelin in obese MC4RKO mice suggest that the capacity of AgRP to stimulate appetite involves the melanocortin system via the antagonistic actions on MC4R [250]. Taken together, these findings indicate that the stimulatory effects of ghrelin on NPY/AgRP neurons are complementary to reduced leptin-sensitive POMC neuronal activity via inhibitory GABA inputs from NPY/AgRP neurons, thus decreasing α-MSH, consequently driving appetite (Figure 1-1). Ultimately, this suggests that ghrelin acts as a functional opposing antagonist to actions of leptin. Previous studies demonstrated that circulating ghrelin levels are decreased in obese humans [235, 251], while peripheral ghrelin injections fail to induce food intake in obese mice [252]. This suggests that access of ghrelin across the BBB may be altered during periods of energy excess. In support of this, obese aged mice showed a reduction in the transport intravenous ghrelin across the BBB [253]. Furthermore, Briggs and colleagues demonstrated that the suppression of the neuroendocrine ghrelin axis during obesity occurs in response to a reduction in circulating acylated and total ghrelin levels, decreased stomach *ghrelin and Goat*, and hypothalamic *Ghsr-1a*, *Npy* and *Agrp* mRNA expression. Moreover, central administration of ghrelin does not promote hypothalamic expression of NPY/AgRP to stimulate food intake in DIO mice [254]. Overall, this suggests that impaired ghrelin transport across the BBB coupled with the reduction in

NPY/AgRP responsiveness to ghrelin as a consequence of a disrupted NPY/AgRP neuronal circuit may exacerbate the development of ghrelin resistance during obesity.

In addition to the influence of ghrelin on appetite and energy balance, acylation of ghrelin exert its stimulatory effects on GH release at both the hypothalamus and pituitary gland [229]. The binding of acylated-ghrelin to GHSR-1a on pituitary somatotrophs activates the PLC/IP3 signaling cascade, resulting in the release of GH (Figure 1-4) [255]. GHSR-1a receptors are also expressed on GHRH neurons [256], suggesting that ghrelin directly increase hypothalamic GHRH-stimulated GH release. In line with this, peripheral infusion of ghrelin potently elicits GH secretion in both humans [237, 257, 258] and rodents [229, 259, 260]. Moreover, GHS treatment enhanced GHRH activity and induces c-fos expression in GHRH neurons [261, 262]. In addition, the actions of ghrelin in regulating GH secretion may occur via peripheral mechanisms. For instance, gastric derived ghrelin signals to the hypothalamus via the vagus nerve to augment the release of GHRH [243]. Given that ghrelin directly activates NPY neurons [239, 263], ghrelin-induced GH secretion may occur, at least in part through hypothalamic actions of NPY. This, however, warrants further investigation.

In normal weight humans, the co-administration of GHRH with ghrelin or GHS elicits a synergistic release of GH, greater than that evoked by GHRH alone [238]. By contrast, in obese individuals where ghrelin levels are low [251, 254, 264], combined therapy of these peptides induced a lower GH response compared to that observed in lean population [265-267]. This suggests a diminished capacity of the ghrelin axis in obese state. As discussed above, the reduction in circulating ghrelin is coupled with the decline in *Ghsr-1a* and *Ghrh-r* mRNA expression in the pituitary gland, as well as hypothalamic NPY/AgRP in DIO mouse [254]. Likewise, the stimulatory effects of ghrelin on GH release and food intake are suppressed in DIO mice [254] and obese MC4RKO mice [250], suggesting the development of central ghrelin resistance. Accordingly, systemic administration of GHS peptide into hypophysectomized adult rat induces *c-fos* mRNA expression in hypothalamic GHRH neurons [256]. Furthermore, the reduction in ARC *Ghsr* mRNA expression results in a decreased in the number of hypothalamic GHRH neurons in transgenic (Tg) rats [268], presumably due to the feedback mechanism of the regulation of GH secretion. In this context, the hyposecretion of ghrelin coupled with alterations in GHSR distribution may, in part, contribute to an overall ghrelin resistance as well as impaired GH secretion in obesity. It should be noted, however, that GH measurements in the DIO [254] and MC4RKO [250] mouse models were based on one off measurements, and thus do not provide a reliable measure of GH release. Furthermore, observations of reduced GH in response to ghrelin

were limited to aged female MC4RKO mice [250]. Given that GH release is pulsatile, that GH pulsatility may vary considerably between male and female mice (Chapter 1.4.1), and that GH release declines with age (Chapter 1.5.1), the role of ghrelin in mediating GH secretion relative to disrupted melanocortin system may be misinterpreted in aging female MC4RKO mice [250]. Details regarding the role of melanocortin system in the regulation of GH will be addressed in this thesis.

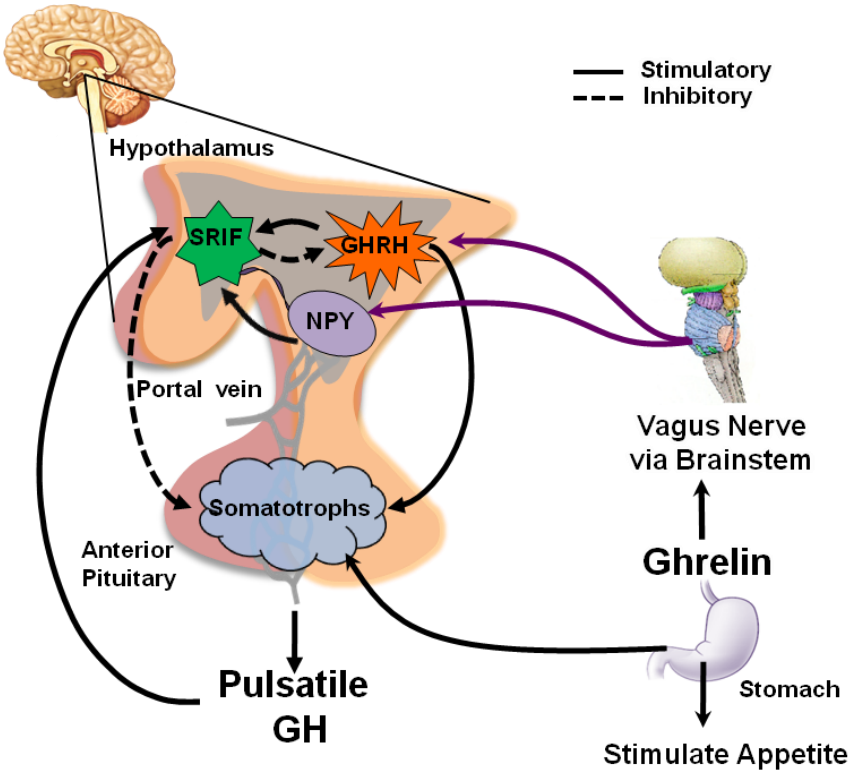

Figure 1-7: Schematic diagram illustrating the peripheral and central roles of ghrelin in mediating GH secretion at the level of the anterior pituitary gland and hypothalamus.

Within the anterior pituitary gland, stomach-derived ghrelin acts directly on somatotrophs via GHSR-1a to stimulate GH release. Within the hypothalamus (where GHSR-1a are localized on GHRH and NPY neurons), ghrelin stimulates GHRH-induced GH secretion, and directly activates NPY neurons via the vagus nerve to regulate feeding. Abbreviations; growth hormone releasing hormone; GHRH, somatostatin releasing inhibitory factor; SRIF, neuropeptide Y, NPY, growth hormone; GH.

1.8.1.3 Insulin

Insulin is a pivotal anabolic hormone that regulates blood glucose homeostasis and lipid mobilization. This is essential to promote the removal and storage of excess glucose in the liver or in muscle via glycogenesis (Figure 1-8) [269, 270]. In addition, insulin plays a critical role in lipid mobilization by promoting adipogenesis [270, 271], lipogenesis [272], and inhibiting lipolysis, thereby promoting normal lipid metabolism [270]. The release of

insulin from pancreatic β-cells occurs in response to a glucose load (following food intake). Consequently, pancreatic β-cells would compensate for insulin resistance by hypersecretion of insulin (hyperinsulinemia) and increased pancreatic β-cells mass to maintain plasma glucose within normal range [273]. Therefore, increased metabolic demand for insulin in the face of insulin resistance (peripherally or systemically in tissues) usually precedes the development of hyperglycemia. In this instance, excessive calorie intake could lead to pancreatic β-cell dysfunction in the early phase of Type 2 diabetes (T2D), culminating in insulin resistance [273].

Several studies demonstrate that insulin exerts an inhibitory effect on GH release. In obese adults, circulating insulin levels are inversely correlated with measures of GH when compared to healthy lean individuals [16-18], while circulating levels of insulin increase proportionate to the decrease in pulsatile GH secretion in mice during progressive weight gain [97, 98]. Similar observations of suppressed GH secretion was reported in obese hyperinsulinemic Zucker rats [100]. Importantly, hyperinsulinemia associated with excess calorie consumption in healthy individuals impairs GH secretion, and this precedes dietary induced weight gain [14]. Anecdotal evidence from Type 1 diabetes (T1D) patients further demonstrates the inverse relationship between circulating GH and insulin secretion, whereby GH release is markedly elevated relative to healthy controls [274, 275]. Moreover, intensive insulin treatment reduced excess GH secretion in these diabetic patients. Collectively, observations are indicative of the inhibitory role of insulin on GH secretion and suggest that elevated insulin in response to hyperphagia may potentially contribute to reduced GH output.

The mechanisms by which insulin regulates GH release are not completely clarified. Given that insulin receptors (InsR) are expressed in the hypothalamus [276], the inhibitory actions of insulin on GH release may be mediated through altered hypothalamic pathways. Insulin may stimulate the release of catecholamines (neurotransmitters) including norepinephrine, epinephrine and dopamine [277], which may in turn activate hypothalamic SRIF neurons to inhibit GHRH-induced GH secretion [278]. The direct involvement of the hypothalamus in the suppression of GH release during obesity however, has not been assessed, and thus the premise that altered GH release in obesity occurs via the hypothalamic pathway remains unsubstantiated. Regardless, hypothalamic gene expression of GHRH and SRIF is preserved in DIO rats, regardless of pituitary insensitivity to GHRH actions [172]. Furthermore, GHRH pretreatment with arginine fail to improve somatotroph sensitivity to recover GH release in obese patients [279]. Consequently, impaired GH secretion in obesity likely occur independent of alterations in hypothalamic

control of GH secretion. Accordingly, compelling evidence demonstrate that insulin may act directly on pituitary somatotrophs to inhibit GH release during the obese state. In isolated rat pituitary somatotrophs or pituitary tumors, GH secretion is reduced following insulin exposure [280-282]. Similarly, insulin acts in the pituitary gland via InsR to inhibit GH release from pituitary somatotrophs of DIO mice [170]. This suppression of GH in response to insulin is accompanied by a decreased *Gh*, *Ghrh* and *ghrelin* mRNA expression in DIO mice. These effects persisted despite the development of systemic insulin resistance, suggesting that somatotrophs remain insulin responsive in obesity. While not assessed alongside pulsatile GH release, subsequent observations demonstrate that the loss of somatotroph-specific InsR in mice resulted in an increase in circulating GH levels (based on single measures) [283]. However, the loss of somatotroph-specific InsR function could not completely prevent the reduction in GH release during dietary-induced weight gain [283]. Thus, suppressed GH secretion in obesity may not occur predominantly in response to systemic factors acting directly on somatotrophs. Observations do not however, clarify the inhibitory effect of insulin on somatotroph function during obesity. Thus, the loss of somatotroph-specific InsR on the recovery of pulsatile GH release during obesity warrants further investigation.

Impaired insulin sensitivity commonly associated with excessive calorie intake or obesity [14, 284] is attributed to several factors including elevated NEFAs and pro-inflammatory cytokines released from adipose mass [285, 286]. In addition, chronic exposure to GH impairs insulin-stimulated glucose uptake and suppresses hepatic glucose production, resulting in insulin resistance in both humans [287-289] and rodents [290, 291]. A secondary effect of a concomitant rise in NEFAs (usually suppressed by antilipolytic actions of insulin) as a consequence of GH-driven lipolysis is thought to further compromised insulin sensitivity. In this context, the balance between GH and insulin regulation is thought to be essential in sustaining lipid metabolism (Figure 1-7). Recent clinical findings confirmed that the suppression of GH secretion following excess calorie intake precedes body weight gain [14], and is thought to ameliorate insulin resistance, thereby improving meal tolerance [71]. Thus, the maintenance of insulin sensitivity during periods of GH deficiency following excess food consumption is believed to be a physiological adaptation that promotes insulin-driven lipogenesis to prevent dyslipidemia [71]. The relationship between GH and insulin in regulating GH release and fatty acid homeostasis in response to excess calorie intake will be addressed in this thesis.

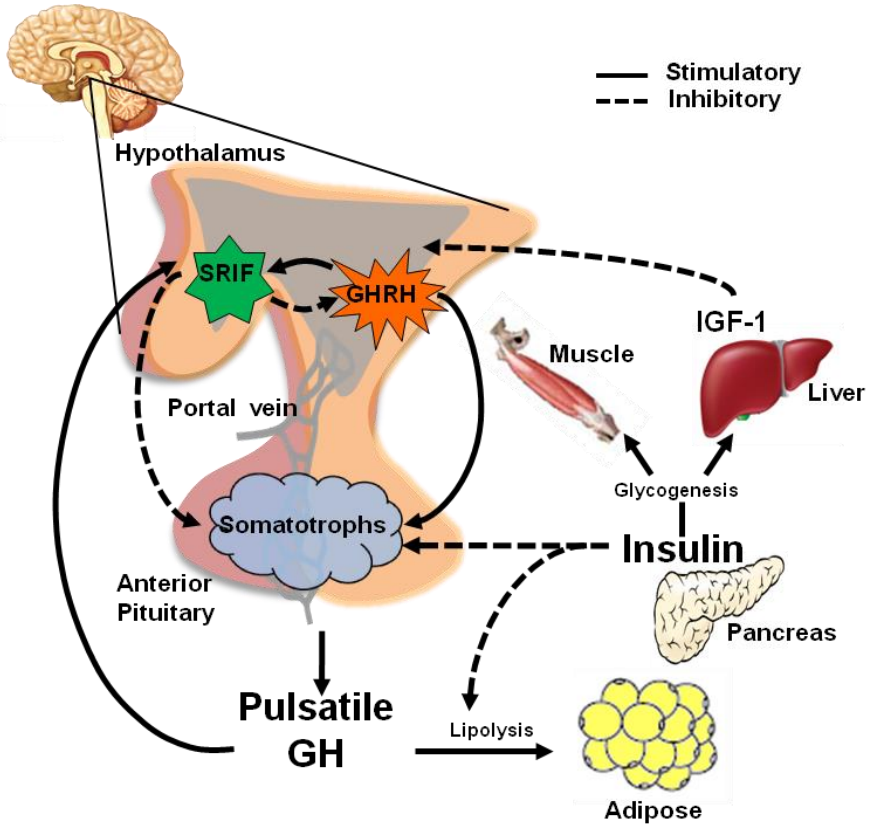

Figure 1-8: Schematic diagram illustrating the metabolic roles of insulin in promoting lipid mobilization while suppressing GH.

Insulin secreted by the pancreas inhibits the release of GH directly at the level of pituitary gland. The balance between GH and insulin overall maintains glucose homeostasis by promoting the uptake of glucose into liver and muscle via glycogenesis. Abbreviations; growth hormone releasing hormone; GHRH, somatostatin releasing inhibitory factor; SRIF, growth hormone; GH.

1.8.2 Metabolites as regulators of GH

1.8.2.1 Non-esterified free fatty acids (NEFAs)

The most prominent metabolic effect of GH is the stimulation of lipolysis and release of NEFAs [125]. NEFAs in turn exert feedback on GH release by acting at the level of pituitary gland and hypothalamus to inhibit GH secretion (Figure 1-9). In rodents, lipid-heparin infusion confirms that NEFAs suppress GH release by acting directly on pituitary somatotrophs [292-294]. This does not, however, reflect the impact of endogenous NEFAs on GH release in obesity. Whereas the rise in NEFAs during obesity coincides with the suppression of GH, pharmacological blockage of lipolysis (by acipimox) to reduce NEFAs concentrations restores spontaneous and stimulated GH release in elderly and obese individuals [295, 296]. This occurs alongside the reversal of hyperinsulinemia and improved insulin sensitivity [297, 298]. Given the proposed interactions of the inhibitory actions of insulin on GH (Chapter 1.8.1.3), the decline in circulating insulin associated with

acipimox treatment may alter GH secretion. Thus, the inhibitory effects of NEFAs on GH secretion in obesity may be misinterpreted. Moreover, recent observations challenged this notion. Observations demonstrated that a chronic reduction in NEFAs levels preceded impaired insulin sensitivity in GH deficient humans following excess calorie consumption [299]. In addition, a recent meta-analysis reported a relatively stable circulating NEFAs regardless of the severity of obesity [285]. Thus, NEFA levels may not directly contribute to impaired GH secretion in obesity. Given that chronic elevated NEFA concentrations are closely associated with insulin resistance in obesity [285, 300], the associated suppression of GH in obesity may reflect counterregulatory response to normalize NEFAs flux to ameliorate insulin resistance. In this context, GH deficiency during obesity may be a physiological adaptation that sustains insulin sensitivity. The relationship between metabolic actions of GH in modulating adiposity and NEFAs will be addressed in Chapter 5.

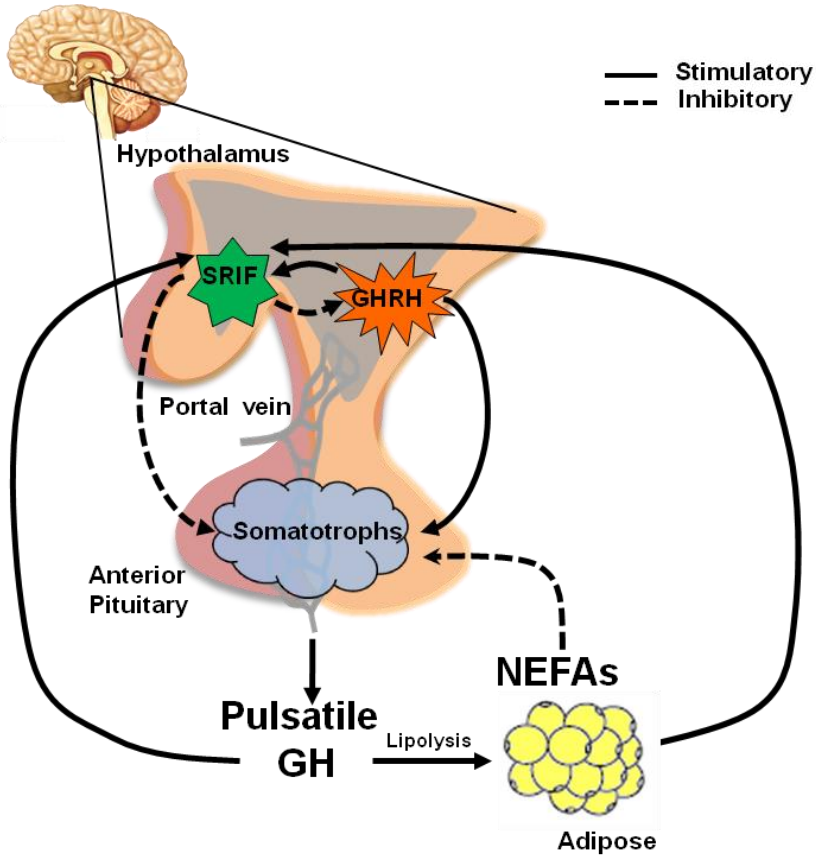

Figure 1-9: Schematic diagram illustrating the proposed role of NEFAs in regulating GH release.

Under physiological circumstances, the lipolytic action of GH on adipose tissue leads to the release of NEFAs flux which may serve as a negative regulator of GH. To suppress GH secretion, NEFAs may act directly on somatotrophs to inhibit GH release. Abbreviations: non-esterified fatty acids; NEFAs, growth hormone; GH.

1.8.2.2 Glucose

Glucose homeostasis is maintained through complex counterregulatory neuro-hormonal systems. The disposal of postprandial or fasting glucose appears to be mediated through insulin-dependent and insulin-independent pathways [301]. Following food intake or calorie excess, postprandial insulin would preferentially metabolize glucose as glycogen in the liver or muscle or as fat in adipose tissue, thus lowering circulating levels of glucose. In contrast, the fasting-induced reduction in insulin augments the utilization of lipids (to yield NEFAs) and decrease glucose uptake. Given that the metabolic actions of GH are antagonistic to the actions of insulin, GH decreases glucose oxidation and promotes lipid mobilization in defense against hypoglycaemia [124].

Perturbations in blood glucose relative to energy balance may impact GH secretion. In view of this, insulin-induced hypoglycaemia potently stimulates GH release in humans [164, 302]. Similar observations are reported in sheep following intravenous insulin infusion to mimic hypoglycaemia [303, 304], and this is thought to be mediated via GHRH-induced GH release [164, 303]. Although the exact glucoregulatory mechanisms of GH regulation are not completely elucidated, findings suggest that neuroregulatory effects of insulin-induced hypoglycemia GH release in sheep are, somewhat, similar to that observed in humans. In contrast, insulin-induced hypoglycaemia or calorie restriction (fasting) suppressed pulsatile GH secretion, and perturbates GH response to GHRH stimuli in adult rats [85, 225]. This was accompanied by a concomitant increase in *Srif* mRNA and content while hypothalamic *Ghrh* mRNA remained unchanged [48, 225, 305, 306]. Subsequent glucose clamp studies confirmed that hyperglycemia does not impact spontaneous and stimulated GH secretion (by GHRH, arginine (to inhibit SRIF), and hexarelin (GHS)) in humans [267, 307, 308]. Thus, hyperglycemia appears to have a modest impact on GH secretion [305]. Rather, observations suggest that blunted GH release in response to hypoglycemia may be attributed to enhanced inhibitory actions of SRIF. This is supported by observations demonstrating an increase in hypophyseal-portal SRIF concentrations in sheep following systemic insulin injections [304]. Thus, while not directly addressed, the effects of indirect stimulation of hypothalamic SRIF may account for the inverse relationship between insulin and the suppression of GH release in response to altered glucose balance. Unlike observations in rats, limited data demonstrate that insulin-induced hypoglycaemia does not alter pulsatile GH secretion in mice [309]. This occurred alongside sustained hypothalamic *Ghrh* and *Srif* mRNA expression [309], suggesting that hypoglycaemia may not directly activate GHRH or SRIF neurons to inhibit GH secretion in mice. Rather, the release of counterregulatory glucose-sensitive hormones that defend

against hypoglycaemia may be involved in the indirect modulation of GHRH and SRIF neurons. To this extent, it appears that the glucoregulatory effects of GH regulation may vary considerably between mammalian systems. Thus, neuroendocrine mechanisms involving insulin-glucose studies requires concomitant measurements of hypophyseal-portal GHRH and SRIF outputs awaits further investigation.

1.9 Aims and Hypothesis

GH is a key regulator of postnatal linear growth during childhood. Given that GH deficiency is associated with short stature, it is thought that an upregulation in endogenous GH secretion contributes to rapid linear growth in humans associated with dysfunction of MC4Rs. Accordingly, assessment of GH secretion in obese hyperphagic MC4R deficient adults demonstrated a partial increase in pulsatile GH secretion relative to obese individuals of similar BMI, regardless of increased adiposity [68]. While not directly assessed, this suggests that rapid linear growth observed in these adults may occur as a consequence of pubertal GH excess. In addition, metabolic alterations such as elevated insulin secretion in response to excess calorie consumption (including hyperphagia and obesity) associated with MC4R deficiency may alter GH release. Given the proposed interactions between GH and insulin, the premise that increased GH release contributes to enhanced linear growth in hyperphagic MC4R deficient children challenge current opinions regarding the impact of obesity on GH release, and the proposed metabolic actions of GH in mediating NEFA flux. Thus, it is uncertain whether GH hypersecretion accounts for rapid linear growth in MC4R deficient adults. Moreover, observations of pulsatile GH release during pubertal growth spurt in MC4R deficient children have not been assessed to date. Consequently, the potential role of GH in promoting linear growth in pubertal MC4R deficient children remains to be determined. Of interest, insulin may promote growth directly by acting through IGF-1 receptors. *It is therefore hypothesized that rapid pubertal growth associated with MC4R deficiency occur secondary to metabolic changes associated with hyperphagia. This may occur at the expense of GH and insulin release in response to excess energy consumption.* Thus, the main aim of this project is to investigate role of melanocortin system in modulating pulsatile GH secretion, relative to linear growth during hyperphagia associated weight gain. To address this, the aims of this project are:

1. To characterize the change in pulsatile GH secretion that occurs relative to rapid linear growth from puberty throughout adulthood in WT mice (Addressed in Chapter 3, published data);

2. To determine whether changes in inhibitory hypothalamic *Srif* mRNA distribution contributes to altered GH release that occurs between puberty and adulthood in mice (Addressed in Chapter 4, published data);
3. To determine whether increased pulsatile GH secretion contribute to rapid pubertal growth associated with MC4R dysfunction in hyperphagic MC4RKO mice (Addressed in Chapter 5);
4. To determine whether MC4Rs are colocalized with the primary regulators of GH (somatotrophs and GHRH) to modulate GH release (Addressed in Chapter 5);
5. To investigate whether the prevention of hyperphagia will normalize rapid linear growth and GH secretion in MC4RKO mice (Addressed in Chapter 6).

CHAPTER TWO: GENERAL METHODOLOGY

2. General Methodology

This section includes general experimental protocols conserved throughout this thesis. Detailed information on specific experimental designs will be addressed in relevant chapters.

2.1 Animal Care

WT C57/BL6 male mice were obtained from The University of Queensland Biological Resources, Australian Institute for Bioengineering and Nanotechnology (UQBR, AIBN). Male transgenic MC4RKO mice on a C57/BL6 background with a targeted deletion of functional neuronal MC4R were originally provided by Professor Michael Cowley (Monash University, Melbourne, Australia). Male homozygous MC4RKO mice and age-matched WT LM were obtained from heterozygote parents breeding pairs. Transgenic adult C57/BL6 mice expressing GFP in GH-secreting cells (GH-GFP mice) and hypothalamic GHRH neurons (GHRH-GFP) were provided by Professor Iain Robinson (MRC National Institute for Medical Research, London) and these are maintained on a homozygote breeding colony for in vitro experiments. All mice were group housed (n=2-3) in a temperature-controlled room (22 ± 2°C), maintained on a 12-h light/dark cycle (lights on at 0630 h and off at 1830 h). Animals were handled daily and had free access to food and water unless otherwise specified. All experiments and procedures were approved by the University of Queensland Animal Ethics Committee.

2.2 Genotyping

Homozygous MC4RKO mice and WT LM used in this project were obtained using a heterozygous breeding strategy. To verify the genotype of the MC4RKO animals, the tip of the toe (1 mm) was collected and genomic DNA was isolated using the REDExtract-N-Amp TISSUE PCR kit (XNATR; Sigma, St. Louis, MO, USA). The genotype of MC4RKO mice was identified using the following published MC4R primer sequences [250]: (forward) 5'- GGA AGA TGA ACT CCA CCC ACC -3', (reversed) 5'- GAC GAT GGT TTC CGA CCC ATT -3' and PGKR3: 5'- TTC CCA GCC TCT GAG CCC AGA -3'. Genotypes were confirmed by the presence of 313bp for the WT LM allele and 405bp for the MC4RKO allele.

2.3 Blood sampling

2.3.1 Serial blood sampling or assessment of pulsatile GH release

To assess pulsatile GH secretion, blood samples were collected and processed as previously described. Care was taken to minimize potential stress-induced disruption of pulsatile GH secretion [95] (Figure 2-1). 36 sequential tail tip blood samples (4 µl) were collected from each mouse at 10-minute intervals for a period of 6 hours. Mice were handled in a circular cardboard roll, and held by the tail. The distal 0.5 mm of the tail was excised using a sterile surgical blade (ProsciTech, Australia). For each sample, 4 µl of whole blood was collected using a 10-µl Gilson pipette, and placed directly into 116 µl of 0.05% PBS-Tween (PBST, w/v). Given an approximate total blood volume of 2.1 ml per mouse (based on estimated 7% blood volume relative to body mass [310]), blood loss was restricted to less than 5.5% of total blood volume over the 6 hours sampling period. This is well below blood loss deemed sufficient to have a physiological impact [311]. Given the average size and weight of a 4-week-old mouse, a similar approach could not be considered. Therefore, to ensure the welfare of 4-week-old mice, blood collection was limited to 2 µl per sample. Following blood collection, gentle pressure was applied to the tail-tip to stem blood flow and mice were returned to the cage. For repeated collection, the tail-tip was briefly immersed in physiological saline (0.9% sodium chloride, Baxter, Australia), and gently wiped dry with a paper towel. If necessary, the dried blood was carefully removed using a surgical blade and gentle pressure was applied along the base of the tip of the tail to encourage blood flow. Samples were mixed by vortex, immediately placed on dry-ice and transferred to -80 °C for storage prior to future batch analysis.

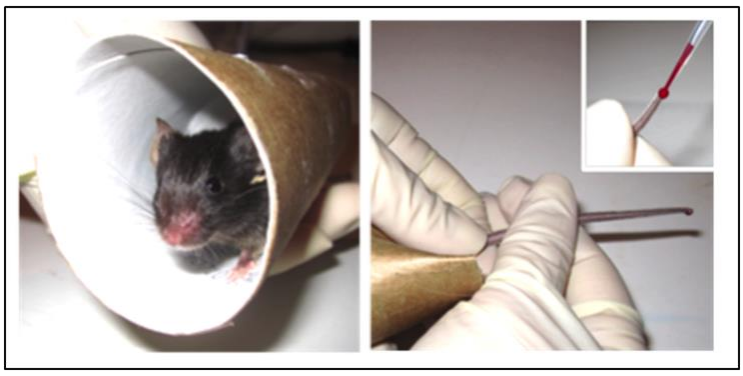

Figure 2-1: Illustration demonstrating mouse tunnel handling method and tail-tip blood sample collection.

Mice were handled within a cardboard roll and held by the base of the tail. A distal 0.5 mm section of the tail end was excised and whole blood samples were collected from the tail tip (inset).

2.3.2 One-off blood sampling from tail tip sampling for assessment of metabolites

To assess circulating metabolites (glucose, insulin, NEFAs) from freely moving mice (un-anesthetized), blood samples were collected from the tail tip as detailed in section 2.3.1. A 20 µl tail-tip blood sample was collected using a heparinized pipette tip (100 IU/ml), and plasma was separated via centrifugation (6000 rpm for 3 mins at room temperature). Aliquoted plasma was placed on dry ice, and stored at −80 °C for future batch analysis.

2.3.3 Terminal blood sampling for assessment of metabolites

Terminal blood samples were collected at the time of sacrifice (between 0700h and 0900 h) via cardiac puncture following procedures as described previously [311]. Mice were anesthetized with a single intraperitoneal (ip) injection of sodium pentobarbitone (32.5 mg/ml, 1PO643-1; Virbac Animal Health, Milperra, NSW, AUS), and tested for corneal and toe-pinch withdrawal reflex prior to blood collection. Approximate 1 ml of whole blood was collected into ethylenediamine tetra-acetate (EDTA) tubes (1.6 mg/ml blood) (D51588; Sarstedt Australia Pty Ltd, SA, AUS). Plasma was separated via centrifugation (6000 rpm for 3 mins at room temperature). Aliquoted plasma was placed on dry ice, and stored at −80 °C for future analysis.

2.4 Assessment of metabolic status by Glucose and Insulin Tolerance Test (GTT/ITT)

For *in vivo* assessment of glucose tolerance, mice were fasted overnight for 12 hours starting at 1800 h, with free access to water. At 0600 h, mice were injected with glucose (ip, 1.0 g per kg body weight). During GTT, an additional single 20 µl tail-tip blood sample was collected using a heparinized pipette tip (100 IU/ml), and plasma was aliquoted for the assessment of insulin at similar time points. For ITT, starting at 0800 h, mice were fasted 6 hours, with free access to water. At 1400 h, mice were injected with insulin (ip, 0.2 units per kg body weight; I9278; Sigma, St. Louis, MO, USA). Immediately prior to the ITT, a single 20 µl tail-tip blood sample was collected using a heparinized pipette tip (100 IU/ml), and plasma was aliquoted for the assessment of fasting insulin. A tail-tip blood sample was collected immediately before the injection and at 15, 30, 45, 60, 90, and 120 mins following the injection. Blood glucose concentration in tail-tip blood samples was determined using the Accu-Chek Performa blood glucose meter (Roche Diagnostics, Indianapolis, IN, USA). A change in blood glucose concentration in response to the glucose injection was compared relative to starting blood glucose levels for each mouse.

2.5 Analysis of Hormones and Metabolites

2.5.1 Measurement of GH via in-house GH ELISA

Analysis of GH was determined using an in-house sandwich ELISA. This assay was optimized and validated for measuring GH from 2 µl whole blood samples [95]. A 9-point standard curve was generated using a mouse GH (mGH) reference preparation (AFP-10783B, National Institute of Diabetes & Digestive & Kidney Diseases -NIDDK-NHP) by two-fold serial dilution (8 ng/ml to 0.03 ng/ml), diluted in PBST supplemented with bovine serum albumin (0.2% BSA-PBST). Briefly, a 96-well plate (Corning Inc., Corning, NY) was coated with 50 µl of capture antibody (NIDDK-monkey anti-rat GH (rGH)-IC-1, AFP411S, NIDDK-NHPP) at a dilution of 1:40 000 and incubated at 4 °C overnight. To reduce non-specific binding, each well was incubated with blocking buffer (5% skim milk powder in 0.05% PBST) for 2 hours at room temperature. Following blocking, standards and samples were loaded into a pre-coated plate in duplicates and incubated for 2 hours at room temperature. Bound standards and samples were incubated with 50 µl of detection antibody (rabbit antiserum to rat GH, AFP5672099, NIDDK-NHPP) at a final dilution of 1:40 000. Bound complex were then incubated with 50 µl of horseradish peroxidase (HRP)-conjugated antibody (anti-rabbit, IgG, GE Healthcare, UK) at a dilution of 1:2000. Addition of 100 µl of O-phenylenediamine (OPD, 00.2003, Invitrogen, CA) substrate to each well resulted in an enzymatic colorimetric reaction. This reaction was stopped by the addition of 50 µl of 3 M hydrochloric acid (HCL, Sigma). The absorbance was read at a wavelength of 490 nm (reference wavelength set at 650 nm) on a TECAN Sunrise 96-well monochromatic microplate reader (TECAN, Switzerland). The concentration of GH in each well was calculated against a non-linear regression of the standard curve.

2.5.2 Measurement of hepatic triglyceride content

To determine hepatic triglyceride content, liver samples were digested by saponification in ethanolic KOH and neutralized with $MgCl_2$ as previously described [312]. Briefly, 100 mg of liver tissue were incubated with 350 µl ethanolic KOH overnight at 55 °C. The volume of sample mixture was adjusted to 1000 µl with H_2O:EtOH (1:1) and centrifuged (6000 rpm for 5 mins at room temperature). The resulting supernatant was transferred to a new tube and the volume was adjusted with H_2O: EtOH (1:1) to a final volume of 1200 µl. 200 µl of the new diluted mixture was incubated with 215 µl 1 M $MgCl_2$ for 10 mins at room temperature. Following centrifugation, the supernatant was transferred to a new tube for assessment of glycerol content. Glycerol content was determined using a glycerol standard (G7793, Sigma, St. Louis, MO) and free glycerol reagent (F6428, Sigma). Total

liver triglyceride content was derived from the following equation: Liver triglyceride content (mg of triglyceride/gram of liver) = *Triolein equivalent glycerol concentration (mg/dL)*(415/200)*0.012(dL)/weight of liver tissue (g)*standard dilution factor*.

2.5.3 Measurement of hepatic and muscle glycogen content

To determine liver and muscle glycogen content, liver and muscle samples were digested in 30% KOH and precipitated in anhydrous ethanol as previously described [313]. Glycogen content was determined using a glucose standard (concentration at 1 mg/ml) and 0.2% anthrone reagent (0.2 g in 100 ml 98% H_2SO_4). Briefly, 100 mg of liver/muscle tissue were incubated in 500 µl of 30% KOH for 30 mins at 100 °C. The tissue mixture was further diluted with 30% EtOH (final volume = 500 µl) (liver samples required higher dilutions; 100x dilution for liver and 10x for muscle. Following dilution, 1 ml of 100% EtOH was added to the new mixture and centrifuged (6000 rpm for 15 mins at room temperature). Following centrifugation, the resulting pellet was resuspended in 500 µl deionized water, and incubated in 1 ml 0.2% anthrone reagent for 30 mins at room temperature. Glycogen content was derived from the following equation: Glycogen (mg/g tissue) = *glucose equivalent (µg/ml)*1.11*0.001*dilution factor*0.5 ml/weight of tissue (g)*.

2.5.4 Measurement of hormones and metabolites using commercial assays

Assessments of endocrine profiles were performed using commercial ELISA assay kits. Assay procedures strictly adhered to methodology supplied with these kits. Commercial kits used are listed in Table 2-1.

Table 2-1: Commercial assay kits used to determine circulating hormones and metabolites.

Hormones/Metabolites	Assay Kits	Company	Catalogue ID
Leptin	Mouse Leptin	Millipore	EZML-82K
Insulin	Rat/Mouse Insulin	Millipore	EZRMI-13K
IGF-1	Rat/Mouse IGF-1	R&D Systems	SMG-100
IGFBPs	Mouse Metabolic Magnetic Bead Panel	Millipore	MIGFBPMAG-43K
Metabolism Multiplex Assay	Mouse Metabolic Hormone Magnetic Bead Panel	Millipore	MMHMAG-44K
NEFAs	NEFAs C Assay	Wako	279-75401
Glucose	Glucose Assay	Cayman	10009582

2.6 Analysis of hypothalamic GHRH and SRIF expression by quantitative polymerase chain reaction (qPCR)

To quantify the amount of hypothalamic *Ghrh* or *Srif mRNA* expression relative to GH output throughout pubertal maturation and in adulthood, hypothalamic micropunch biopsies containing pooled regions of PVN, PeVN and the ARC were isolated from mice. Mice were anesthetized with a single ip injection of sodium pentobarbitone (32.5 mg/ml, 1PO643-1). The brain from each mouse was immediately removed, snap-frozen on dry ice, and stored at -80 °C. Micropunch biopsies were collected from 300 µm thick coronal brain sections using fine-gauge needles (23G x 11/4") (Terumo® Syringe) following established methodology [48]. Tissue biopsies representative of the PVN, PeVN and ARC located between bregma -0.34 and -2.18 (Figure 2-2) were suspended in 1 ml TRIzol (15596-026; Invitrogen, Carlsbad, CA). Tissue was stored at -80 °C prior to quantitative polymerase chain reaction (qPCR) analysis. Total RNA was isolated using a PureLink RNA Mini kit (12183-018A; Invitrogen). Total RNA in each sample was quantified using a spectrophotometer (Nanodrop 2000, Thermo Scientific) and treated with DNase (AM2235; Invitrogen) to remove possible genomic DNA contamination. 100 ng of RNA were transcribed into cDNA using a SuperScript® VILO™ Master Mix (11755050; Invitrogen). qPCR was performed using Taq polymerase (Taqman) probes and the following primers: *Ghrh*, assay ID: Mm00439100_m1; *Srif*, assay ID: Mm00436671_ml; Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), assay ID: Mm9999915_gl. All primers were purchased from Applied Biosystems (Carlsbad, CA). The experiment was set up according to the protocol and reagents provided. Amplification in a 10 µl reaction volume was assessed using the QuantStudio 7 system (Applied Biosystems, Australia). Data were displayed as an amplification plot and analysis was done by ViiA7 Software v1.2 (Applied Biosystems). Changes in cycle threshold of the gene of interest were corrected to the housekeeping gene (*Gapdh*), and expressed relative to their age-matched controls.

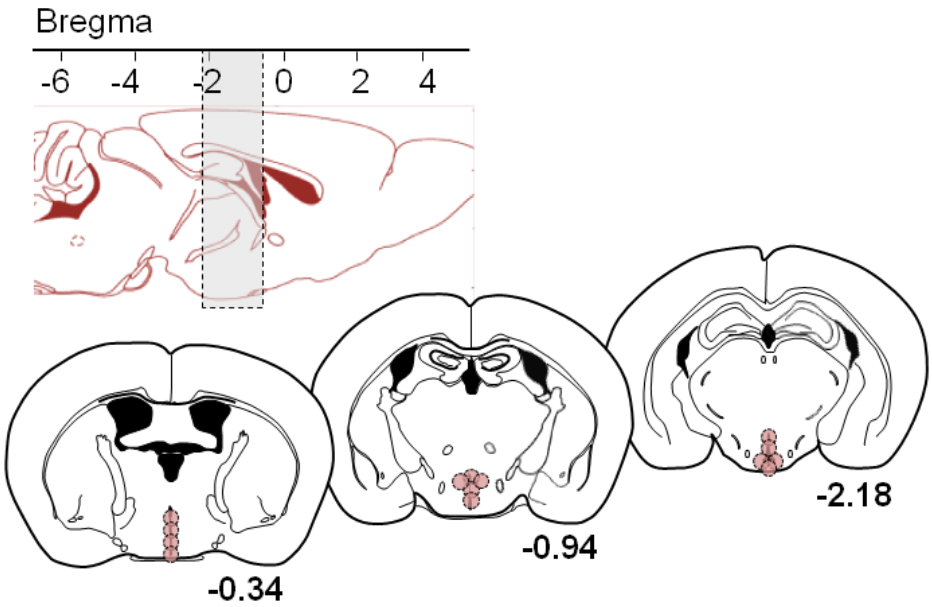

Figure 2-2: Regions of brain (red) illustrating the collection of micropunch biopsies (between bregma -0.34 to -2.18) from mice. Tissue collected was representative of the arcuate nucleus, periventricular nucleus and paraventricular nucleus complex.

2.7 Data and statistical analysis

All data are presented as mean ± SEM unless otherwise stated. Age-related differences between WT mice between 4, 8 and 16 weeks of age were analyzed by one-way ANOVA with Turkey's multiple comparison tests. The rate of progressive weight gain and linear growth between genotypes were assessed by linear regression. Comparisons between genotypes (MC4RKO mice and WT LM) were analyzed by Student's unpaired t-test and restricted to age. All measures (excluding deconvolution analysis) were performed using GraphPad Prism 6.0c (GraphPad, Inc., San Diego, CA, USA). The threshold level for statistical significance was set at $P \leq 0.05$. The quantitative features underlying GH secretion and clearance associated with the observed concentration profiles were determined by deconvolution analysis following parameters established previously [135, 314].

CHAPTER THREE: EXPERIMENTS TO ADDRESS AIM 1

3. ALTERED PUBERTAL GROWTH RATE OCCURS ALONGSIDE THE ESTABLISHMENT OF ALTERED GH SECRETION PROFILES IN THE MOUSE.

- *This chapter is reproduced from published observations (Tan et al. 2013, Journal of Neuroendocrinology)*

3.1 Introduction

The administration of GH to normal or hypophysectomized young rats promote or restore growth, respectively [315]. GH is characterized as a key anabolic hormone that promotes longitudinal bone and skeletal muscle growth, and thus is a key factor in regulating somatic maturation in early adulthood [11]. The secretion of GH is characterized by low basal GH levels dispersed by several dominant pulses that occur at periodic intervals [96]. In humans, the pubertal increase in spontaneous pulsatile GH secretion is attributed to an enhancement of GH pulse amplitude [316-319], a change that coincides with peak periods of linear growth [1]. While not directly addressed, the same phenomenon is thought to occur in mice throughout puberty. However, due to challenges encountered with frequent blood sampling in mice, measures of pulsatile GH release in pubertal mice do not exist. Using a novel approach to assess pulsatile GH secretion from serial blood sampling in mice [95], this study aimed to determine changes in pulsatile GH secretion relative to linear growth in mice.

An increase in GH secretion is observed following the loss of the MC4R in MC4R deficient individuals. It is therefore thought that an elevation in GH release enhance rapid linear growth in MC4R deficient children. Measures of pulsatile GH secretion were restricted to MC4R deficient adults when linear growth slowed, and thus observed GH measures may not accurately define GH secretion during periods of dynamic linear growth. This thesis seeks to characterize the role of GH in promoting rapid pubertal growth in MC4RKO mice. To do this, it is critical to first establish the release of GH during pubertal growth in WT mice. *The characterization of pulsatile GH secretion in early puberty throughout adulthood in WT mice relative to linear growth will define fundamental paradigms critical for the assessment of pubertal changes in GH release relative to rapid linear growth in MC4RKO mice (this is further discussed in Chapter 5).*

3.2 Materials and Methods

3.2.1 Characterization of rate of linear growth in WT mice from puberty into early adulthood

To monitor the rate of linear growth, weekly body length (nasal-anal distance) of WT mice was monitored from puberty (4 weeks of age) to adulthood (20 weeks of age).

3.2.2 Assessment of pulsatile GH secretion from puberty into early adulthood

Starting at 3 weeks of age, weaned mice were relocated to the procedure room and allowed 1 week to acclimate to new housing conditions before the commencement of all experiments. Animals remained in the same room for the duration of the experiment. Changes in pulsatile GH secretion were assessed in mice at 4, 8 or 16 weeks of age. Starting at 0700 h, 36 sequential tail-tip blood samples were collected from mice at 10-minute intervals (as detailed in section 2.3.1). Given the average size and weight of a 4-week-old mouse, a similar approach could not be considered. Therefore, to minimize potential stress from blood sampling in younger mice, blood collection was limited to 2 µl per sample in 4-week-old mice. Following collection of blood samples, mice were returned to their home cage and given two days to recover, prior to sacrifice and collection of tissue for additional measures.

3.3 Results

3.3.1 Rate of linear growth peaks between 5 and 10 weeks of age

The rate of linear growth in WT mice was monitored by assessing weekly body length. Measures were collected between 4 and 20 weeks of age. The growth curve of total body length throughout this period is illustrated in Figure 3-1. Body length increased rapidly between (A) 5 and 8 weeks of age and slowed between (B) 9 and 11 weeks of age at which animals reached near adult body length. This was followed by a further slowing in linear growth rate between (C) 12 and 20 weeks of age. By 20 weeks of age the average body length was 8.2±0.14 cm. The slope corresponding to rate of growth between (A) 5 to 8 weeks of age, (B) 8 to 12 weeks of age, and (C) 12 to 20 weeks of age are significantly different ($P < 0.001$).

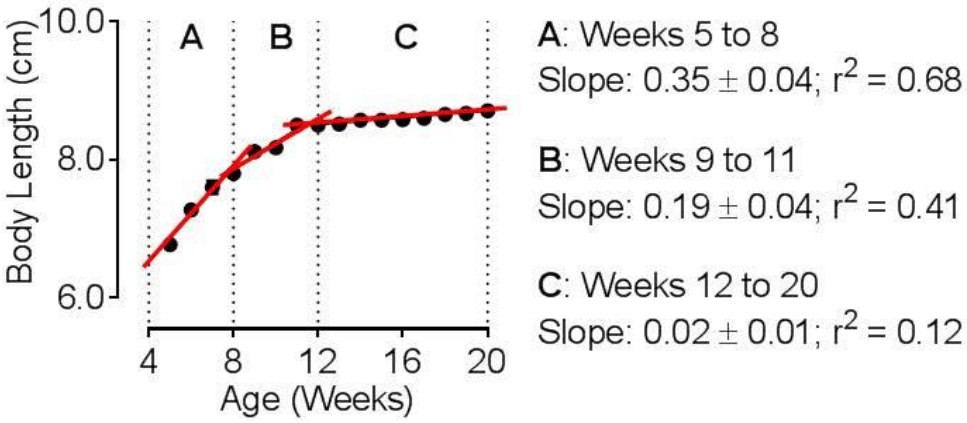

Figure 3-1: Growth curve illustrating weekly rate of linear growth in WT mice from 4 to 20 weeks of age.

The slopes (corresponding to the rate of growth) was determined by linear regression, corresponding to data points between (A) 5 to 8 weeks of age, (B) 9 to 11 weeks of age, and (C) 12 to 20 weeks of age. Slopes (A to C) are significantly different ($P < 0.001$). Data are presented as mean ± SEM. A P value < 0.05 was accepted as significant; N=11 per group. Abbreviation: wild-type; WT.

3.3.2 Peak pulsatile GH secretion declines alongside the slowing in linear growth rate

Pulsatile GH secretion was assessed at three ages corresponding to the 3 identified phases of linear growth; 4 weeks (corresponding to the observed onset of rapid linear growth), 8 weeks (during the period characterized by the slowing of linear growth) and 16 weeks of age (during the period characterized by a further slowing of linear growth as animals reach adult body length). Representative secretory profiles illustrating pulsatile GH secretion in mice at 4, 8 and 16 weeks of age are presented in Figure 3-2.

As previously observed [48, 95, 97], peak periods of GH secretion were dispersed by periods of low basal levels of secretion. At 4 weeks of age, the pattern of GH secretion was characterized by frequent bursts in GH release often approaching 200 ng/ml. Relative to 8 and 16 weeks of age, peak GH secretion at 4 weeks of age was greater at 4 weeks of age. Consistent with humans observations [1, 11, 128, 317], higher peak GH levels at 4 weeks of age was characterized by a high pulsatile secretion rate and a high mass of GH secreted per burst. Elevated pulsatile GH secretion at 4 weeks of age preceded the peak period of linear growth. Relative to 4 weeks of age, deconvolution analysis of pulsatile measures of GH release confirmed a significant decline in GH secretion at 8 and 16 weeks of age. This was characterized by a reduction in total GH secretion rate (Figure 3-2C; 472±75.6 ng/ml per 6 h & 306 ± 68.3 ng/ml per 6 h versus 1062 ± 166 ng/ml per 6 h),

pulsatile GH secretion rate (Figure 3-2D; 396±64.1 ng/ml per 6 h & 266±62.0 ng/ml versus 1015± 62 ng/ml per 6 h), the mass of GH secreted per pulse (MPP) (Figure 3-2E; 148±44.7 ng/ml per 6 h & 68.0±12.8 ng/ml per 6 h versus 186±22.3 ng/ml per 6 h), and the number of GH secretory bursts (Figure 3-2F; 3.30±0.42 pulses per 6 h & 3.90±0.41 pulses per 6 h versus 5.50±0.50 pulses per 6 h). The mode of GH secretion was not altered across all ages. Overall, data demonstrate that rapid pubertal linear growth in mice occurred alongside the establishment of altered GH secretion profiles. Second to this, a decline in the mass and frequency of GH secretion correlate with the progressive slowing of linear growth in mice from puberty into early adulthood. Comparisons of GH secretory parameters after deconvolution analysis are summarized in Table 3-1.

Table 3-1: Deconvolution analysis of output values pulsatile GH release in whole blood tail-tip samples collected from mice at 4, 8 and 16 weeks of age. Samples were collected at 10-minute intervals between 0700 h and 1300 h.

	4Wks (N=8)	**8Wks (N=10)**	**16Wks (N=10)**
Total GH secretion rate (ng/ml per 6 h)	1062±166	472±75.6*	306±68.3*
Pulsatile GH secretion rate (ng/ml per 6 h)	1015±162	396±64.1*	266±62.0*
Basal GH secretion rate (ng/ml per 6 h)	46.5±22.7	75.9±27.9	39.4±7.30
Mass of GH secreted/burst (MPP, ng/ml)	186±22.3	148±44.7	68.0±12.8*
Number of secretory burst/6 h	5.50±0.50	3.30±0.42*	3.90±0.41*
Mode of GH secretion	13.5±1.08	14.4±1.12	13.4±1.03

Data are presented as mean ± SEM by one way ANOVA. A P-value of <0.05 was accepted as significant. N=8-10 per group.

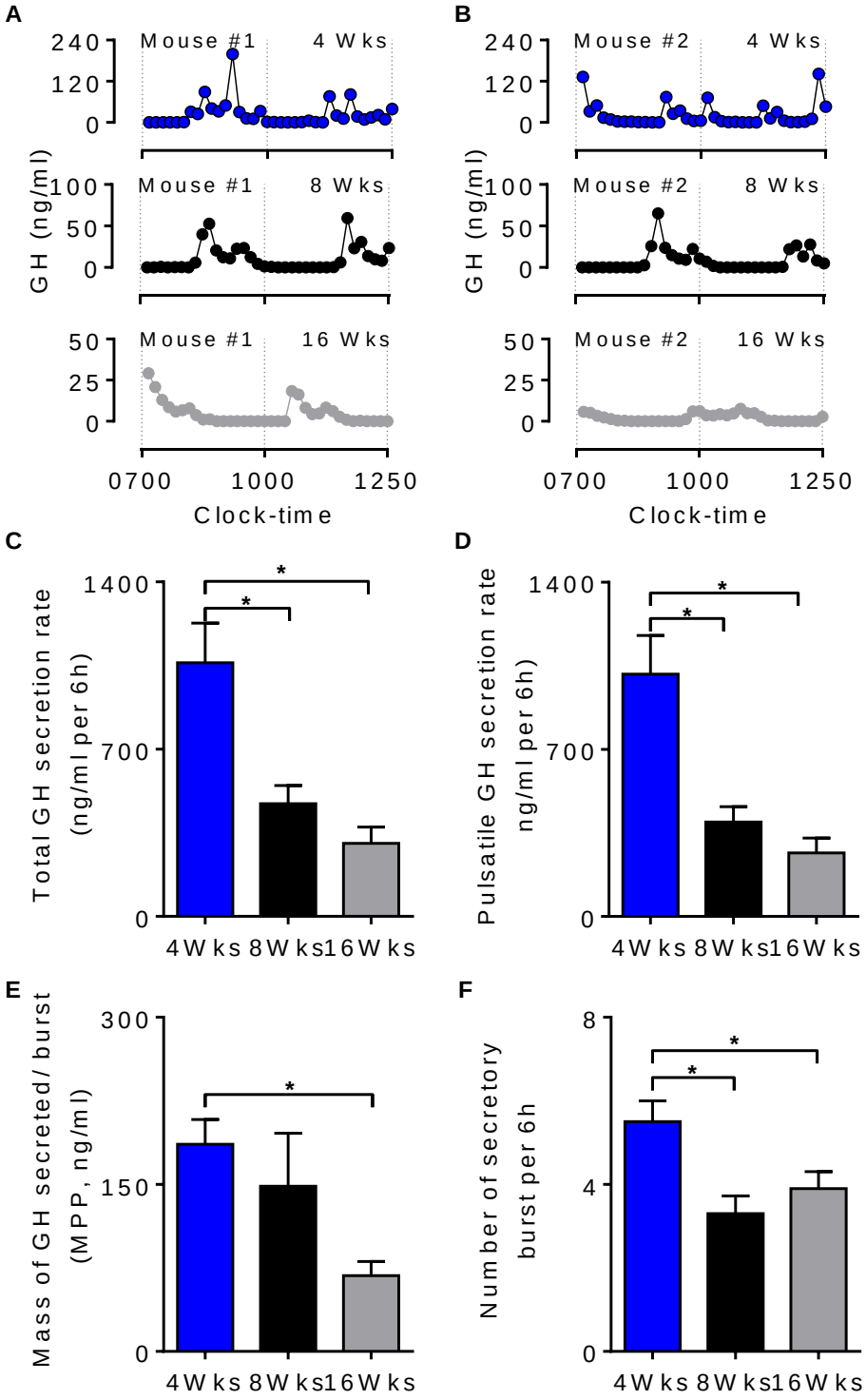

Figure 3-2: Representative profiles of pulsatile GH secretion of WT mice throughout rapid pubertal linear growth.

(A,B) Representative examples of pulsatile GH secretion from WT mice at 4 (blue), 8 (black) and 16 (grey) weeks of age. Compared to 4 weeks of age, a decline in (C) total GH, (D) pulsatile GH, (E) the mass of GH secreted per burst, and (F) the number of GH secretory bursts (F) by 16 weeks of age was observed. This was confirmed by deconvolution analysis (details summarized in Table 2-1). Data presented as mean±SEM. A P value < 0.05 was accepted as significant; N= 8 to 10 per group. Abbreviations: wild-type; WT, growth hormone; GH.

3.4 Discussion

An elevation in pubertal GH secretion is necessary to sustain rapid linear growth characteristics of puberty in humans [1, 317]. While not directly addressed, this observed phenomenon is thought to be conserved across all mammalian species including mouse models. Given the historical challenges in measuring pulsatile GH secretion in mice, prior observations of GH secretion in mice were limited to one-off measures [93, 94]. Consequently, measures of pulsatile GH secretion from pubertal mice do not exist. The divergence in linear growth rate begins during puberty. Given that GH is primarily associated with rapid linear growth, changes in the dynamics of pulsatile GH release may contribute to the assessment of enhanced rapid pubertal growth in MC4RKO mice. Thus, it is important to determine pubertal changes in pulsatile GH secretion relative to linear in normal WT mice. In the present study, 3 phases of linear growth were identified; onset of rapid linear growth occurring between 5 and 8 weeks of age, slowing linear growth between 9 and 11 weeks of age, and a further slowing in linear growth rate when mice reached adulthood between 12 and 20 weeks of age. Given that the maximal GH release is critical in promoting the pubertal growth spurt [1, 317], pulsatile GH secretion was assessed throughout early pubertal and early adulthood in mice relative to linear growth.

During pubertal human development, an increase in GH secretion is accompanied by a significant increase in GH peak amplitude [128, 320], suggesting that puberty is associated with increased GH secretion. Similar observations have been confirmed in rats [321], demonstrating that the marked increase in GH secretion during periods of rapid pubertal growth occur in response to an elevation in GH pulse amplitude. In agreement with these findings, pulsatile GH secretion in pubertal mice at 4 weeks of age was elevated when compared to 8 and 16 weeks of age. This coincided with peak periods of linear growth (between 5 and 8 weeks of age). This perceived rise in GH secretion at 4 weeks of age corresponded to an increase in total, pulsatile GH secretion and the MPP. Furthermore, an increase in pulse frequency was observed throughout the sampling period, suggesting that increased GH secretion throughout the peak periods of pubertal growth in mice may occur as a consequence of increased pulse amplitude and frequency. It should be noted, however, that assessment of pulsatile GH release prior to 4 weeks of age was not possible, and thus it remains unknown whether pulsatile GH secretion at 4 weeks of age in mice rise relative to a younger age.

Relative to 4 weeks of age, the progressive decline in GH release observed by 8 and 16 weeks of age corresponded a slowing in the rate of linear growth. This was characterized by the overall reduction in total, pulsatile, MPP as well as the number of GH

secretory pulses. While observations propose that the synchronization pattern of pulsatile GH release established by adulthood and an increase in GH pulse frequency may underlie the promotion of rapid linear growth in mice, the decline in pubertal GH secretion throughout adulthood may account for the slowing in somatic growth rate into adulthood Thus, changes in GH release throughout adulthood, and possibly the role of GH in promoting linear growth may be coupled to alterations in GH secretory dynamics. To date, measures of pubertal changes in GH secretion are scarce and are limited to rat models [321]. Moreover, pubertal changes in GH release relative to growth velocity throughout adulthood in mice do not exist, and thus extrapolation regarding the changes in the dynamics of GH secretory pattern relative to linear growth requires further exploration.

While not directly assessed, it has been proposed that the synchronization of the somatotroph network at puberty will promote enhanced coordinated pulsatile GH secretion, resulting in accelerated pubertal growth [75]. Interestingly, the arrangement of this network appears to be dynamic, in that pubertal modifications in the arrangement of somatotrophs are reversed by approximately 16 weeks of age in mice [75]. It was anticipated that structural changes in the somatotroph network would result in the coordinated secretion of GH, and thus reflect an overall elevation in pulsatile GH secretion throughout pubertal development and the subsequent decline of GH secretion into early adulthood. Indeed, immunocytochemical and morphological observations revealed that the number and/or size of GH-secreting cells of the anterior pituitary gland decrease with age in humans [322] and in mice [323, 324]. Furthermore, Bonnefont and colleagues confirmed observations of altered capacity constituting the somatotroph network, in that the volume of somatotroph cells transiently decreased from puberty into early adulthood in mice [75]. To this extent, in this study, measures of pulsatile GH secretion in the mouse perfectly complement the changes and timing of altered GH secretion proposed by Bonnefont and colleagues, confirming that attainment of peak GH secretion and the subsequent decline of GH secretion is a product of morphological changes in somatotroph network connectivity. Irrespective of its causes, pulsatile GH secretion declines following the attainment of peak adult body length. This was characterized by an overall reduction in peak pulsatile GH secretion. Given that the functional activity of somatotrophs are directly under the influence of hypothalamic factors, it is likely that altered integrity of the somatotroph network and subsequent changes in GH secretion from puberty into early adulthood occur in response to alterations in central or peripheral mechanisms known to modulate somatotroph activity. While evidence exist for the contribution of age-associated changes in GHRH activity to modulate the age-associated changes in GH release, the role of SRIF neurons in

contributing to pubertal changes in GH release throughout adulthood remains unclarified. Hypothalamic contributions to these age-associated changes in GH release are discussed in Chapter 4, wherein the role of SRIF neurons in modulating GH release from puberty throughout adulthood was further addressed.

Collectively, observations confirmed that the slowing of rapid pubertal linear growth into adulthood is accompanied by a decline in total GH secretion. This is characterized by an overall reduction in peak GH secretion, as well as the number of GH secretory events. Importantly, while GH release declines in adulthood, observations clearly demonstrate that rapid pubertal linear growth in mice occurred alongside the establishment of altered GH secretion profiles. This pattern of GH release throughout puberty into adulthood is thought to be paramount to the changes in linear growth rate. Using these established parameters of GH secretion relative to linear growth in mice, observations were extended to evaluate the role of GH in promoting rapid pubertal linear growth in MC4RKO mice (addressed in Chapter 5).

CHAPTER FOUR: EXPERIMENTS TO ADDRESS AIM 2

4. ALTERED GH RELEASE FROM PUBERTY THROUGHOUT ADULTHOOD IN MICE DOES NOT OCCUR ALONGSIDE CHANGES IN HYPOTHALAMIC SOMATOSTATIN DISTRIBUTION.

- *This chapter is reproduced from published observations (Tan et al. 2013, Journal of Neuroendocrinology)*

4.1 Introduction

Alterations in the pattern of pulsatile GH secretion are thought to occur relative to growth and ageing, with the most prominent change characterized between the transition of puberty into early adulthood [325]. As discussed previously, the slowing of rapid pubertal linear growth in the mouse is accompanied by a gradual decline on peak GH release, characterized by a reduction in total and pulsatile GH secretion between 4 and 16 weeks of age (Chapter 3, Figure 3-2). Importantly, observations also demonstrate a significant change in pulse dynamics, defined by a reduction in pulse irregularity and number, resulting in the establishment of characteristic adult GH secretion profiles. These observations are in agreement with prior measures, demonstrating that the secretion of GH declines progressively with age [12, 97, 326]. While not assessed alongside pulsatile GH secretion, existing observations show a rise in circulating levels of GHRH during human pubertal development, and a decline during late puberty when linear growth ceased [327]. Similar observations were reported in rats, whereby the administration of neonatal monosodium glutamate to impair GHRH release abolished the pubertal increase in GH mRNA and pituitary GH content and significantly impaired growth rate [134]. These observations suggest that GHRH may, at least, in part contribute to enhanced pubertal GH secretion, and thus somatic development at this time. Assessment of pulsatile GH release relative to pubertal linear growth or advanced age has not been documented in mouse models. Consequently, it remains unknown whether enhanced pubertal GH release contributes to rapid linear growth in mice. Following pubertal growth, GH secretion declines with approximately 14% per decade [12]. Studies in humans and rodents demonstrate that the age-associated decrease in GH release is associated with the age-dependent decrease in endogenous hypothalamic GHRH output, thus resulting in decreased GH pulse amplitude [82, 138, 141, 328]. Consequently, alterations in hypothalamic GHRH-induced GH secretion, together with differences in GH network structure, may be responsible for the age-associated loss of GH release.

The role of SRIF in modulating pubertal or age-associated alterations in GH secretion is less defined, and measures of age-related changes in SRIF immunoreactivity are limited to the ageing brain. While increased somatostatinergic tone is thought to contribute to the progressive reduction in GH secretion with age [329, 330], conflicting observations of structural changes in SRIF expression mar efforts to confirm a direct involvement. For instance, the observed loss in SRIF immunoreactivity in aged rats [139, 145] is contradicted by observations showing sustained SRIF immunoreactivity regardless of age [81]. Moreover, the assessment of *Srif* mRNA expression within the brain has not been performed alongside assessment of pulsatile GH secretion, while measures directly assessing the role of SRIF in modulating pubertal changes in GH secretion do not exist. Consequently, no credible information exists to address the potential role of SRIF in accounting for changes in pulsatile GH secretion observed from puberty into adulthood. Because SRIF inhibits pulsatile GH release, it is important to determine the role of SRIF in regulating changes in GH release relative to linear growth from puberty into adulthood. Observations in this study will contribute to the assessment of pubertal GH release relative to rapid linear growth in MC4RKO mice (Chapter 5). *Thus, this study aims to characterize the role of SRIF in modulating the change in pulsatile GH secretion in WT mice throughout puberty and into early adulthood.*

4.2 Methods

4.2.1 Assessment of age-related changes in *Srif* mRNA expression

Srif mRNA expression was quantified relative to observed pubertal and age-related changes in pulsatile GH secretion that reflect altered linear growth (Chapter 3). Consequently, mice were sacrificed at 4, 8 and 16 weeks of age. Mice were anesthetized with an ip sodium pentobarbitone dosage. Whole brain tissues were rapidly extracted and fixed in RNase-free 4% paraformaldehyde (PFA, Sigma-Aldrich, Australia) at 4 °C overnight and processed for in situ hybridization (ISH) as detailed below.

4.2.2 Generation of *Srif* RNA probes

Primers specific (with T7 and T3 promoter sequences added to each gene specific sequences at the 5’ end) for mouse *Srif* (GenBank accession: NM-009215) mRNA were generated. Standard PCR was performed to obtain *Srif* template cDNA. Briefly, a mouse hypothalamus was used to obtain total RNA, which was reversed transcribed and used to amplify *Srif* cDNA amplicons. The amplification protocol was: initial cycle at 94 °C for 1 min, 38 cycles of 94 °C for 1 min, 66.4 °C for 45 sec and 72 °C for 1 min, followed by a final

extension for 10 mins at 72 °C. Correct product size was documented on agarose gel (1%). The *Srif* cDNA template is a 521 base pair (bp) fragment of mouse *Srif* cDNA, corresponding to nucleotides 7-527 of mouse *Srif* full-length cDNA. For *in vitro* transcription of antisense and sense RNA probes, digoxigenin (DIG)-RNA labeling was performed according to the manufacturer's instructions (Roche, Mannheim, Germany). Approximately 200 ng of purified DNA was incubated for 3 hours at 37 °C in a mixture of reagents containing 10x transcription buffer, DIG reaction mix, RNase Inhibitor, T3 RNA polymerase (sense probe) and T7 RNA polymerase (antisense probe) (Promega, Madison, Wisconsin, US). The reaction was stopped with 0.02 M ethylenediaminetetracetic acid (EDTA, Invitrogen) followed by the addition of 0.5 M lithium chloride (Sigma-Aldrich, Australia) and 2.5 volumes of 100% ethanol, mixed and incubated overnight at -20 °C to precipitate the cRNA probes. The mixture was then centrifuged at maximum speed for 20 mins at 4 °C, the pellet was washed with 70% ethanol, air-dried and resuspended in 20 µl RNAse-free water. Due to the incorporation of DIG, DIG-labelled RNA antisense probes show a shift in molecular weight. Probes were run on 1% agarose gels to verify size. Concentrations of probes were quantified by spectrophotometer (Nanodrop 2000, Thermo Scientific, USA) prior to use. RNAse-free conditions were maintained up to post-hybridization steps. All solutions were made with 0.05% diethyl pyrocarbonate (DEPC, Sigma-Aldrich, Australia) treated water. Probes were stored at -80 °C for later use.

4.2.3 Tissue Preparation and In situ Hybridization (ISH)

PFA (4% in phosphate buffer solution (PBS), pH 7.4, 0.1 M) fixed whole brains were washed in PBS and passed through graded sucrose solutions (15% and 30% in PBS) prior to embedding in OCT (Tissue-Tek® OCT™ Compound, Sakura Finetek, USA). Sections were cut on a cryostat (16 µm, Hyrax C60, Carl Zeiss, Germany), mounted on slides (SuperFrost® Plus Microscopic Slides, Menzel-Gläser, Germany), and stored at -80 °C. For ISH, sections were re-hydrated in PBS, post-fixed in 4% PFA for 10 mins, treated with Proteinase K (10 mg/ml, Roche, Mannheim, Germany) for 6 mins at room temperature, acetylated for 10 mins (acetic anhydride, 0.5%; Sigma-Aldrich, Australia), and hybridized with DIG-labelled Srif probes overnight at 65 °C. Hybridization buffer contained 1x salt (200 mM sodium chloride, 13 mM Tris, 5 mM sodium phosphate monobasic, 5 mM sodium phosphate dibasic, 5 mM EDTA), 50% formamide, 10% dextran sulphate, 1 mg/ml yeast tRNA (Roche, Mannheim, Germany), 1x Denhardt's (1% w/v bovine serum albumin, 1% w/v Ficoll, 1% w/v polyvinylpyrrolidone), and DIG-labelled probe (final dilution of 1:2000 from a 20 µl reaction starting with 200 ng template cDNA). Two post-hybridization washes

(1x SSC, 50% formamide, 0.01% Tween-20) were carried out at 65 °C for 30 mins, followed by two 1x MABT washes (150 mM sodium chloride, 100 mM maleic acid, 0.01% Tween-20 – pH 7.5) at room temperature for 30 mins, and 30 mins RNase treatment (400 mM sodium chloride, 10 mM Tris pH7.5, 5 mM EDTA, 20 µg/ml RNAse A) at 37 °C. Sections were blocked in 1 x MABT, 2% blocking reagent (Roche), 20% heat-inactivated horse serum at room temperature for 1 hour, and incubated with anti-DIG antibody (Roche) diluted in blocking solution (1:2500) at 4 °C overnight. Following four 20-minute 1 x MABT washes, slides were rinsed in 1 x NTMT (100 mM sodium chloride, 50 mM magnesium chloride, 100 mM Tris – pH 9.5, 0.1% Tween-20) at room temperature for 10 mins, and incubated with NBT/BCIP in NTMT containing 100 mg Levamisole (Sigma) according to manufacturer's instruction (Promega). Slides were counterstained with nuclear fast red, dehydrated and cleared in xylene, and mounted in cytoseal mounting medium (VWR). Probe specificity was confirmed by substitution of antisense RNA probes with an equivalent amount of labelled sense RNA probes, and the inclusion of positive and negative tissue controls to confirm the specificity of RNA probes

4.2.4 Image acquisition and analysis

As an anatomical guide, sections were matched using the mouse brain atlas of Franklin and Paxinos [331]. Serial sections representing regions throughout the PeVN, ARC and PVN at 4, 8 and 16 weeks of age were selected from all mice and analyzed for *Srif* mRNA expression. The juvenile mouse brain is considerably smaller than the adult mouse brain, and thus assessment of neuron counts for brain nuclei were collected relative to landmarks corresponding to adult bregma, and presented accordingly. For the PeVN, assessment was performed according to adult bregma -0.4 to -0.5, adult bregma -0.7 to -0.8, adult bregma -0.9 to -1.0, and adult bregma -1.3 to -1.4. For the juvenile ARC, sections were selected according to adult bregma -1.3 to -1.4, and adult bregma -1.8 to -1.9. For the juvenile PVN (including areas matching the PaLM, PaMM and PaV), sections were selected according to adult bregma -0.7 to -0.8.

Sections were imaged on Aperio ScanScope XT slide scanner (Aperio Technologies, Vista, CA, USA). Extracted images were processed in ImageJ using the following commands: (i) Image > type > 8-bit, (ii) Image > Adjust > Threshold (corrected to 0/70), (iii) Process > Binary > Watershed. The function of watershed segmentation that automatically splits closely touching cells was applied to all counted sections. Thus, *Srif* mRNA expressing cells that cannot be separated by threshold was separated by watershed function to avoid counting of overlapping cells. The resulting particles were

counted using the particle analysis cell counter option (Analyze > Analyzed Particles). Particles smaller than 200 pixels were excluded from the analysis. In general, this limited counts of cells to those with a diameter in excess of 5 μm. This counting procedure followed guidelines established previously [332].

4.2.5 Real-time quantitative PCR measurement of hypothalamic *Srif* mRNA expression

To quantify the relative amount of hypothalamic *Srif* mRNA expression throughout pubertal maturation and in adulthood, hypothalamic micropunch biopsies containing pooled regions of PeVN, PVN and the ARC were isolated from WT mice at 4, 8 and 16 weeks of age detailed in section 2.6. Results were normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA expression, and expressed relative to *Srif* mRNA expression at 4 weeks of age.

4.3 Results

4.3.1 Distribution of *Srif* mRNA expression in mouse brain

To determine whether the changes in pulsatile GH secretion between 4 and 16 weeks of age (as confirmed in Chapter 3) correlate with age-related changes in SRIF, *Srif* mRNA expression was assessed within the ARC/PeVN complex. Figure 4-1A illustrates a sagittal view along the rostrocaudal axis of the mouse brain (between bregma levels -0.46 to 1.82) along which *Srif* mRNA expression was assessed. Representative coronal sections showing the distribution of *Srif* mRNA at corresponding bregma levels are illustrated in Figure 4-1B to D. The specificity of positive *Srif* hybridization was determined using a sense *Srif* probe, the complimentary probe sequence to *Srif*, which produced no hybridization signal (Figure 4-1E). To further confirm the quality and specificity of hybridization *Srif* mRNA signal, WT mouse spinal cord (Figure 4-1F) and pituitary gland tissue (Figure 4-1G) were included as positive- and negative-*Srif* mRNA expressing controls, respectively.

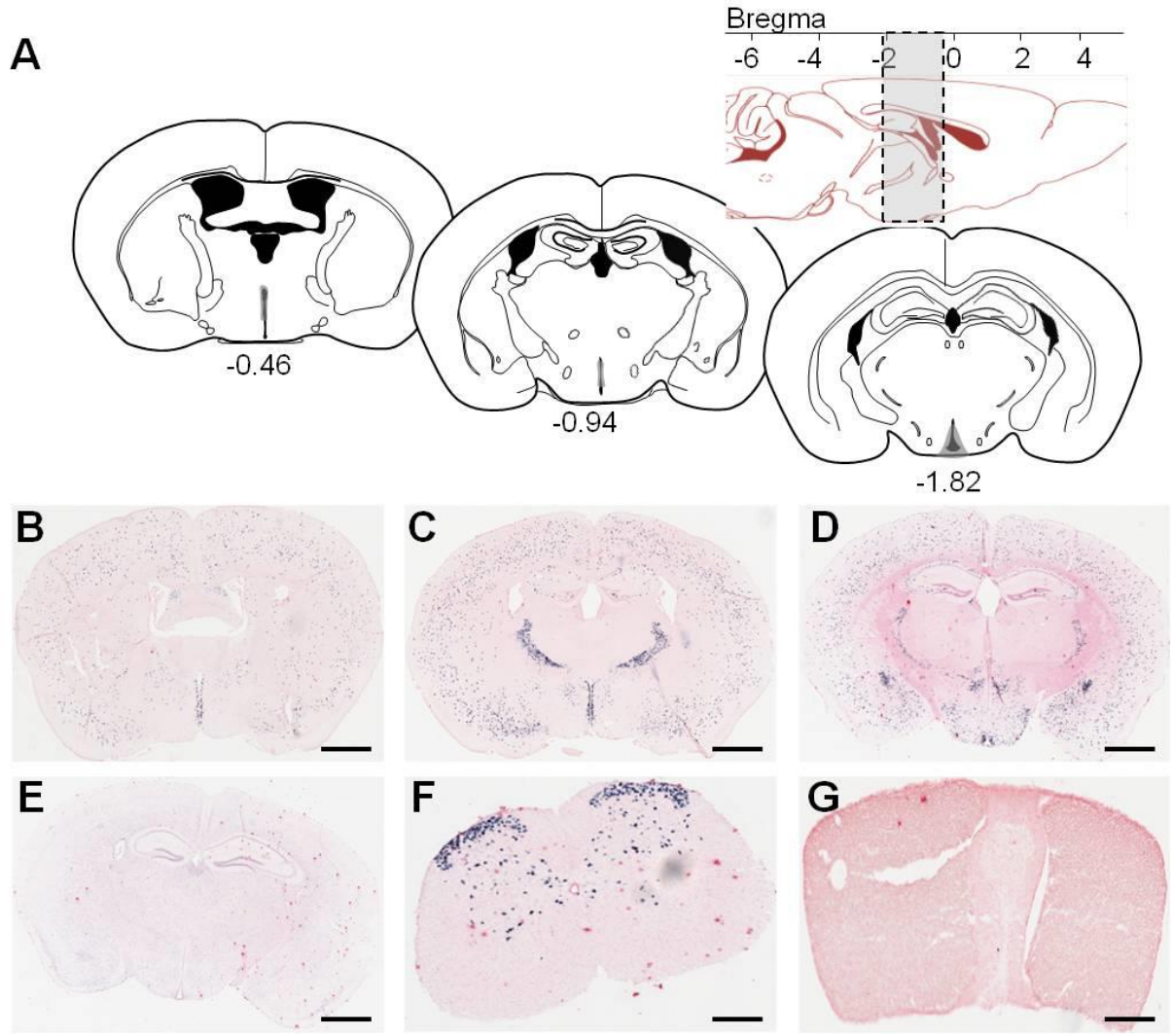

Figure 4-1: Distribution of *Srif* mRNA expression within the WT mouse brain.

(A) Schematic illustrating the sagittal view of the mouse brain at 8 weeks of age (grey shading illustrates bregma regions in which *Srif* mRNA distribution are observed). Images are representative of the level of the mouse brain corresponding to regions throughout which *Srif* mRNA distribution observed between bregma levels -0.46 and -1.82. (B) - (D) Representative coronal sections showing the distribution of *Srif* mRNA by ISH using DIG-labelled *Srif* RNA probe. (E) Represents *Srif*-labelled sense control. Representative (F) positive and (G) negative expression for *Srif* mRNA in the mouse spinal cord and pituitary gland, respectively. Representative sections illustrating *Srif* mRNA expression are verified across 3 animals. Images were representative of 1x scanned magnification (on the Aperio ScanScope XT slide scanner) to capture the entire area of the tissue. Abbreviation: Somatostatin; *Srif*, wild-type; WT, in situ hybridization; ISH, digoxigenin; DIG.

4.3.2 *Srif* mRNA expression within the PeVN/ARC-ME Complex

As seen in rats [333], *Srif* mRNA is widely expressed throughout the mouse brain. While majority of the *Srif* mRNA expressing neurons are found within the PeVN (Figure 4-2D), *Srif* mRNA is also highly expressed in the ARC-ME complex (Figure 4-2H). In addition, *Srif* mRNA expression is apparent in several hypothalamic nuclei including the lateral

magnocelluar paraventricular nucleus (PaLM; Figure 4-2A), medial magnocelluar paraventricular nucleus (PaMM; Figure 4-2B), ventral paraventricular nucleus (PaV; Figure 4-2C), hippocampus (HippoC) (Figure 4-2E), central nucleus of amygdala (CeA; Figure 4-2F) and dorsomedial hypothalamus (DMH; Figure 4-2G).

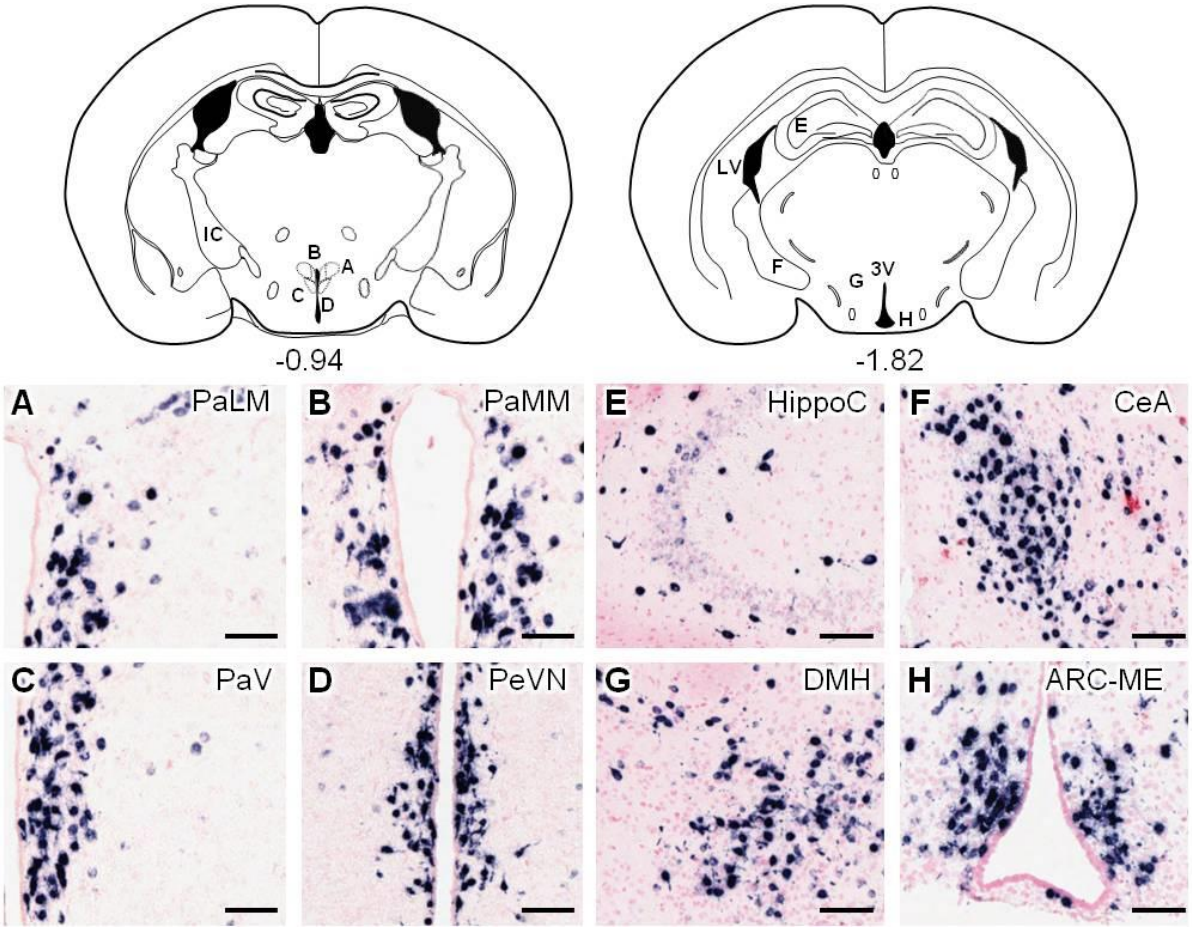

Figure 4-2: Schematic coronal diagrams of mouse brain mapping *Srif* mRNA distribution by ISH between bregma -0.94 and -1.82.

Srif mRNA is expressed in the (A) PaLM, (B) PaMM, (C) PaV, (D) PeVN, (E) HippoC, (F) CeA, (G) DMH and (H) ARC-ME. Scale bar = 0.1 mm. Representative images illustrate *Srif* mRNA expression are verified across 3 animals. Abbreviations: Lateral ventricle; LV, internal capsule; IC, third ventricle; 3V, lateral magnocelluar paraventricular nucleus; PaLM, medial magnocellular paraventricular nucleus; PaMM, ventral paraventricular nucleus; PaV, periventricular nucleus; PeVN, hippocampus; HippoC, central nucleus of the amygdala; CeA, dorsomedial hypothalamus; DMH, arcuate nucleus-median eminence complex; ARC-ME.

4.3.3 Changes in *Srif* mRNA expression within the ARC/PeVN/PVN complex do not occur alongside changes in pulsatile GH secretion between 4 and 16 weeks of age

To determine whether the observed decline in pulsatile GH secretion between pubertal and early adult mice occur in response to age-related changes in SRIF distribution, *Srif* mRNA expression were assessed in the hypothalamic regions of mice at 4, 8 and 16 weeks of age. Regions assessed include the PeVN (relative to adult bregma -0.4 to -0.5 (Figure 4-3B), adult bregma -0.7 to -0.8 (Figure 4-3C), adult bregma -0.9 to -1.0 (Figure 4-3D), and adult bregma -1.3 to -1.4 (Figure 4-3E)) and the ARC (relative to adult bregma -1.3 to -1.4 (Figure 4-3F) and adult bregma -1.8 to -1.9 (Figure 4-3G)). Given that *Srif* mRNA expressing neurons located within the PVN may contribute to the neuroendocrine regulation of GH release from the pituitary somatotrophs [334], assessment of *Srif* mRNA expression were extended to subnuclei of the PVN (including areas matching the PaLM, PaMM and PaV; relative to adult bregma -0.7 to -0.8). Representative *Srif* mRNA expression in the PeVN (top panels) and ARC (bottom panels) regions throughout 4, 8 and 16 weeks of age are illustrated in Figure 4-4A. The number of *Srif* mRNA expressing neurons within all assessed areas of the PeVN did not change between 4 and 16 weeks of age (Figure 4-4B-E). Similarly, the number of *Srif* expressing neurons within all assessed areas of the ARC did not differ significantly between 4 to 16 weeks of age (Figure 4-4F,G). A decrease in *Srif* mRNA expressing neurons in the PVN was observed. This did not reach significance until 16 weeks of age (Figure 4-4). No differences in the number of *Srif* mRNA expressing neurons are observed within the PaMM and PaV nuclei between 4 and 16 weeks of age. Compared to mice at 4 weeks of age, the numbers of PaLM *Srif* mRNA expressing cells were reduced by 16 weeks of age. The collective decline in *Srif* mRNA expressing neurons within the PaLM and PaV contributed to the overall decline in the number of cells expressing *Srif* mRNA within the PVN by 16 weeks of age.

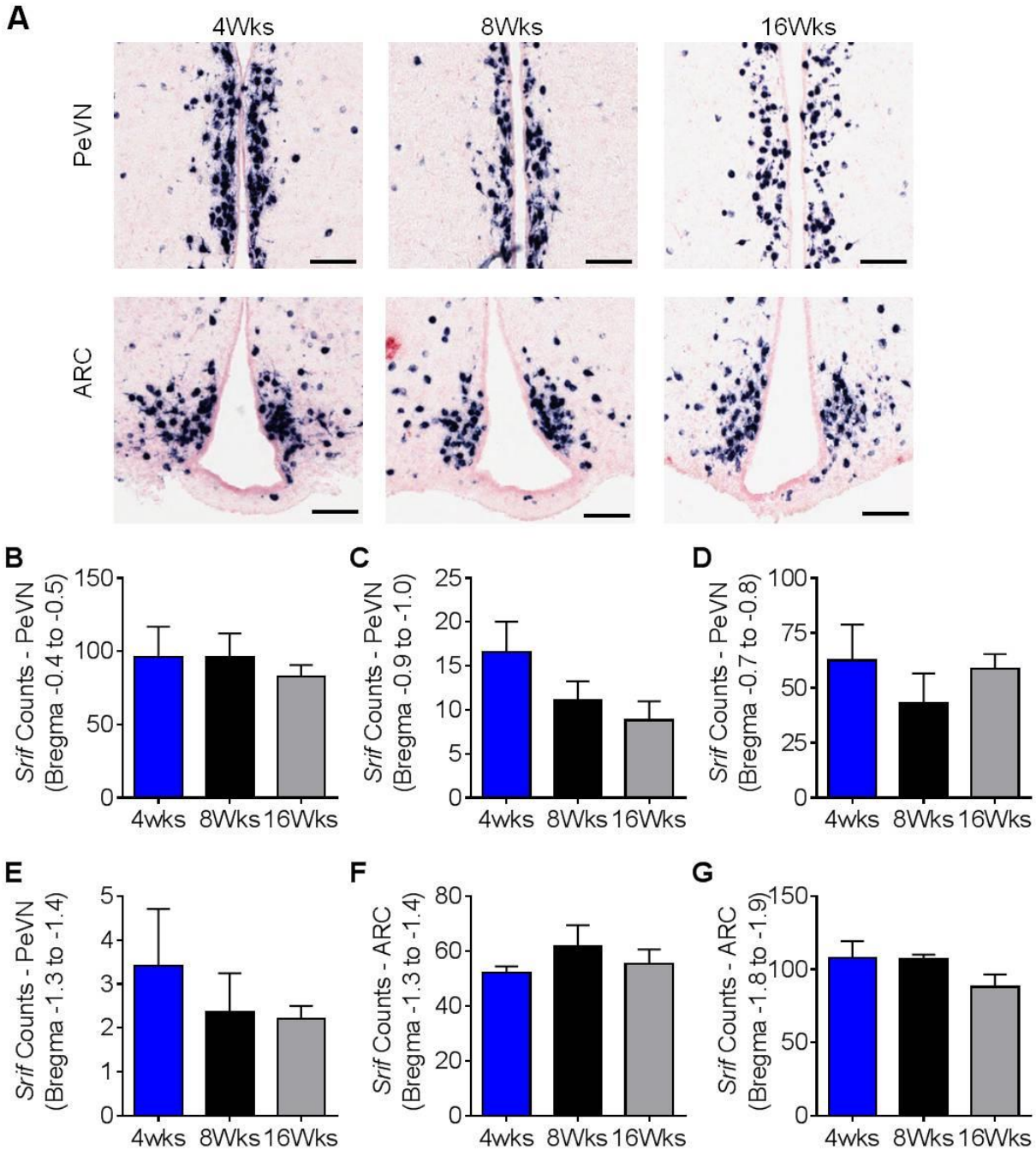

Figure 4-3: Representative examples of *Srif* mRNA expressing cells within the periventricular nucleus (PeVN relative to adult bregma -0.7 to -0.8, top panels) and arcuate nucleus (ARC relative to adult bregma -1.3 to -1.4, bottom panels) at 4, 8 and 16 weeks (Wks) of age.

Corresponding bar graphs show the (A) number of *Srif* mRNA expressing cells relative to the PeVN and ARC at levels corresponding to adult bregma (B) -0.4 to -0.5, (C) -0.7 to -0.8, (D) -0.9 to -1.0, (E) -1.3 to -1.4 and (F) -1.8 to -1.9. The number of *Srif* mRNA expressing neurons in the PeVN and ARC regions (across all levels of assessment) did not differ significantly between 4 (blue), 8 (black) and 16 (grey) weeks of age. Data presented as mean ± SEM. A P value < 0.05 was accepted as significant. Scale bar = 0.1 mm. N=4 per age group. Abbreviations: periventricular nucleus; PeVN, arcuate nucleus-median eminence complex; ARC-ME.

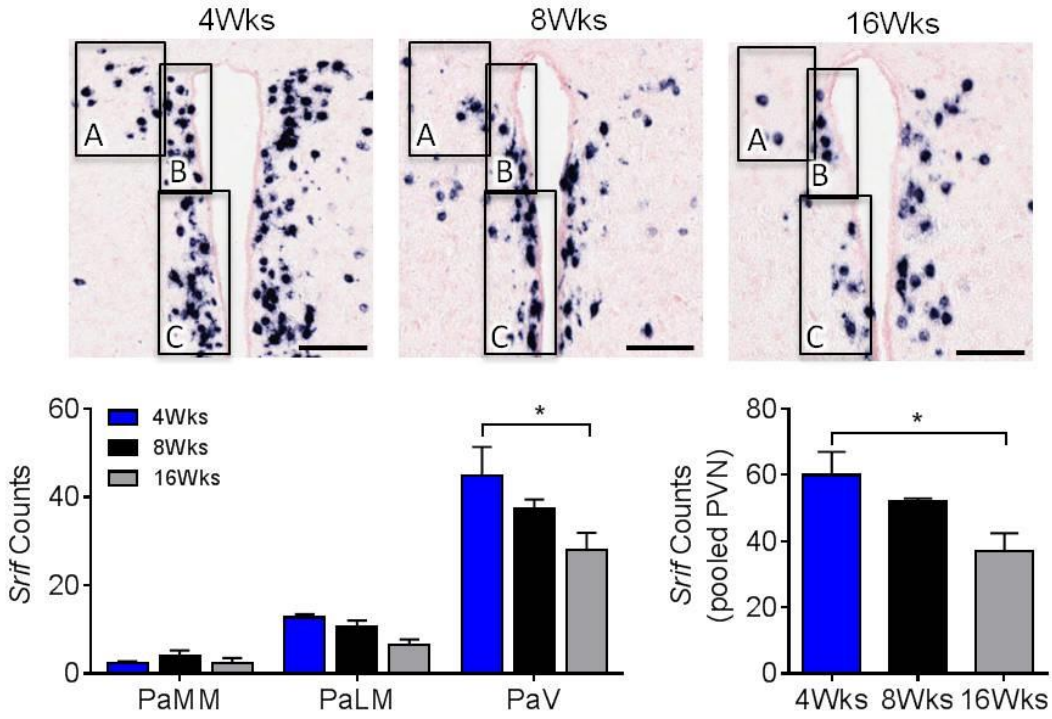

Figure 4-4: Representative sections showing the distribution of *Srif* mRNA expressing cells within the PVN and subnuclei of the PVN (A - PaLM, B - PaMM and C - PaV) at 4, 8 and 16 weeks of age.

Compared to 4 weeks of age (blue), the number of *Srif* mRNA expressing neurons within the PaLM was reduced by 16 weeks of age (grey). This resulted in an overall decrease in the total number of *Srif* mRNA expressing neurons within the PVN by 16 weeks of age. Data presented as mean ± SEM. A P value < 0.05 was accepted as significant. Scale bar = 0.1mm. N=4 per age group. Abbreviations: Lateral magnocelluar paraventricular nucleus; PaLM, medial magnocellular paraventricular nucleus; PaMM, ventral paraventricular nucleus; PaV, ventral paraventricular nucleus; PVN.

4.3.4 Quantitative analysis confirmed that hypothalamic *Srif* mRNA expression in the mouse does not change from puberty to adulthood

To verify assessment of *Srif* mRNA expression by ISH, micropunch biopsies of the PeVN/PVN/ARC complex were isolated and the abundance of *Srif* mRNA expression was determined by qPCR (as described in section 2.6). No significant changes in *Srif* mRNA expression between 4, 8 and 16 weeks of age (Figure 4-5) were observed. Overall, observations confirmed that the alterations in pubertal GH secretion are not matched by an overall change in the abundance of *Srif* mRNA expression within the mouse PeVN/PVN/ARC complex.

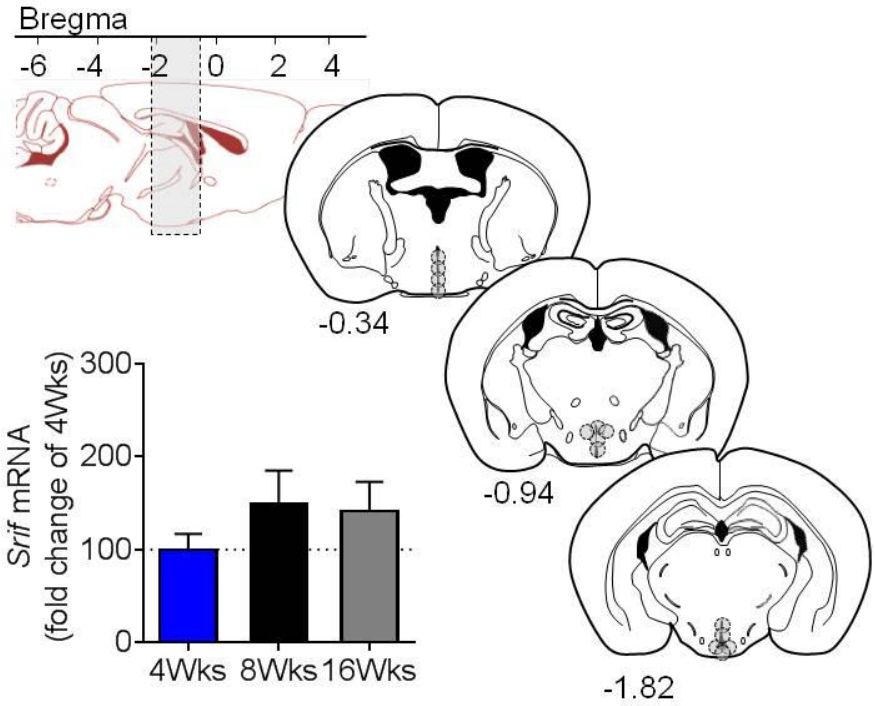

Figure 4-5: *Srif* mRNA expression from pooled hypothalamic micropunch biopsies from mice at 4, 8 and 16 Wks of age.

Hypothalamic tissues containing the PeVN, PVN and ARC were collected from 300 µm thick frozen brain sections. Micropunch biopsies were obtained from brain sections located between bregma -0.34 and -1.82 (tissue collected at representative levels corresponding to the PeVN, PVN and ARC nucleus complex outlined and shaded in grey). *Srif* mRNA copy numbers were normalized to GAPDH mRNA and expressed relative to mice at 4 weeks of age. Data presented as mean ± SEM. A P value < 0.05 was accepted as significant; N= 6 per age group. Abbreviations: Periventricular nucleus; PeVN, paraventricular nucleus; PVN, arcuate nucleus-median eminence complex; ARC-ME.

4.4 Discussion

Alterations in the secretory pattern of pulsatile GH release are thought to occur relative to growth and ageing, with the most prominent change characterized between transitions of puberty into early adulthood. Following cessation of pubertal growth and the attainment of peak adult height, GH secretion declines by approximately 14% per decade [12]. While changes in the abundance of hypothalamic GHRH neurons [323, 324], and a rearrangement of the clustering of somatotrophs within the anterior pituitary gland [75] are thought to contribute to this decline in GH secretion, the exact cause for changes in GH secretion during pubertal development throughout adulthood is still under investigation. Moreover, the role of SRIF neurons in modulating this change in GH secretion is not well defined.

As discussed above, the release of GH from somatotrophs is regulated primarily by stimulatory GHRH and inhibitory SRIF neurons [80]. Observations specific to the pubertal maturation of interactions between GHRH and SRIF neurons are limited, and thus extrapolation of the interrelationship between the GHRH and SRIF network relative to age-associated changes in GH release are largely based on observations from the ageing rats and primates. Initial observations demonstrated impaired GHRH-stimulated GH secretion in aged versus young rats [335], while subsequent studies in humans confirmed that GH response following GHRH treatment coupled with arginine (SRIF antagonist to suppress endogenous SRIF release) does not differ with age [143, 336, 337]. SRIF inhibits GH secretion directly via the suppression of GH release from the somatotroph, or indirectly via the inhibition of GHRH induced GH secretion [80]. Thus, it seems that the age-associated decline in GH secretion occur independent of SRIF activity. Accordingly, the release of GHRH output in aged female rhesus monkeys is three to four times lower compared to younger animals while the release of SRIF in aged rhesus monkeys is twofold higher compared to young female adults [328]. Consequently, the decline in pulsatile GH release throughout early adulthood is thought to be attributed to a substantial decrease in hypothalamic GHRH and an increase in SRIF release. To this extent, while not assessed alongside pulsatile GH secretion, existing evidence supports the observed age-associated decline in GHRH in modulating age-associated decline in GH release. Conversely, the role of SRIF relative to altered GH release throughout adulthood remains unclarified. Moreover, observations regarding age-related changes in GH secretion are limited to adulthood (humans, rats and primates) when pubertal development had ceased. Furthermore, data specific to the mouse brain is non-existent. Thus, it is important to decipher the role of SRIF in modulating pubertal changes in GH secretion throughout adulthood in mice.

Based on the premise that SRIF directly inhibits GH release, alterations in SRIF release may mediate changes in GH secretion throughout adulthood. Hence, the assessment of SRIF in modulating GH release in mice (Chapter 3, Figure 3-2) further complement measures of pulsatile GH output throughout puberty and adulthood.

Observations in this study confirm that the number of *Srif* mRNA expressing neurons within the PeVN/ARC complex of the mouse does not largely differ throughout the transition from puberty into early adulthood. Similarly, the abundance of *Srif* mRNA expression within the PeVN/PVN/ARC complex (as assessed by qPCR) does not vary significantly throughout this time. Consequently, changes in pulsatile GH secretion from puberty through adulthood may not be mediated by age-related changes in the distribution of *Srif* mRNA expressing neurons throughout the PeVN or the ARC regions. Of interest, a decline in the number of *Srif* mRNA expressing neurons within subnuclei of the PVN (predominantly the PaLM) was observed. This coincided with the decline in pulsatile GH secretion. To date, the action of SRIF neurons within the PVN relative to the pulsatile release of GH has not been assessed. Thus, information to clarify the direct relationship between SRIF neurons within the PVN and GH secretion does not exist. Given that SRIF neurons located within the PeVN may innervate the PVN [334], changes in the distribution of *Srif* mRNA expressing neurons within the hypothalamic PVN region may contribute to alterations of GH release throughout the transition into adulthood. It is however, possible that the abundance or morphological changes of *Srif* mRNA expression may not reflect active SRIF neuronal firing to inhibit GH release. Thus, observations might be interpreted to mean that age-associated changes in GH release throughout early adult mice may not be mediated by age-related changes in SRIF expression. Nonetheless, observations within this study are limited to 16 weeks of age. It cannot be excluded, however, that age-related changes in SRIF in mice occur with progressively ageing. This requires further investigation.

Collectively, observations suggest that pubertal and adulthood changes in GH secretion do not occur alongside changes in the abundance of *Srif* mRNA expressing neurons located within the PeVN/ARC complex, and thus morphological changes in *Srif* mRNA expressing neurons specific to the PeVN and ARC may not contribute to altered GH secretion at this time. It should be noted, however, that the abundance of *Srif* mRNA expression does not necessarily reflect overall SRIF release, and that collection of portal blood to determine SRIF release into portal circulation in mice is current not available. Accordingly, the role of SRIF may not be entirely excluded. Given that SRIF-deficient mice exhibit normal pubertal growth in comparison to WT LM [338], altered SRIF release may

not be of critical importance in mediating pubertal and adulthood changes in GH secretion relative to linear growth. Observations confirmed in this study were incorporated into the assessment of pubertal and adulthood changes in GH release relative to linear growth in MC4RKO mice. Given observations, it seems unlikely that changes in hypothalamic SRIF would alter pubertal GH release to promote rapid linear growth in MC4RKO mice (addressed in Chapter 5).

CHAPTER FIVE: EXPERIMENTS TO ADDRESS AIMS 3 AND 4

5. RAPID PUBERTAL GROWTH ASSOCIATED WITH MC4R DYSFUNCTION DOES NOT OCCUR AS A CONSEQUENCE OF GH/IGF-1 HYERSECRETION.

5.1 Introduction

While GH secretion declines with age (discussed in Chapter 3), the release of GH is exacerbated with increased adiposity, resulting in GH deficiency in obesity. It remains unknown how metabolic disruptions associated with obesity, contribute to reduced GH output. Assessment of GH secretion in obese MC4R deficient individuals demonstrate a recovery in pulsatile GH release relative to obese individuals of similar BMI [68], thus suggesting that the MC4R may, somewhat, contribute to altered GH release in obesity. This may occur through direct interactions with somatotrophs, or indirect interactions with GHRH and/or SRIF neurons. To date, there have been no studies on the localization of MC4R expression on pituitary GH-secreting cells or hypothalamic GHRH in rodents, and evidence for direct mediation of MC4R action on pituitary somatotrophs is lacking. Consequently, it remains unknown whether the MC4R directly modulate pulsatile GH release. Interestingly, as with childhood onset obesity [339], obese MC4R deficient adults present with increased adult height [68]. Thus, while defective MC4R signaling appears to contribute to the recovery of GH release in obesity, it is thought that hypersecretion of GH in this population may contribute to rapid linear growth during puberty. There is, however, no evidence to support this proposed concept. Given the arduous nature of assessing pulsatile GH secretion during pubertal growth in children, it remains uncertain whether pubertal GH hypersecretion contributes to rapid pubertal linear growth in hyperphagic MC4R deficient children. To this extent, the demonstration of a functional GH/IGF-1 axis in animal models that emulate the effects of loss of MC4R signaling is of critical importance, as this may validate the proposed role of MC4R in mediating GH release relative to adiposity and the anticipated role of GH in promoting rapid linear growth. Moreover, such studies may provide critical information to define the role of GH and insulin in regulating substrate clearance in hyperphagic individuals, while identifying the interrelationships between GH, insulin and growth during periods of energy excess.

GH secretion declines with increased adiposity in both humans and in mice [12, 97]. This loss of GH release is thought to facilitate the actions of insulin, thereby enhancing fatty acid uptake and storage [14, 71]. Accordingly, the premise that increased GH secretion contributes to increased linear growth in MC4R deficient children contradict current conventions regarding the metabolic actions of GH relative to insulin in sustaining

endogenous fatty acid and glucose homeostasis. Moreover, as insulin and IGF-1 signal through common pathways, insulin may interact with IGF-1 receptors (IGF-1R) to potentially promote IGF-1 mediated growth in MC4R deficient individuals. Using the MC4RKO mouse model, this chapter aims to address whether MC4R directly contributes to altered GH release during hyperphagia-induced weight gain. *This study will address the hypothesis whether GH hypersecretion contributes to rapid pubertal growth associated with loss of MC4R signaling, while briefly addressing the potential mechanisms that may contribute to altered GH release and rapid linear growth in MC4RKO mice. Finally, this study will demonstrate the inverse relationship between GH and insulin in facilitating fatty acid and glucose homeostasis throughout hyperphagia-induced weight gain in MC4RKO mice*.

5.2 Methods

5.2.1 Phenotypic characteristics of MC4RKO mice

To monitor the rate of rapid growth, body weight and body length (nasal-anal distance) of WT LM and MC4RKO mice were monitored from puberty (4 and 5 weeks of age, respectively) to adulthood (20 weeks of age), on a weekly basis. To monitor the development of hyperphagia, food consumption was measured daily starting from 5 weeks of age. A pre-weighed amount of food was placed into the food hopper and the remaining food was weighed daily. Measurements were conserved for all animals across all experiments.

5.2.2 Characterizing the metabolic profile of MC4RKO mice

To assess *in vivo* metabolic status following the development of hyperphagia, WT LM and MC4RKO mice underwent a glucose tolerance test (GTT) at 6 and 16 weeks of age. Following GTT, animals were given free access to food and allowed 2 days to recover from fasting before assessment of insulin sensitivity (through ITT, as detailed in section 2.4). Following recovery from ITT, WT LM and MC4RKO mice were sacrificed between 0900 and 1000 h as detailed in section 2.3.3. Whole blood was collected into tubes containing EDTA, thereby preventing the degradation of peptides including glucagon-like peptide-1 (active GLP-1). Tissues and plasma were collected and stored at -80 °C for future analysis. To determine the adiposity of mice, gonadal fat pads, inguinal fat pads and interscapular fat pads were isolated by dissection and the fat pad mass was determined.

5.2.3 Characterizing pulsatile GH secretion in MC4RKO mice

For measures of pulsatile GH secretion, blood samples were collected and processed as detailed in section 2.3.1. For assessment of changes of pulsatile GH secretion from puberty into early adulthood, weaned WT LM and MC4RKO mice at 3 weeks of age were relocated to the procedure room, individually housed and allowed 1 week to acclimate to sampling conditions before the commencement of all experiments. Changes in pulsatile GH secretion throughout pubertal growth were assessed in WT LM and MC4RKO mice at 4, 8 and 16 weeks of age. Following collection of blood samples, mice were returned to their home cage and given 2 days to recover before assessment of hormone/metabolite profile.

5.2.4 Assessment of IGF-1/Insulin receptor protein expression

To determine whether altered InsR or IGF-1R expression correspond to periods of rapid linear growth, muscle-specific InsR and IGF-1R protein expression were determined at 4, 10 and 20 weeks of age. Gastrocnemius muscle of WT LM and MC4RKO mice were lysed in buffer containing 50 mM Tris-HCl, 150 mM NaCl, 10 mM NaF, 10 mM Na4P2O4, 1 mM Na3VO4, 1% Nonidet P-40, and protease inhibitor (Roche, Basel, SUI). 50-70 µg of protein were resolved by SDS-PAGE (7-10%) and transferred to nitrocellulose membranes. Membranes were blocked in 5% skim milk-Tris buffered solution-0.1% Tween 20 and incubated overnight with anti-InsR (SC-711, Santa Cruz, CA, USA) or anti-IGF-1Rβ (SC-713, Santa Cruz) and detected with donkey anti-rabbit IgG HRP (NA934, Amersham, NJ, USA). Blots were stripped and reprobed with anti-GAPDH (MAB374, Millipore) and detected with sheep anti-mouse IgG HRP (NA931, Amersham) to verify equal protein loading. Protein bands were visualized with enhanced chemiluminescence detection kit (Pierce, Thermo Fisher Scientific, PA, USA). Densitometry analyses of immuno-reactive bands were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The integrated pixel value of each protein band was obtained by multiplying its intensity value by its area value. The normalized integrated pixel values of MC4RKO bands were compared to WT LM.

5.2.5 Isolation of mouse somatotrophs

Adult GH-GFP transgenic male mice (8 weeks old) were decapitated and whole pituitary glands were collected aseptically into a tissue culture dish (BD Biosciences, CA, USA), containing ice cold Ca2+ and Mg2+ free-Hank's buffered solution (HBSS, 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na2HPO4, 0.4 mM KH2PO4, 4.2 mM NaHCO3, 1.0 mM d-glucose,

pH 7.2). Tissues were digested in pre-warmed filtered protease solution (10 µl/ml, Aspergillus Oryzae, Sigma) and incubated at 37 °C for 30 mins. Dissociated tissues were incubated in HBSS-supplemented bovine serum albumin (BSA) (Bovogen, VIC AUS) at room temperature for 5 mins and filtered using sterile 50 µm nylon gauze, and centrifuged at 1300 rpm for 5 mins. The cell pellet was resuspended in 1 ml warmed-Dulbecco's Modified Eagle Medium (DMEM, Sigma). Fluorescence activated cell sorting (FACS, BD FACS Aria Influx Cell Sorter, BD Biosciences) was used to specifically isolate somatotrophs amongst other pituitary cells. Clusters of cells or cell doublets were omitted to ensure only GFP-expressing cells were isolated. Cells were collected in TRIzol (Invitrogen, CA, USA) and processed immediately for RNA analysis.

5.2.6 Total RNA isolation and reverse transcriptase-polymerase chain reaction

Total RNA was extracted from freshly dissected hypothalamus, anterior pituitary glands, sorted GH-GFP somatotrophs and liver tissues followed methodology detailed in Chapter 2.5. To generate first-stranded cDNA, 1 µg total RNA was transcribed using an iScript cDNA synthesis kit (Biorad Laboratories Inc., CA, USA). The intensity of the PCR bands was visualized on a Molecular Imaging GelDoc XR System (Biorad Laboratories Inc., AUS). The expression of MC4Rs in the hypothalamus, pituitary gland and somatotrophs was determined by conventional RT-PCR using MC4R primers (detailed in section 2.2), with GAPDH as an internal control. Mouse hypothalamus and liver tissues were used as positive and negative controls respectively.

5.2.7 Perfusion and tissue preparation for Immunohistochemistry

WT C57/BL6 male mice, transgenic GH-GFP and GHRH-GFP mice were anesthetized with a single ip injection of sodium pentobarbitone (32.5 mg/ml, Virbac). Animals were flushed with 10 ml of ice-cold saline through the left ventricle, followed by 10 ml of freshly prepared ice-cold 4% paraformaldehyde (PFA, Sigma-Aldrich, Australia) in phosphate buffer (0.1 M PB, pH 7.4). Whole brain and pituitary glands were collected and post-fixed in 4% paraformaldehyde overnight at 4 °C. Fixed tissues were washed and underwent graded sucrose hydration in 15% and 30% sucrose respectively. Prior to cutting, whole pituitary gland were equilibrated in 100% Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) for 4 h at 4 °C. For immunohistochemistry, coronal sections of pituitary glands were cut at a thickness of 8 µm, using a cryostat (Hyrax C60, Carl Zeiss, Oberkochen, DEU). Sections were mounted on glass slides (SuperFrost® Plus Microscopic Slides, Menzel-Gläser, DEU) and stored at -20 °C. Coronal brain sections of

GHRH-GFP mice were cut at a thickness of 30 µm on a cryostat (LEICA CM 1850, USA). Serial sections through the hypothalamus was collected and stored in cryoprotectant containing 30% sucrose, 1% polyvinyl pyrolidone and 30% ethylene glycol in 0.1M phosphate buffer (PB), at -20 °C. Prior to use, sections were warmed to room temperature for 30 mins.

5.2.8 Detection of MC4R on somatotrophs or GHRH by Immunofluorescence

Immunofluorescence was performed to detect the colocalization of MC4R on somatotrophs and GHRH neurons from transgenic mice expressing GFP. For colocalization of MC4R with somatotrophs, sections were incubated in blocking buffer containing 3% normal goat serum (NGS, Sigma-Aldrich, Australia) / 0.5% BSA in 0.1 M PB-Tween 20 before incubating in rabbit polyclonal primary MC4R antibody (Abcam, Cambridge, ENG) overnight. Following incubation in primary antibody, sections were incubated with an Alexa Fluoro® 555-conjugated secondary goat anti-rabbit antibody (Invitrogen) at room temperature for 2 h. For colocalization of MC4R with GHRH neurons, sections were processed for antigen retrieval containing 10 mM sodium citrate (pH 6.0, Sigma) at 80 °C for 30 mins. Sections were incubated with rabbit MC4R (Abcam) and mouse GFP (Santa Cruz, SC-9996) diluted in blocking buffer containing 10% normal donkey serum (NDS, Sigma) for 72 hours at 4 °C on an orbital shaker. Following incubation in primary antibodies, sections were washed and incubated with secondary antibodies (Alexa Fluoro® 555-conjugated donkey anti rabbit antibody, and Alexa Fluoro® 488-conjugated donkey anti mouse 1:800, Invitrogen). Negative controls were treated by omitting the primary antibody and replacing with blocking buffer. To visualize immunoreactivity, sections were air-dried, mounted with Golden anti-fade reagent with DAPI (Invitrogen), and examined under a 60-fold oil immersion objective (numerical aperture 1.35) and imaged using a confocal laser-scanning microscope utilizing 488 nm laser source for GFP and 555 nm laser source for red fluorescence signals (Olympus FluoView™ FV1000 Confocal Microscope, US).

5.3 Results

5.3.1 Rapid weight gain in pubertal hyperphagic MC4RKO mice occurs alongside the progressive accumulation of adiposity and rapid linear growth

The rate of weight gain in age-matched WT LM and MC4RKO mice was monitored starting from 4 weeks of age through to 20 weeks of age. Deletion of MC4R in mice results in hyperphagia, starting from a young age (Figure 5-1A). This was observed alongside an

increase in weight gain (Figure 5-1B,C, Table 5-1), and the accumulation of adipose fat mass including epididymal (Figure 5-1D), inguinal (Figure 5-1E) and interscapular fat mass (Figure 5-1F). Circulating levels of leptin corresponded to increased adiposity, reflecting an overall increase in fat mass relative to rapid weight gain (Figure 5-1G).

As seen in humans [68], impairment of MC4R signaling in mice resulted in an increase in linear growth rate (Table 5-1), and the consequential attainment of increased in adult height (Figure 5-1H,I). The rate of rapid linear growth velocity between 5 and 8 weeks and between 8 and 16 weeks of age in MC4RKO mice was significantly greater than that of WT LM (Figure 5-1H, Table 5-1). The growth rate in MC4RKO mice slowed to that seen in WT LM by 16 weeks of age (Figure 5-1H, Table 5-1), confirming that increased linear growth rate in MC4RKO mice is limited to puberty and early adulthood.

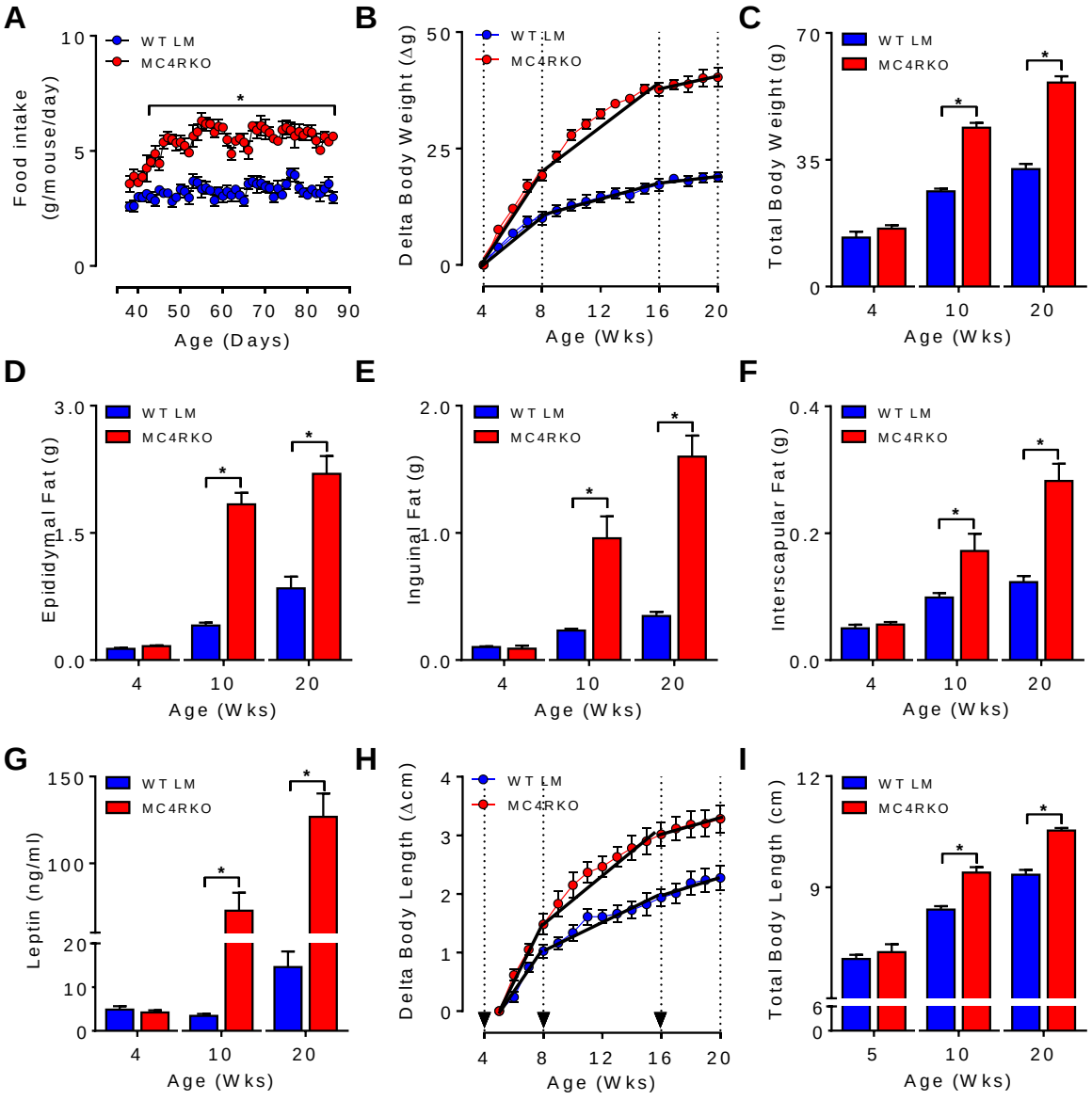

Figure 5-1: Loss of MC4R in mice results in hyperphagia, contributing to rapid weight gain and increased adiposity, and accelerated rate of linear growth, resulting in increased adult length.

(A) Hyperphagia was documented by 5 weeks of age. (B) Growth curve illustrating cumulative weekly body weight gain and (C) total body weight change in MC4RKO mice compared to WT LM. Relative to 4 weeks of age, hyperphagic MC4RKO mice show an early increase in accumulation of (D) epididymal, (E) inguinal and (F) interscapular fat (fueled by hyperphagia) at 10 and 20 weeks of age relative to WT LM. (G) Circulating leptin correspond to increased adiposity. (H) Growth curve illustrating cumulative weekly body length gain; black arrow heads indicates assessment of pulsatile GH secretion relative to rapid linear growth 4, 8 and 16 weeks of age. (I) total body length change in MC4RKO mice compared to WT LM. Data are presented as mean ± SEM. A P value < 0.05 was accepted as significant; WT LM: blue, MC4RKO: red, N=6-8 per group. Abbreviations: Melanocortin 4 receptor knockout mice; MC4RKO mice, wild-type littermates; WT LM.

Table 5-1: Rate of linear growth (slope) relative to cumulative body weight and body length in *ad libitum* fed WT LM and MC4RKO mice at 4 to 12, and 12 to 20 weeks of age.

Body Weight	**WT LM (slope)**	**MC4RKO (slope)**	**P value**
Wks 4 to 8	2.57±0.43	4.78±0.45	<0.001
Wks 8 to 16	0.83±0.15	2.29±0.15	<0.001
Wks 16 to 20	0.36±0.32	0.66±0.49	0.604
Body Length	**WT LM (slope)**	**MC4RKO (slope)**	**P value**
Wks 5 to 8	0.36±0.04	0.49±0.05	0.033
Wks 8 to 16	0.11±0.02	0.18±0.02	0.013
Wks 16 to 20	0.09±0.06	0.06±0.07	0.744

Data presented as mean±SEM. N=6-8 per group.

5.3.2 Rapid linear growth velocity in hyperphagic MC4RKO mice does not coincide with the hypersecretion of GH release

Hypersecretion of GH is thought to promote rapid pubertal linear growth in MC4R deficient individuals [68]. Given that rapid linear growth velocity in hyperphagic MC4RKO mice predominantly occurred between 5 and 8 weeks of age and between 8 and 16 weeks of age (Figure 5-1H,I), it was anticipated that hypersecretion of GH would occur at this time. Accordingly, pulsatile GH secretion was assessed in WT LM and MC4RKO mice from early puberty through adulthood. Assessed ages corresponded to periods associated with the initial onset of rapid growth velocity (4 weeks of age), sustained rapid growth velocity (8 weeks of age), and the eventual slowing of growth velocity to that seen in adult WT LM (16 weeks of age). Pulsatile GH secretory events in WT LM and MC4RKO mice occurred at all ages, and were characterized by periods of increased GH secretion flanked by periods of low basal GH release. This is in consistent with prior observations from WT

mice, demonstrating that rapid pubertal linear growth in WT LM and MC4RKO mice occurred alongside the establishment of altered GH secretion profiles throughout adulthood. For WT LM, age-associated changes in GH secretion matched secretion profiles described previously (Chapter 3, Figure 3-2). As a characteristic of GH release, the pattern of GH secretion and timing of the onset of secretory events in mice was highly variable between animals, regardless of age and genotype (Figure 5-2A (4 weeks of age), Figure 5-2D (8 weeks of age), and Figure 5-2G (16 weeks of age)).

Representative examples of pulsatile GH secretion in WT LM and MC4RKO mice are presented in Figure 5-2. While male mice exhibit pulsatile release of GH, pulsatile GH profiles varied amongst mice (Figure 5-2). GH secretion patterns and the amount of GH released in 4-week-old MC4RKO mice were comparable to that observed in WT LM (Figure 5-2A, Table 5-2). GH secretion in MC4RKO mice was markedly reduced by 8 weeks of age (Figure 5-2D, Table 5-2), regardless of sustained linear growth at this time. This reduction in pulsatile GH secretion preceded the gradual slowing in linear growth, suggesting that the absence of GH release is unlikely to sustain rapid linear growth in MC4RKO mice relative to WT LM. This was characterized by a significant reduction in total (Figure 5-2B) and pulsatile (Figure 5-2E) GH release, and the mean peak of GH release per pulse (Figure 5-2H). The suppression of GH release in MC4RKO mice at 8 and 16 weeks of age are in consistent with changes in pulsatile GH profiles observed in high fat diet fed mice showing increased adiposity during weight gain [97, 98]. Of importance, independent of alterations in the GH secretory events (Figure 5-2C,F,I), the reduction in GH release in MC4RKO was greatly exaggerated relative to WT LM. Given that GH declines with increased adiposity [12], and that MC4RKO mice show increased fat mass (Figure 5-1D to F), observations suggest that potential peripheral factors in modulating adiposity may account for this change. Moreover, the slowing in the rate of linear growth in MC4RKO mice between 16 and 20 weeks of age was comparable to that seen in WT LM, suggesting that the slowing of growth rate in these mice did not coincide with the gradual withdrawal of GH release. Observations suggest that increased linear growth rate in hyperphagic MC4RKO mice occur alongside the progressive development of GH deficiency. Comparisons of GH secretory parameters after deconvolution analysis are summarized in Table 5-2.

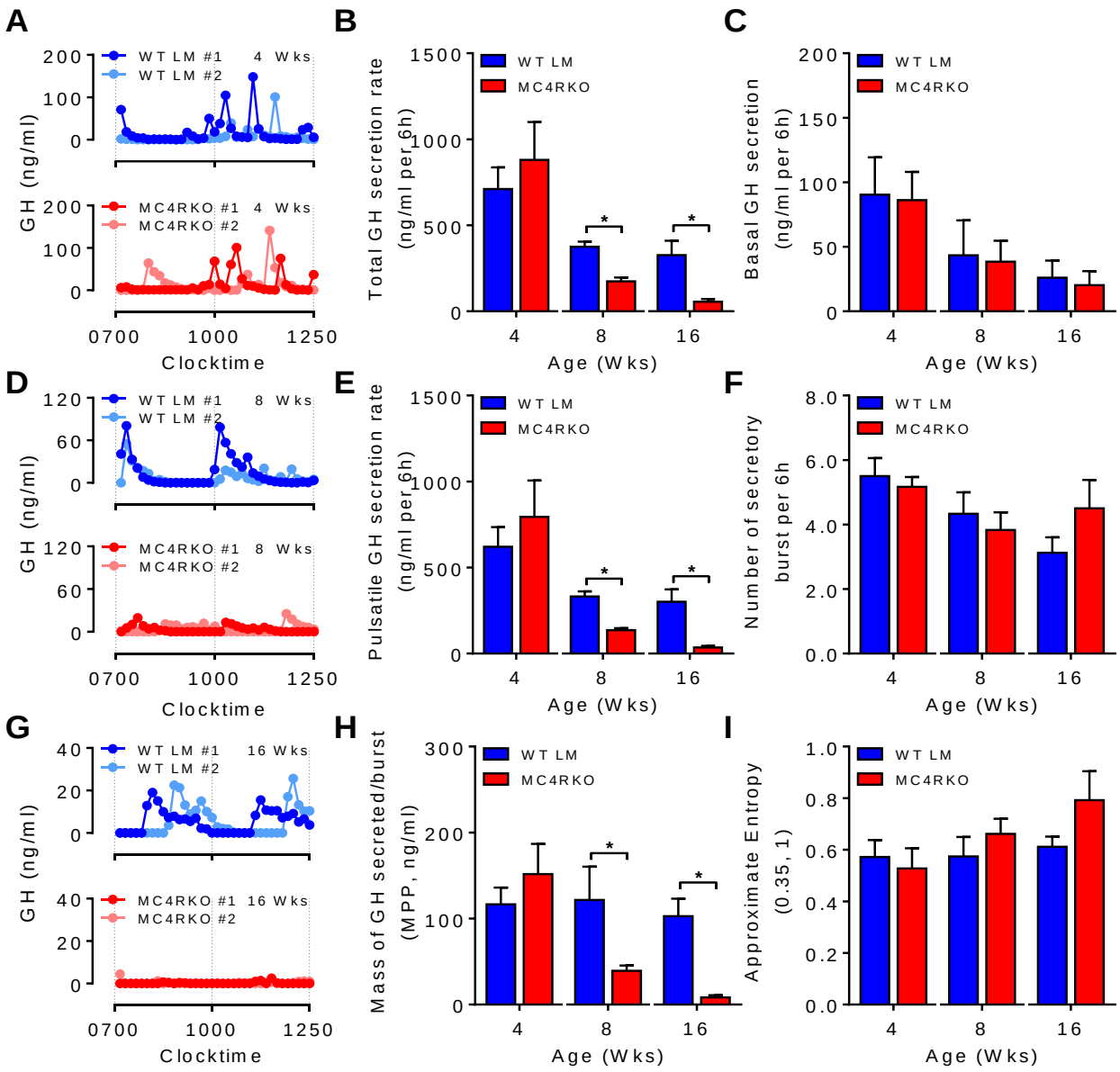

Figure 5-2: MC4RKO mice developed GH deficiency during sustained periods of rapid pubertal linear growth. The magnitude of the reduction in GH release from 8 weeks of age was greater in MC4RKO mice compared to WT LM.

Representative examples of pulsatile GH profiles in WT LM and MC4RKO mice at (A) 4, (D) 8 and (G) 16 weeks of age. Compared to 4 weeks of age, a decline in (B) total GH secretion, (E) pulsatile GH secretion and (H) mass of GH secreted per burst was dramatically exaggerated in MC4RKO mice at 8 week of age when compared to WT LM. This decline in GH release occurred prior to the slowing of rate of linear growth in MC4RKO mice. (C) Basal GH secretion, (F) GH secretory events and (I) Approximate entropy (regularity of GH release) in MC4RKO mice remained unchanged throughout periods of altered GH release compared to WT LM. Data are presented as mean ± SEM. A P value < 0.05 was accepted as significant; WT LM: blue, MC4RKO: red, N=6-8 per group. Abbreviations: Melanocortin 4 receptor knockout mice; MC4RKO mice, wild-type littermates; WT LM, growth hormone; GH.

Table 5-2: Deconvolution analysis of output values of pulsatile GH release in whole blood tail-tip samples collected from WT LM and MC4RKO mice maintained on a standard diet. Samples were collected at 10-minute intervals between 0700 h and 1300 h.

4 Wks of age	**WT LM**	**MC4RKO**	**P value**
Total GH secretion rate (ng/ml per 6h)	711±126	880±219	0.518
Pulsatile GH secretion rate (ng/ml per 6h)	621±115	794±212	0.487
Mass of GH secreted/burst (MPP, ng/ml)	117±19.5	152±35.0	0.399
Basal GH Secretion rate (ng/ml)	90.4±28.9	86.1±21.9	0.908
Number of secretory burst/6h	5.50±0.56	5.17±0.31	0.210
Approximate Entropy (0.35,1)	0.54±0.07	0.056±0.08	0.918
8 Wks of age	**WT LM**	**MC4RKO**	**P value**
Total GH secretion rate (ng/ml per 6h)	375±29.3	175±22.2	<0.001
Pulsatile GH secretion rate (ng/ml per 6h)	332±29.6	136±11.9	<0.001
Mass of GH secreted/burst (MPP, ng/ml)	84.3±12.6	39.4±5.99	0.010
Basal GH Secretion rate (ng/ml)	43.4±27.1	38.5±16.2	0.880
Number of secretory burst/6h	4.33±0.67	3.83±0.54	0.574
Approximate Entropy (0.35,1)	0.55±0.06	0.66±0.06	0.200
16 Wks of age	**WT LM**	**MC4RKO**	**P value**
Total GH secretion rate (ng/ml per 6h)	327±83.4	55.5±16.1	0.018
Pulsatile GH secretion rate (ng/ml per 6h)	300±72.6	35.3±9.34	0.009
Mass of GH secreted/burst (MPP, ng/ml)	103±20.4	8.33±2.63	0.002
Basal GH Secretion rate (ng/ml)	26.0±13.3	20.3±10.9	0.754
Number of secretory burst/6h	3.13±0.48	4.50±0.89	0.169
Approximate Entropy (0.35,1)	0.611±0.04	0.79±0.012	0.114

Data are presented as mean±SEM. A P value of < 0.05 was accepted as significant. N=6-8 per group.

5.3.3 Hypothalamic *Ghrh* or *Srif* mRNA expression is unaltered in MC4RKO mice

To investigate whether the reduction in GH secretion was due to altered hypothalamic control of GH release, hypothalamic tissue of WT LM and MC4RKO mice containing PeVN and ARC regions (Figure 5-3A) were isolated for mRNA analysis. Compared to WT LM, no discernible changes in *Ghrh* (Figure 5-3B) or *Srif* (Figure 5-3C) mRNA expression was observed within the PeVN/ARC complex of MC4RKO mice, suggesting that impaired GH secretion in MC4RKO mice may occur independent of changes in hypothalamic control of GH secretion. The lack of change in hypothalamic mRNA expression may not accurately reflect circulating GHRH or SRIF output. Consequently, assessment of *Ghrh* and *Srif* mRNA expression may not reflect hypothalamic control of GH release. While measures of GHRH and SRIF release into the portal circulation to modulate GH release has been demonstrated in rhesus monkeys and sheep [328, 340], assessment of GHRH and SRIF output in portal blood from mice do not exist. Regardless, observations confirmed that suppressed GH release in MC4RKO mice does not correspond to changes in *Ghrh* or *Srif* mRNA expression, suggesting that factors regulating GH release in MC4RKO mice may act independent of the hypothalamus.

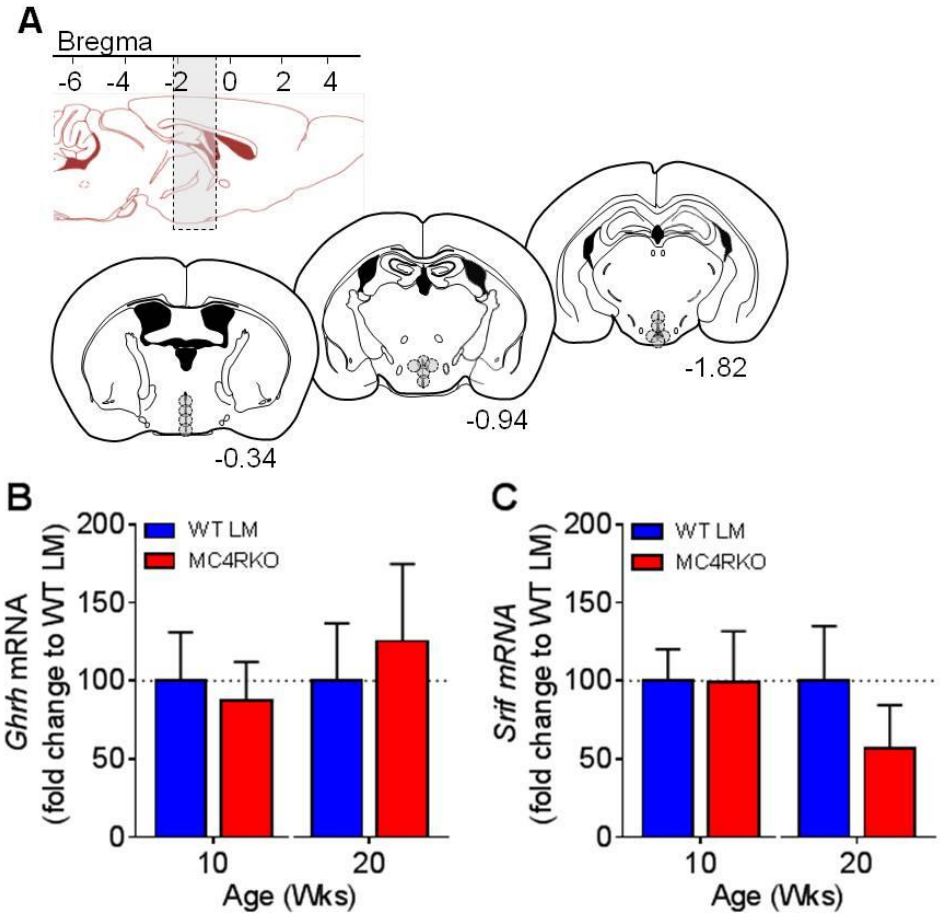

Figure 5-3: Hypothalamic *Ghrh* and *Srif* mRNA expression in PeVN/ARC complex of MC4RKO mice

(A) Hypothalamic tissues containing the PeVN, PVN and ARC were collected from 300 µm thick frozen brain sections. Micropunch biopsies were obtained from brain sections located between bregma -0.34 and -1.82 (tissue collected at representative levels corresponding to the PeVN, PVN and ARC nucleus complex outlined and shaded in grey). No differences in (B) *Ghrh* or (C) *Srif* mRNA expression were observed between WT LM and MC4RKO mice. Data are presented as mean±SEM. A P value of < 0.05 was accepted as significant. WT LM: blue, MC4RKO: red, N=6 per group. Abbreviations: Growth hormone releasing hormone; GHRH, somatostatin; SRIF, melanocortin 4 receptor knockout mice; MC4RKO mice, wild-type littermates; WT LM, periventricular nucleus; PeVN, paraventricular nucleus; PVN, arcuate nucleus; ARC.

5.3.4 Rapid pubertal linear growth velocity in MC4RKO mice does not occur in response to increased circulating or local IGF-1 hypersecretion

GH promotes linear growth indirectly via autocrine/paracrine effects of IGF-1 [129]. This is largely dependent on pubertal associated changes in GH secretion [1, 11]. Moreover, IGF-1 is a major determinant of postnatal growth [185-188]. Thus, increased bio-available and tissue-specific IGF-1 expression in the presence of GH deficiency suggests that IGF-1 may promote growth in MC4RKO mice independent of GH. To determine whether rapid linear growth in MC4RKO mice coincide with a parallel rise in IGF-1, total circulating IGF-1, IGF-1/IGFBP3 molar ratio (indicator of free IGF-1 bioavailability), and IGF-1 binding protein (IGFBPs) were determined at 4, 10 and 20 weeks of age.

Circulating IGF-1 (Figure 5-4A) levels did not rise alongside rapid linear growth in MC4RKO mice relative to WT LM (Figure 5-4D). IGF-1/IGFBP3 molar ratio (Figure 5-4B) is elevated at 10 weeks of age however, this did not reach statistical significance relative to WT LM (Figure 5-4E). Regardless, IGFBP3, the major carrier for IGF-1, was reduced in MC4RKO mice (Figure 5-4C) when compared to that of WT LM (Figure 5-4F). Given that the release of IGFBP3 is GH-dependent [341], the gradual decline in circulating IGFBP3 levels occurred, presumably due to the loss of GH release in MC4RKO mice, although this did not reach statistical difference when compared to WT LM. Circulating IGFBP1 (Figure 5-4G,J) and IGFBP2 levels (Figure 5-4H,K) declined throughout rapid linear growth in MC4RKO mice relative to WT LM. Changes in the abundance of circulating IGFBP1 and IGFBP2 in MC4RKO mice are usually reflective of their underlying insulin resistance in response to their concomitant hyperinsulinemia (Figure 5-5A,B). This is in accordance with that seen in humans following extended periods of energy consumption [14, 192, 342]. Consequently, it was anticipated that rapid pubertal linear growth in MC4RKO mice may be mediated through autocrine/paracrine effects of local IGF-1. Accordingly, a progressive loss of muscle (Figure 5-4I) and liver (Figure 5-4L) specific IGF-1 expression was observed in MC4RKO mice relative to WT LM. Observations are congruent with the

progressive development of GH deficiency in MC4RKO mice (Figure 5-4, Table 5-2). Technical limitations prevented the assessment of bone IGF-1 levels, and thus it remains unknown whether changes in bone IGF-I content contribute to altered linear growth in MC4RKO mice. This warrants further investigation. Collectively, observations demonstrate sustained rapid linear growth in MC4RKO mice does not occur in response to increased GH/IGF-1 hypersecretion.

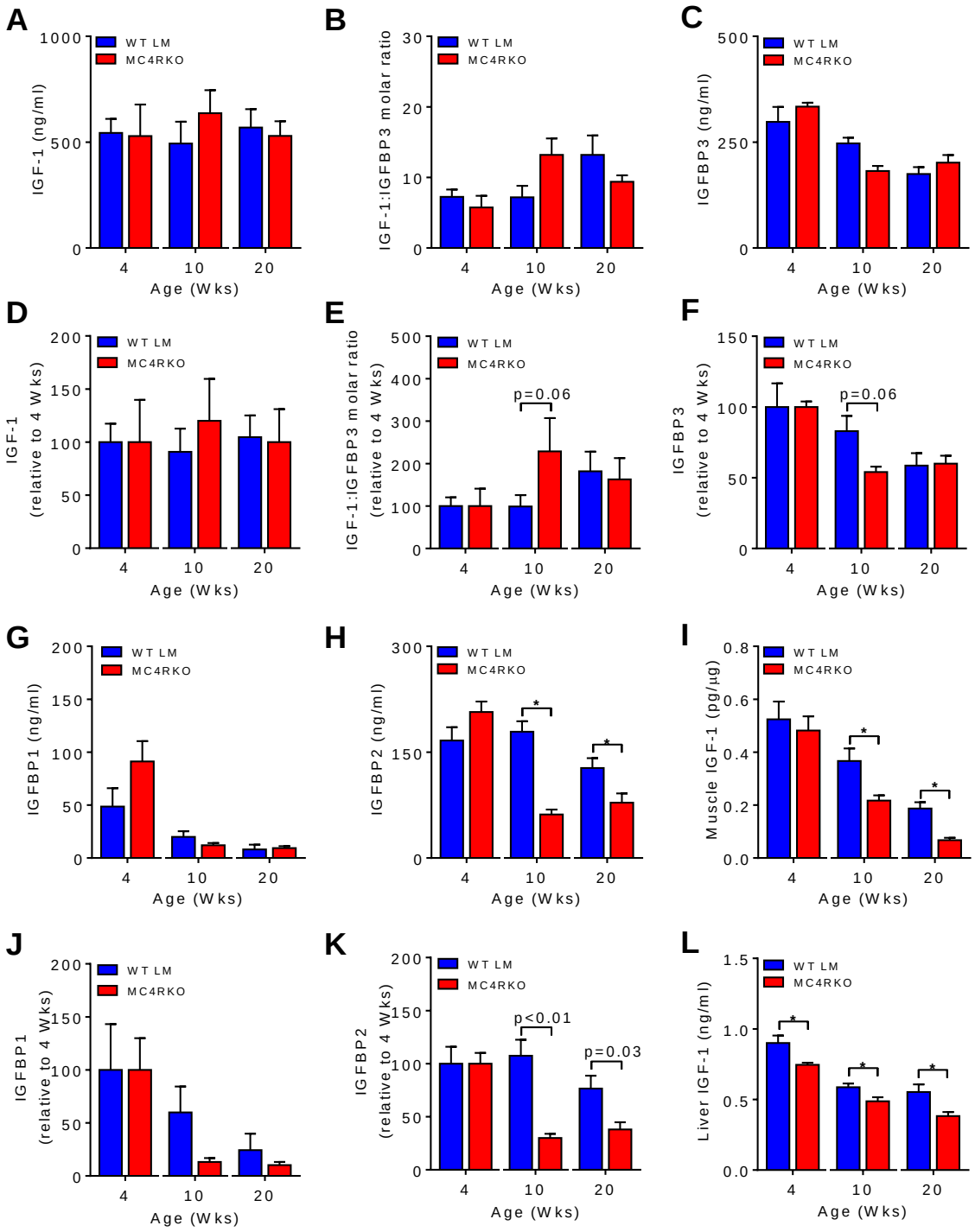

Figure 5-4: Rapid pubertal growth in MC4RKO mice does not occur as a consequence of increased circulating or local IGF-1 levels.

(A) Circulating levels of IGF-1, (B) molar ratio of IGF-1:IGFBP3 and (C) IGFBP3 in MC4RKO mice at 4, 10 and 20 weeks of age. (D) Circulating IGF-1, (E) molar ratio of IGF-1:IGFBP3 and (F) IGFBP3 in MC4RKO mice relative to WT LM. (G) Circulating levels of IGFBP1, (H) IGFBP2 and (I) muscle-specific IGF-1 in MC4RKO mice at 4, 10 and 20 weeks of age. (J) Circulating IGFBP1 and (K) IGFBP2 in MC4RKO mice relative to WT LM. (L) Liver-specific IGF-1 in MC4RKO mice at 4, 10 and 20 weeks of age. Data are presented as mean ± SEM. A P value < 0.05 was accepted as significant; WT LM: blue, MC4RKO: red, N=5-8 per group. Abbreviations: Melanocortin 4 receptor knockout mice; MC4RKO mice, wild-type littermates; WT LM, insulin-like growth factor-1; IGF-1, IGF binding proteins; IGFBPs.

5.3.5 Hyperphagic MC4RKO mice develop hyperinsulinemia and insulin resistance while sustaining plasma NEFAs and glucose homeostasis

Recent observations demonstrate that the progressive rise in insulin levels relative to an adipose-specific decline in pulsatile GH secretion [98]. In this regard, reduced GH secretion relative to weight gain is thought to aid insulin action, thereby sustaining fatty acid flux during periods of excess energy consumption. As in obesity, hyperphagia is associated with increased insulin secretion and decreased GH secretion. This is preceded by the gain in body weight [14]. Disruption of this GH/insulin balance by pharmacological GH replacement to prevent the loss of GH release results in impaired insulin signaling and impaired fatty acid and glucose homeostasis [71]. Given similarities between obesity and hyperphagia, it is anticipated that the progressive loss of GH release in MC4RKO mice may occur alongside the hypersecretion of insulin (hyperinsulinemia), and that this is essential for sustained maintenance of fatty acid flux. Assuming this is true, it is anticipated that metabolic alterations in response to hyperphagia will result in increased insulin and decreased GH release, and that this would serve as a potential mechanism to override the traditional role of GH in promoting rapid linear growth.

To validate the anticipated role of GH/insulin balance in sustaining fatty acid flux, alterations in insulin secretion and insulin sensitivity alongside the progressive development of hyperphagia was assessed. Observations demonstrate a striking elevation in fed and fasting insulin in MC4RKO mice, with circulating levels of insulin rising between 6 to 20 weeks of age (Figure 5-5A,B). By comparison, glucose tolerance in MC4RKO mice was maintained at 6 and 16 weeks of age (Figure 5-5C,D). Sustained glucose tolerance in MC4RKO mice was facilitated by a greater response to glucose-stimulated insulin secretion (GSIS) (Figure 5-5C,D). In addition, it is likely that hyperinsulinemia (Figure 5-5A) would have sustained glucose tolerance by promoting the corresponding storage of muscle and hepatic glycogen (Figure 5-6E,F). MC4RKO mice developed insulin resistance by 16 weeks of age, coinciding with a slowing in the rate of linear growth velocity (Figure

5-5E,F, 16 weeks of age). To further explore mechanisms underlying sustained glucose homeostasis in MC4RKO mice, circulating insulin levels were measured following acute glucose challenge (GSIS). In response to glucose administration, the first-phase of peak insulin secretion is rapid, occurring between 0 and 15 mins; second-phase release of insulin gradually slows from 15 and 60 mins [343]. MC4RKO mice exhibited significantly higher insulin levels during the first phase of insulin secretion at 15 mins, and during the second phase of insulin secretion at 30 and 45 mins (Figure 5-6A). Therefore, enhanced insulin release in response to glucose challenge appears to contribute to enhanced glucose clearance in MC4RKO mice. Given the proposed role of GH and insulin in maintaining fatty acid flux, circulating levels of NEFAs and storage of liver triglycerides (TG) were assessed. MC4RKO mice maintained circulating NEFAs and glucose within a similar range as seen in WT LM (Figure 5-6B,C). Presumably, this was in response to increased lipid clearance, as demonstrated by the progressive accumulation of hepatic TG content (Figure 5-6D) by 10 weeks of age. Amongst these metabolic alterations in response to hyperphagia, the rise in insulin is the most dramatic and significant. Given the direct association between hyperphagia and rapid linear growth, observations were extended to assess insulin co-secreted peptides following the development of hyperphagia. Circulating GLP-1 is secreted in response to food ingestion and may increase insulin sensitivity [344]. Circulating C-peptide, peptide YY (PYY) and amylin are pancreatic peptides co-secreted with insulin. Circulating GLP-1 (representative of active GLP-1) (Figure 5-7A) and PYY (Figure 5-7C) did not differ between WT LM and MC4RKO mice throughout hyperphagia. Circulating C-peptide in MC4RKO mice did not reach statistical significance following the development of insulin resistance at 20 weeks of age when compared to WT LM (Figure 5-7B). Amylin levels rise alongside insulin (Figure 5-7D). Of interest, amylin promotes linear growth in adult mice [345], and this may act to sustain rapid linear growth in MC4RKO mice in the absence of GH. Collectively, these observations suggest that alterations in GH/insulin balance in MC4RKO mice likely occur in response to hyperphagia, and may facilitate the continued blood glucose clearance, the clearance and storage of NEFAs, and possibly the promotion of rapid linear growth velocity in the absence of GH.

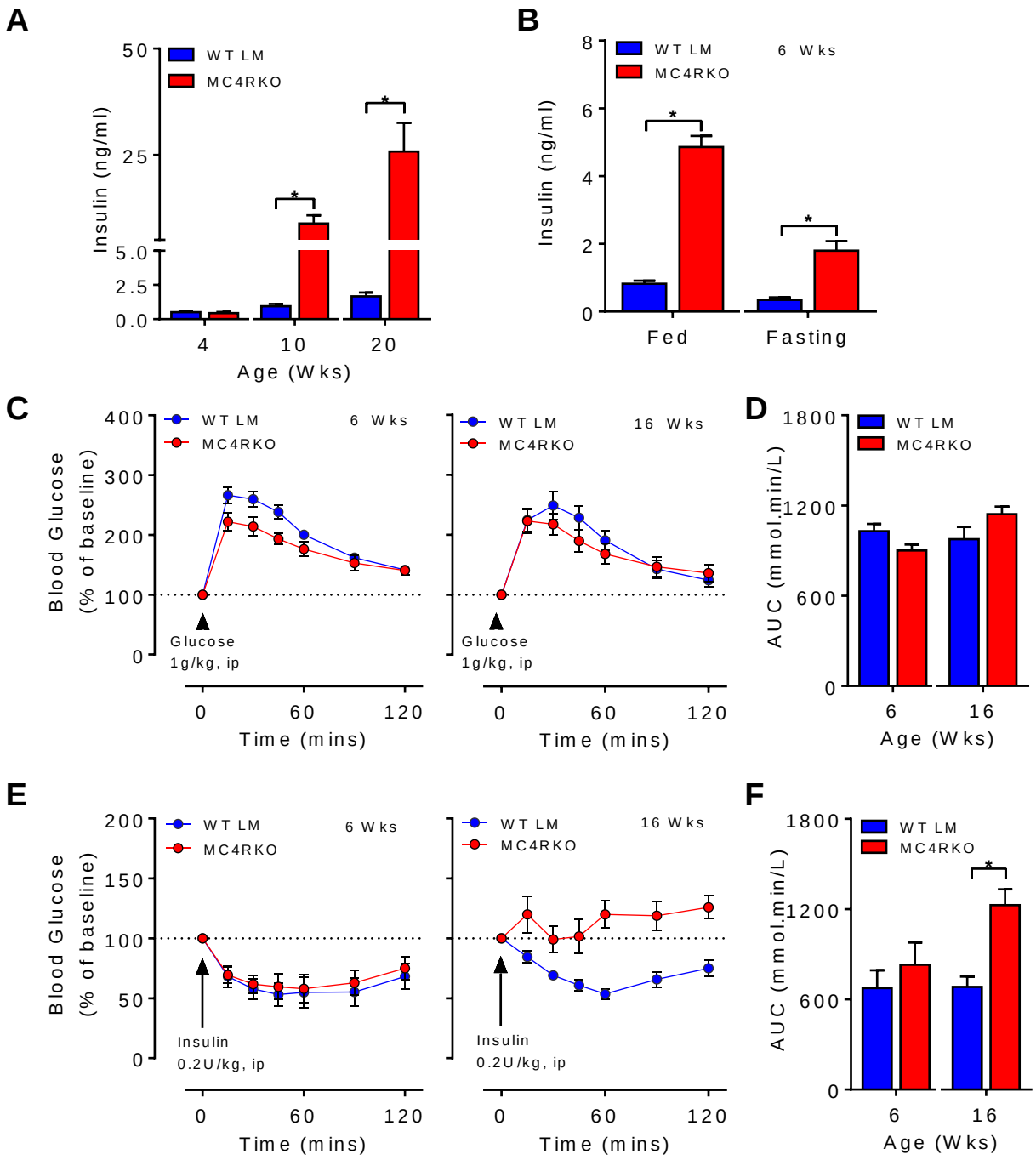

Figure 5-5: Hyperphagic MC4RKO mice developed hyperinsulinemia and insulin resistance, however, glucose clearance is sustained.

(A) Circulating measures of insulin in WT LM and MC4RKO mice at 4, 10 and 20 weeks of age. (B) Fed and fasting insulin levels in *ad libitum* MC4RKO mice were elevated by 6 weeks of age. (C) MC4RKO mice remained glucose responsive at 6 and 16 weeks of age. (D) Area under curve for GTT at 6 and 16 weeks of age. (E) Insulin resistance in MC4RKO mice did not develop until 16 weeks of age. (F) Area under curve for ITT at 6 and 16 weeks of age. Data are presented as mean ± SEM. A P value < 0.05 was accepted as significant; WT LM: blue, MC4RKO: red, N=6-8 per group. Abbreviations: Melanocortin 4 receptor knockout; MC4RKO, wild-type littermates; WT LM, area under curve; AUC.

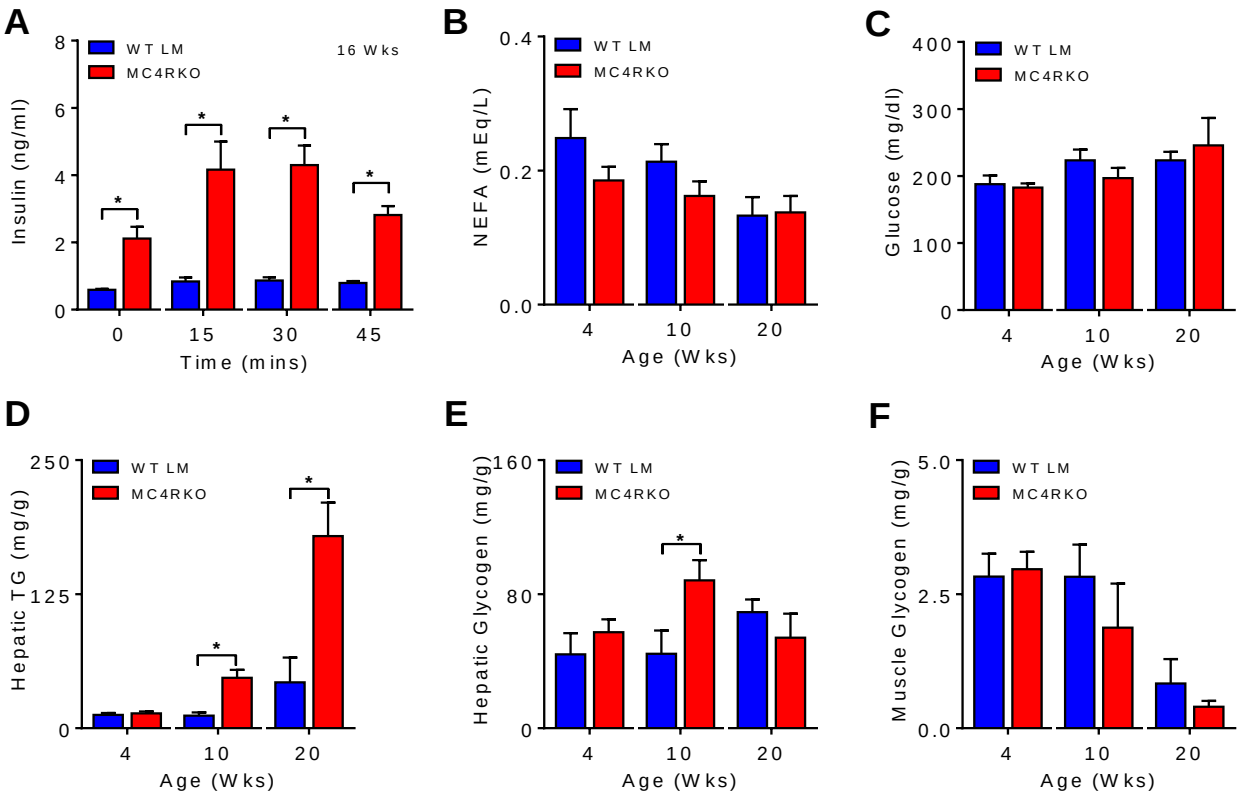

Figure 5-6: Altered GH release and hyperinsulinemic MC4RKO mice occurred alongside sustained glucose and NEFA flux.

(A) *In vivo* insulin response during GTT in MC4RKO mice compared to wild-type littermates (WT LM). (B) Circulating NEFAs, (C) glucose, (D) hepatic TG, (E) muscle glycogen and (F) hepatic glycogen in WT LM and MC4RKO mice at 4, 10 and 20 weeks of age. Data are presented as mean ± SEM. A P value < 0.05 was accepted as significant; WT LM: blue, MC4RKO: red, N=6-8 per group. Abbreviations: Growth hormone, GH, melanocortin 4 receptor knockout; MC4RKO, wild-type littermates; WT LM, glucose tolerance test; GTT, non-esterified free fatty acids; NEFA, triglycerides; TG, area under curve; AUC.

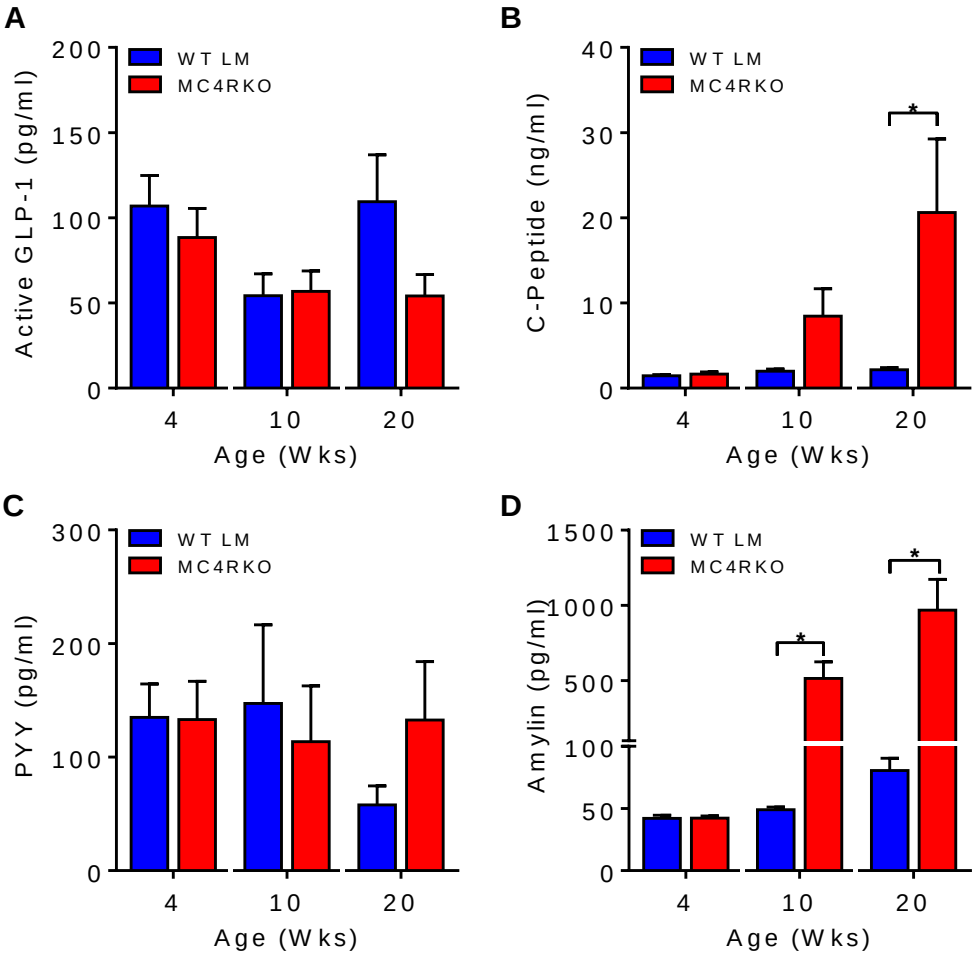

Figure 5-7: Assessment of circulating GLP, C-peptide, PYY and amylin in response to hyperphagia in MC4RKO mice.

Circulating levels of (A) GLP-1 and (C) PYY did not differ between genotypes across all ages assessed. (B) Circulating C-peptide MC4RKO mice was elevated by 20 weeks. (D) Amylin, co-secreted alongside insulin was upregulated in MC4RKO mice and may serve to promote growth. Data are presented as mean ± SEM. A P value < 0.05 was accepted as significant; WT LM: blue, MC4RKO: red, N=6-8 per group. Abbreviations: Glucagon-like peptide; GLP, peptide YY; PYY, melanocortin 4 receptor knockout; MC4RKO, wild-type littermates; WT LM.

5.3.6 The MC4R is not colocalized with somatotrophs or GHRH neurons

To validate the observed changes in altered GH release in MC4RKO mice occur as a consequence of hyperphagia rather than a direct effect of the loss of MC4R signaling, colocalization of MC4R with somatotrophs and GHRH neurons was performed by immunofluorescence. Gene expression of MC4R was examined in the hypothalamus, anterior pituitary gland, liver tissue of WT mice, and somatotrophs isolated from pituitary glands of transgenic mice expressing GH-GFP (Figure 5-8A,B). MC4Rs are abundantly expressed in the hypothalamus of WT mice (Figure 5-8A). Liver do not express MC4Rs

and was used as a negative tissue control. RT-PCR results confirmed that MC4Rs are expressed in the anterior pituitary gland but not on somatotrophs (Figure 5-8B). Accordingly, the hypothalamic-pituitary-thyroid (HPT) axis is involved in the regulation of energy homeostasis [346], and thyroid dysfunction is thought to have debilitating consequences on appetite and body weight [347, 348]. Thus, while not directly addressed, it is possible that the anterior pituitary thyrotrophs [73] express MC4R. To determine if MC4Rs regulate GH secretion via direct interaction with the somatotrophs, the colocalization of MC4Rs with GH-secreting somatotrophs were examined. Immunofluorescence results confirmed that MC4Rs are not expressed on somatotrophs (Figure 5-8C to E), confirming that the melanocortin system does not directly modulate GH release from somatotrophs through the MC4R.

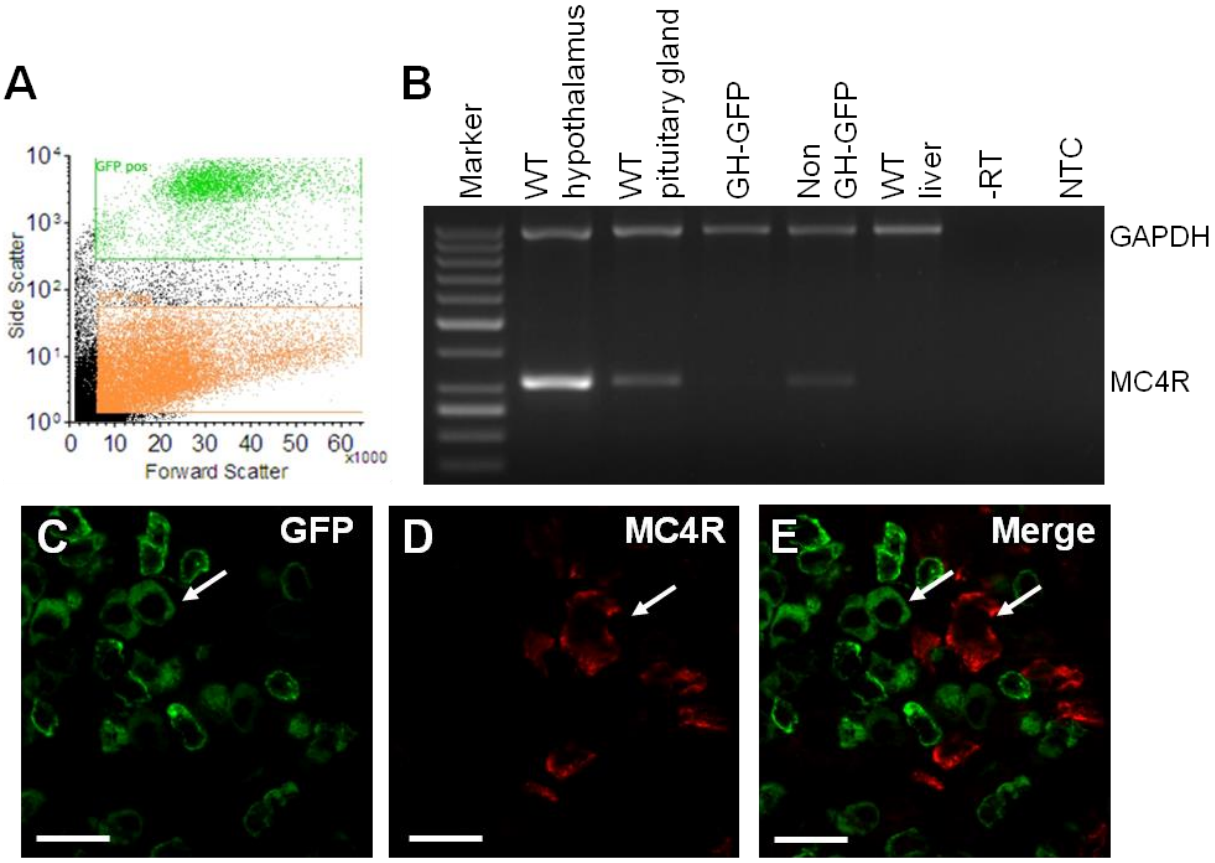

Figure 5-8: Expression of *MC4R* in hypothalamus and isolated somatotrophs of the anterior pituitary gland of WT mice, and MC4R immunoreactivity in the pituitary gland of mice at 8 weeks of age.

(A) FACS of somatotrophs isolated from pituitary glands of transgenic mice expressing GH-GFP. (B) Expression of MC4Rs in WT hypothalamus, anterior pituitary gland and somatotrophs isolated using FACS in GH-GFP mice at 8 weeks of age. Immunofluorescence of MC4R expression in the pituitary gland of transgenic mice expressing GFP in somatotrophs (C-E). MC4Rs (red) are not expressed on somatotrophs (green, E). Scale bar = 100 μm. Representative images illustrate interactions verified across 4 animals. Abbreviations: Melanocortin 4 receptor; MC4R, wild-type; WT,

fluorescence-activated cell sorting; FACS, growth hormone; GH, green fluorescent protein; GFP.

The colocalization of MC4Rs with GHRH neurons was examined to determine if MC4Rs regulate GH secretion via direct interactions with GHRH. Immunofluorescence observations confirmed that the widespread distribution of MC4Rs within the CNS of the mouse was similar relative to rodent brains [35, 349]. An aggregation of positive MC4R immunoreactivity (identified by neuronal cell bodies) was evident in the cortex (Figure 5-9C), hippocampus (Figure 5-9D), and thalamus (Figure 5-9E), PVN (Figure 5-9F) and the DMH (Figure 5-9G). Although at seemingly lower levels, the ARC demonstrate punctuate MC4R immunoreactivity (Figure 5-9H). Observations are in consistent with previous studies characterizing MC4R expression in rodents [35]. While GHRH neurons lies along the ventral hypothalamus (Figure 5-9I), immunofluorescence assessment demonstrated that MC4Rs do not colocalized with GHRH neurons (Figure 5-9K), confirming that the melanocortin system does not directly act via MC4Rs to modulate GHRH-induced GH secretion. Thus, the observed reduction in GH release in hyperphagic MC4RKO mice suggest that altered GH release likely occur in response to metabolic alterations as a consequence of hyperphagia.

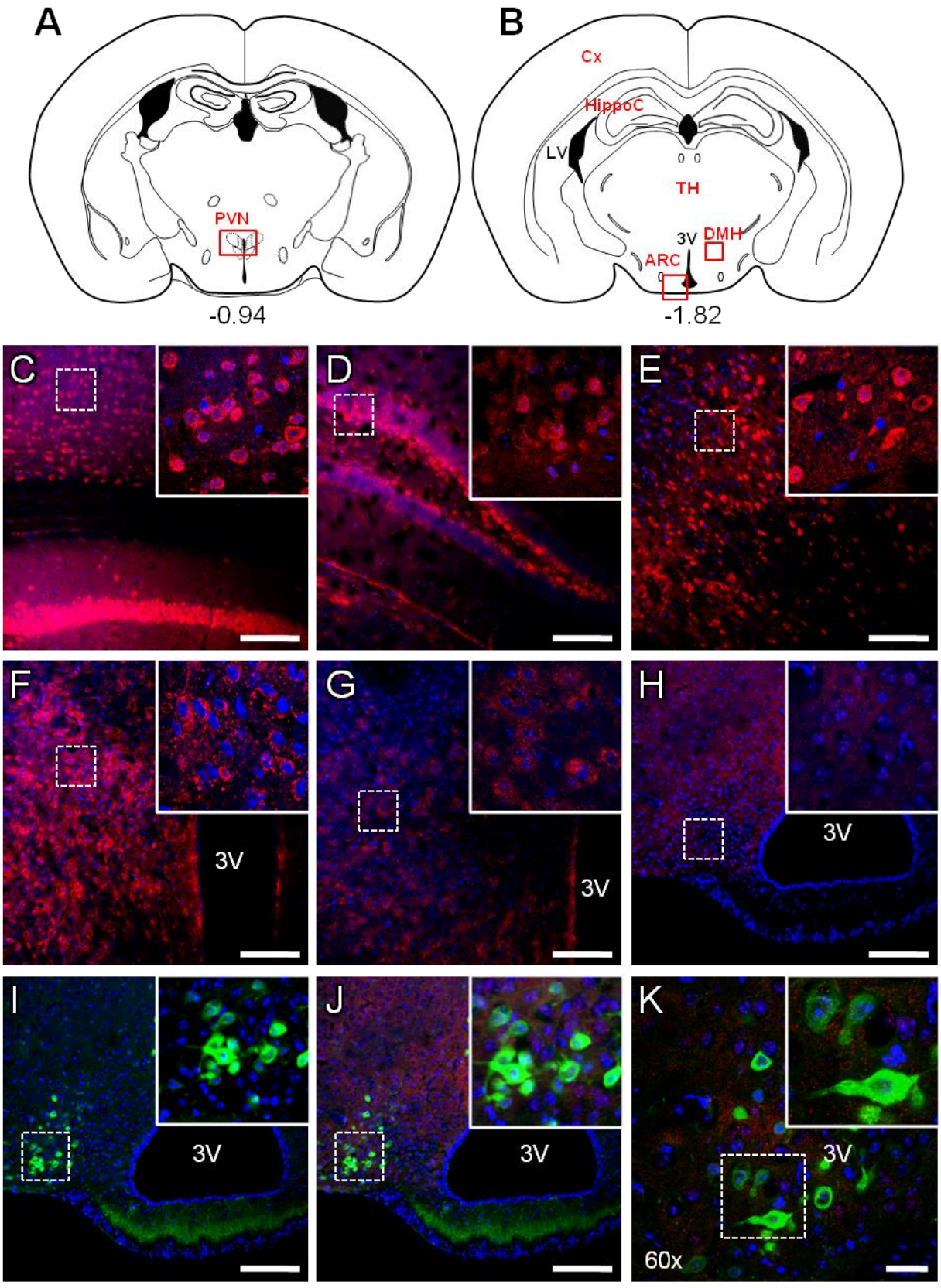

Figure 5-9: Expression of MC4R in 8-week-old transgenic mice expressing GFP in GHRH neurons.

(A-B) Schematic coronal diagrams of mouse brain mapping MC4R immunoreactivity at bregma between -0.94 and -1.82. MC4R immunoreactivity was detected in the (C) Cx, (D) HippoC, (E), Thal, (F) PVN, (G) DMH and the (H) ARC. Immunofluorescence assessment

of GHRH-GFP (green, I) and MC4R (red, J) confirmed that MC4R are not expressed on GHRH neurons (merge, K). (C-J) Scale bar = 25 μm. (K) Scale bar = 50 μm. Inserts illustrate a magnified view of the figures (Scale bar = 100 μm). Representative images illustrate interactions verified across 5 animals. Abbreviations: Melanocortin 4 receptor; MC4R, green fluorescent protein; GFP, growth hormone releasing hormone; GHRH, cortex; Cx, hippocampus; HippoC, thalamus; Thal, paraventricular nucleus; PVN, dorsal medial hypothalamus; DMH, arcuate nucleus; ARC, lateral ventricle; LV, third ventricle; 3V.

5.3.7 Hyperphagic MC4RKO mice show a reduction in muscle-specific InsR alongside the development of insulin resistance

Current data demonstrate a decline in GH release occurring alongside hyperinsulinemia. Moreover, observations confirmed that the suppression of GH release occurred independent of direct MC4R-mediated interactions. While this may underlie sustained fatty acid flux following extended periods of hyperphagia, it remains unknown how rapid linear growth in MC4RKO mice is sustained in the absence of GH. Importantly, insulin and IGF-1 share a number of major structural homologies [350], and insulin can bind to the IGF-1R although at a much lower affinity [351]. Accordingly, hypersecretion of insulin may facilitate rapid growth in MC4RKO mice, acting through the InsR or IGF-1R. To address this, muscle-specific InsR and IGF-1R expression were assessed in WT LM and MC4RKO mice. For insulin and IGF-1, assessment was limited to the expression of intracellular β-subunit as this is representative of the capacity to facilitate downstream signaling [352]. Compared to WT LM, data demonstrate a progressive loss of InsRβ expression, reaching significance by 20 weeks of age (Figure 5-10A,B). In contrast, IGF-1Rβ expression in MC4RKO mice did not change relative to WT LM, regardless of age (Figure 5-10C,D). Observations suggest that altered InsR expression may contribute to the development of systemic insulin resistance observed by 16 weeks of age (Figure 5-5E,F). Moreover, sustained expression of IGF-1R expression throughout hyperphagia may contribute to sustained linear growth velocity in hyperphagic MC4RKO mice in the absence of GH. Thus, given the association between hyperphagia and rapid linear growth, sustained hyperphagia-induced hyperinsulinemia may contribute to altered GH release in MC4RKO mice, and this occurs independent of MC4R-mediated actions. Second to this, sustained hyperphagia-induced hyperinsulinemia may promote for rapid linear growth velocity in the absence of GH in MC4RKO mice. This will be addressed in Chapter 6.

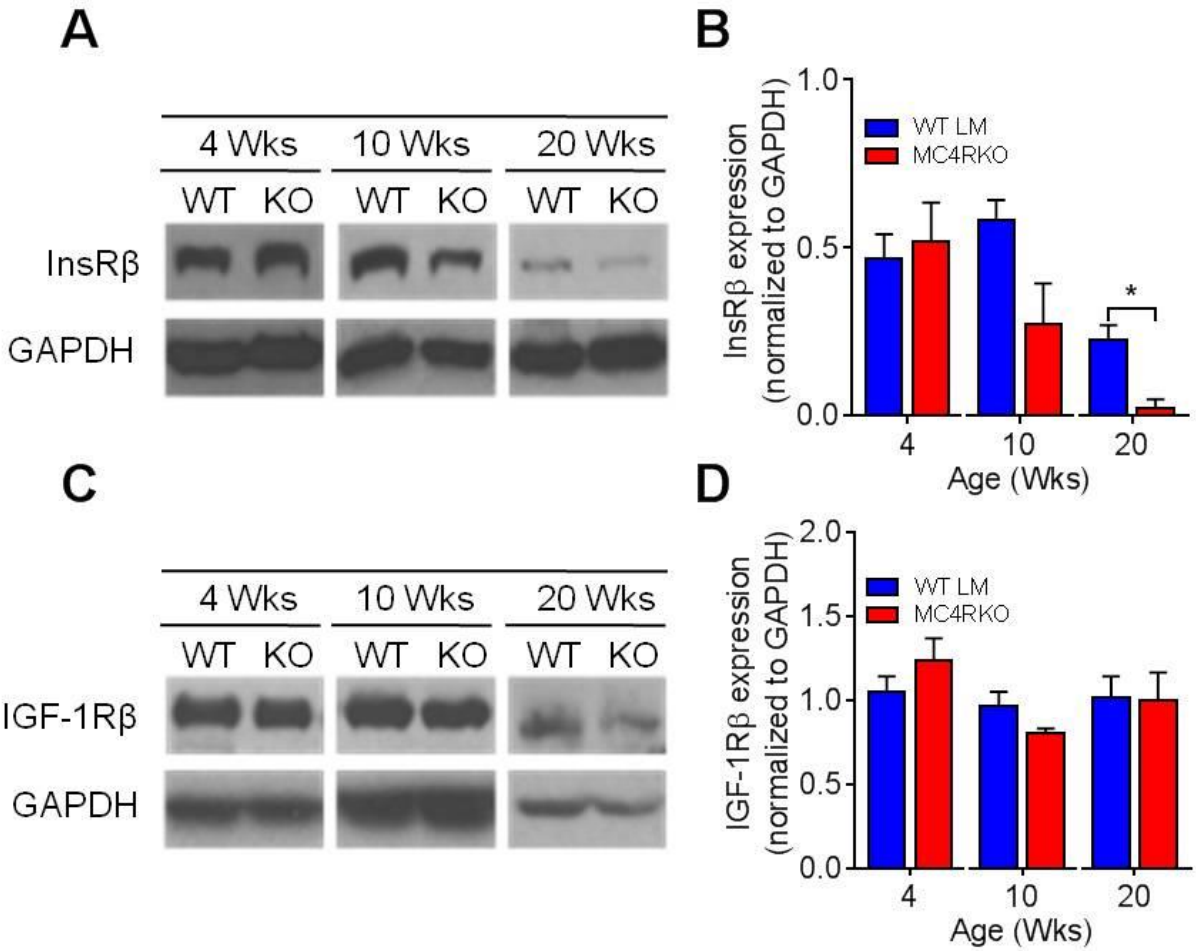

Figure 5-10: Hyperphagic MC4RKO mice show a progressive loss in muscle-specific InsRβ expression while maintaining stable IGF-1Rβ expression.

(A-B) A progressive loss of muscle InsRβ expression was observed in MC4RKO mice by 20 weeks of age. (C-D) The expression of muscle IGF-1Rβ in MC4RKO mice remained stable throughout rapid pubertal growth. Data are presented as mean ± SEM. A P value < 0.05 was accepted as significant; WT LM: blue, MC4RKO: red, N=4 per group. Abbreviations: Melanocortin 4 receptor knockout; MC4RKO, insulin-receptor beta; InsRβ, insulin-like growth factor-1 receptor beta; IGF-1Rβ, wild-type littermates; WT LM.

5.4 Discussion

Increased pulsatile GH secretion is observed in obese adults with defective MC4R signaling when compared to BMI-matched obese humans [68]. Moreover, defects in MC4R signaling in humans [68] and rodents [58] are associated with increased adult linear height/body length. While not directly addressed, these observations suggest that increased pubertal GH secretion following the loss of MC4R signaling may contribute to increased pubertal linear growth. To investigate whether increased pulsatile GH secretion contribute to rapid pubertal growth associated with MC4R dysfunction, pulsatile GH secretion was determined in MC4RKO mice throughout the period of increased pubertal linear growth.

As demonstrated previously [58], deletion of MC4R in mice is associated with early onset hyperphagia resulting in rapid weight gain, increased adiposity, and rapid linear growth. Current data confirms that the rate of linear growth in MC4RKO mice between 5 and 8 weeks of age, and between 8 and 16 weeks of age was significantly greater to that observed in WT LM, confirming that rapid linear growth in MC4RKO mice occurs predominantly during pubertal maturation leading into early adulthood. Given this, it was anticipated that endogenous GH levels would increase prior to 5 weeks of age to facilitate rapid pubertal linear growth. Unexpectedly, the anticipated increase in GH secretion was not observed at this time. Observations demonstrated that MC4RKO mice developed GH deficiency by 8 weeks of age during peak periods of sustained linear growth. Of importance, current observations do not support the original perception of increased GH secretion contributing to accelerated linear growth in MC4R deficient adults. Rather, sustained rapid linear growth in MC4RKO mice occurs regardless of the loss of GH release. GH stimulates the release of IGF-1 [353-355], and thus the loss of pulsatile GH secretion may impact circulating measures of IGF-1. However, the progressive loss of GH release in MC4RKO mice did not coincide with an elevation in circulating or local IGF-1. Rather, circulating IGF-1 levels remained unchanged in hyperphagic MC4RKO mice, suggesting that factors other than GH may sustain circulating measures of IGF-1. This may occur through increased insulin-mediated hepatic GHR to sustain plasma IGF-1 production in MC4RKO mice [356]. Although considerable evidence confirms that GH/IGF-1 mediates linear growth [1, 111], current data do not support the hypothesis that increased linear growth rate in MC4RKO mice occur as a direct consequence of increased GH/IGF-1 release. Rather, rapid linear growth in MC4RKO mice occurred in the absence of GH/IGF-1 hypersecretion.

Assessment of GH release in pubertal WT mice demonstrates a progressive decline in GH secretion preceding the slowing of linear growth rate (Chapter 3, Figure 3-1). The gradual decline in GH secretion is observed in both the WT LM and MC4RKO mice, confirming the change in pulsatile GH secretory dynamics throughout adulthood. Of interest, the anticipated rise in GH release was not observed alongside rapid linear growth rate in MC4RKO mice. Rather, the progressive reduction in GH release relative to age occurred much earlier in MC4RKO mice compared to that seen in WT LM (Figure 5-2) and WT mice (Chapter 3, Figure 3-2). Based on the premise that pulsatile GH secretion was restored in MC4R deficient adults relative to obese controls [68], it is proposed that the suppression of pulsatile GH secretion in obesity may partially be mediated through the melanocortin system (via activation of MC4R) to inhibit GH release [68]. Prior *in vivo* studies demonstrated that central infusion of MC4R non selective agonist melanotan II (MT-II) or antagonist SHU9119 did not impact hypothalamic *Ghrh* mRNA expression, or circulating GH and insulin-like growth factor-1 (IGF-1) in rats, suggesting that antagonism of the central melanocortin system has no effect on the somatotrophic axis [20, 21]. In addition, acute administration of melanocortin receptor antagonists HS014 or AgRP failed to alter the levels of spontaneous GH secretion in rats [22]. Current observations demonstrate that MC4Rs are neither expressed on somatotrophs (Figure 5-8) nor on GHRH neurons (Figure 5-9), confirming that MC4R does not directly modulate pulsatile GH secretion from the somatotroph, or indirectly via GHRH-induced GH release. To this extent, observations are in line with findings that exclude the MC4R as an intermediate in regulating GH release [20-22], thus providing compelling evidence to further support the notion that the melanocortin system (acting via MC4Rs) does not directly modulate the release of GH. While it remains unknown whether SRIF neurons express the MC4R, current observations confirmed that the decline in GH release did not occur in response to altered *Srif* mRNA expression or changes in *Srif* mRNA distribution throughout the PeVN/ARC complex (Chapter 4). Moreover, the decline in GH secretion in MC4RKO mice occur independent of changes in the hypothalamic control of GH release (Figure 5-3C). Thus, it appears unlikely that the MC4R impacts SRIF neurons to inhibit GH secretion. Collectively, data suggests that altered GH release in MC4RKO mice does not occur as a direct consequence of MC4R dysfunction. Rather, the altered endocrine profile associated hyperphagia-induced weight gain may exacerbate the suppression of GH release in MC4RKO mice. Given current observations, it is likely that the rapid decline in GH secretion in MC4RKO mice occurs secondary to hyperphagia-associated metabolic alterations.

Observations from humans show that sustained hyperphagia contributes to decreased GH release and increased insulin secretion [14, 71]. The reduction in pulsatile GH secretion that occurs alongside an elevation in insulin secretion during periods of sustained hyperphagia precedes observable changes in weight gain. Moreover, treatment with GH to prevent the naturally occurring loss of pulsatile GH release in hyperphagic individuals results in impaired insulin action, and disrupted fatty acid and glucose homeostasis [71]. In agreement with findings in humans, glucose tolerance remained normal in MC4RKO mice, regardless of the developed insulin resistance by 16 weeks of age. Moreover, circulating NEFA concentrations in MC4RKO mice was maintained within the same range as seen in WT LM, presumably as a consequence of sustained lipid uptake in adipose (accounting for the rapid accumulation of fat mass) and within the liver (accounting for the accumulation of hepatic triglycerides). Given this, it is likely that the suppression of GH release in MC4RKO mice is an essential physiological adaption that promotes sustained fatty acid and glucose flux in circulation in response to sustained hyperphagia. Presumably, the observed decline in GH release accommodates the rise in insulin to facilitate the continued clearance and storage of dietary substrates in hyperphagic mice. Given the interaction between GH and insulin in sustaining meal tolerance, increased insulin secretion may contribute to the suppression of GH secretion following hyperphagia-induced weight gain and in obesity. Accordingly, insulin is proposed to act directly at the pituitary gland via the InsR to suppress GH release [170, 280-282]. These effects persist despite systemic insulin resistance [170], suggesting that somatotrophs responsivity to insulin is preserved. In this context, it is likely that the progressive suppression of GH release may occur as a consequence of the progressive rise in circulating insulin in response to hyperphagia.

Classically, in obesity, the onset of hyperphagia-induced hyperglycemia is thought to trigger both β-cell dysfunction and insulin resistance, and the interplay between β-cell dysfunction and the development of insulin resistance is highly complex [273]. Accordingly, hyperphagia induces hyperglycaemia in MC4RKO mice [58], and consequently the demand for insulin increases. In this instance, it was predicted that β-cell mass expansion may occur in the face of insulin resistance (following the development of hyperinsulinemia). Thus, while not directly addressed, the compensatory hypersecretion of insulin (demonstrated by GSIS, Figure 5-6A) would suggest an expansion in β-cell mass, and possibly an alteration in profiles of key enzymes in mediating β-cell glucose metabolism. Accordingly, observations in hyperphagic MC4RKO mice confirmed that normoglycemia was achieved at 16 weeks of age (presumably due to enhanced insulin secretion from β-

cell), suggesting that β-cell function and insulin signaling in β-cells and/or glucose-sensing tissues (in liver and muscle) in maintaining glucose homeostasis may be preserved at this time. Observations are in consistent with normal weight patients following short-term excess dietary consumption, demonstrating that impaired pulsatile GH release occurs alongside sustained circulating glucose levels and elevated insulin secretion. This occurred prior to development of insulin resistance [14, 71]. Thus, while definitive mechanisms of hyperinsulinemia in suppressing GH release are limited, observations support the inverse relationship between insulin and GH (further discussed in Chapter 7). It should be noted that current observations in hyperphagic MC4RKO mice are limited to 16 weeks of age. Furthermore, due to technical limitations, β-cell physiology was not addressed in this study. Thus, the potential mechanisms underlying hyperphagia-induced hyperinsulinemia in sustaining physiological glucose homeostasis in hyperphagic MC4RKO mice warrants further investigation.

Insulin and IGF-1 act through converging signaling pathways [357]. Thus, it is likely that hyperphagia-induced hyperinsulinemia may directly enhance rapid linear growth by acting through IGF-1R [339, 358]. Of interest, the slowing in the rate of linear growth velocity in MC4RKO mice coincided with the development of systemic insulin resistance alongside the reduction in muscle-specific InsR. Conversely, muscle-specific IGF-1R expression was preserved throughout periods of rapid linear growth. In addition, insulin modulates hepatic production of IGFBPs and IGF-1, thereby increasing the bioavailability of IGF-1. Thus, hyperphagia-induced hyperinsulinemia may promote rapid growth via IGF-1 mediated mechanisms. However, muscle-specific downstream activators of insulin/IGF-1 signaling were not evaluated throughout rapid linear growth. Moreover, the observed sustained IGF-1/IGFBP3 molar ratio throughout rapid linear growth may not reflect the bioactivity of IGF-1. Thus, the mechanisms by which insulin directly promote rapid linear growth requires further exploration. Regardless, altered GH/insulin balance during periods of energy excess that sustain NEFA and glucose clearance may override the physiological actions of GH to sustain pubertal linear growth.

Collectively, observations confirmed that MC4Rs are not expressed on somatotrophs or GHRH neurons, suggesting that MC4R does not directly modulate pulsatile GH release at the level of pituitary or hypothalamus. Thus, impaired GH secretion in MC4RKO mice does not occur as a direct consequence of loss of MC4R signaling. Rather, the progressive suppression of GH secretion is observed alongside hyperphagia-induced weight gain and hyperinsulinemia. Presumably, altered GH/insulin balance in hyperphagic MC4RKO mice reflects metabolic requirements that sustain NEFAs/glucose

homeostasis in response to excess energy consumption. Thus, this physiological adaptation may mimic the growth-promoting actions of GH to ensure rapid pubertal linear growth in the absence of GH actions. Given that GH deficiency in MC4RKO mice occurred alongside hyperphagia and increased insulin secretion, suppressed pulsatile GH release and altered linear growth in MC4RKO mice likely occur in response to hyperphagia-induced hyperinsulinemia. The impact of hyperphagia-induced hyperinsulinemia on GH release and rapid linear growth will be addressed in Chapter 6.

CHAPTER SIX: EXPERIMENTS TO ADDRESS AIM 5

6. PREVENTION OF HYPERPHAGIA PREVENTS HYPERINSULINEMIA, NORMALIZED ABERRANT LINEAR GROWTH AND RESTORE GH/IGF-1 SECRETION IN MC4RKO MICE.

6.1 Introduction

Dysfunction of MC4R signaling promotes hyperphagia leading to rapid weight gain and accelerated linear growth [68]. As detailed in Chapter 5, current observations suggest that rapid linear growth in hyperphagic MC4RKO mice occur independent of a rise in endogenous GH release. Rather, MC4RKO mice developed GH deficiency by 8 weeks of age (Chapter 5, Figure 5-2). Of interest, the progressive suppression of GH release in hyperphagic MC4RKO mice occurred alongside sustained fatty acid and glucose homeostasis (Chapter 5, Figure 5-6), presumably as a consequence of increased insulin release (culminating in hyperinsulinemia, Chapter 5, Figure 5-5). Given observations demonstrating the functional role of GH withdrawal in hyperphagic adults [14, 71], the observed withdrawal of GH secretion in hyperphagic MC4RKO mice may act to facilitate insulin action to sustain insulin-mediated lipogenesis and glucose homeostasis. Moreover, current observations suggest that hypothalamic MC4Rs are not expressed on somatotrophs and the GHRH neurons of the somatotrophic axis. Therefore, the suppression of GH release in hyperphagic MC4RKO mice may not occur as a direct consequence of MC4R dysfunction.

Hyperphagia is associated with decreased GH and increased insulin secretion [14]. The progressive suppression of GH secretion in MC4RKO mice may occur secondary to metabolic alterations in response to hyperphagia-associated hyperinsulinemia. Moreover, as insulin may directly impact growth, hyperphagia-associated hyperinsulinemia may compensate for the suppressed effects of growth-promoting actions of GH. Ultimately, this will contribute to rapid linear growth in GH deficient MC4RKO mice. *This chapter will address the hypothesis that the prevention of hyperphagia-associated hyperinsulinemia will restore pulsatile GH release and normalize linear growth in MC4RKO mice*.

6.2 Methods

6.2.1 Pair Feeding (PF) intervention

Starting at 4 weeks of age, WT LM and MC4RKO mice were individually housed and food intake was controlled to limit the amount of food consumed by MC4RKO mice. Pair fed MC4RKO (PF MC4RKO) mice were given the equivalent amount of food consumed by

age-matched WT LM. To prevent MC4RKO mice from consuming all food within a single meal, food was supplied as two meals. Mice are nocturnal animals and may show diurnal food anticipatory response. Thus, pair fed MC4RKO mice was compared to WT LM mice where provision of food was restricted to two meals. Food pellets were weighed and placed inside the cage of MC4RKO mice and WT LM twice a day (at 0800 and 1700 h) for a period of 12 weeks. Measures of PF WT LM and PF MC4RKO mice were compared to WT LM and MC4RKO mice with *ad libitum* access to food. Water was provided freely to all groups at all times.

6.2.2 Phenotypic characteristics of PF MC4RKO mice

To monitor body weight and linear growth curves following PF intervention, body weight and body length (nasal-anal distance) of *ad libitum* fed WT LM and MC4RKO mice, and PF WT LM and PF MC4RKO mice were monitored from puberty (4 weeks of age) throughout early adulthood (16 weeks of age). Measures were collected weekly as described in section 5.2.1. Measurements were conserved for all animals across all experiments.

6.2.3 Characterizing the metabolic profile of PF MC4RKO mice

To determine whether PF prevents the development of hyperphagia-associated hyperinsulinemia, WT LM, MC4RKO, PF WT LM and PF MC4RKO mice were assessed for fed and fasting insulin and glucose levels at 6 and 12 weeks of age. Methodology for plasma collection and analysis of circulating insulin levels are detailed in section 2.3.2. Assessment of ITT at 6 and 12 weeks of age was performed as described in section 2.4. WT LM, MC4RKO, PF WT LM and PF MC4RKPO mice were sacrificed at 8 and 16 weeks of age (following 4 and 12 weeks of PF intervention). At the time of sacrifice (between 0900 and 1000 h), mice were anesthetized as detailed in section 2.3.3 and plasma were stored at -80 °C for analysis of NEFAs and glucose. To determine adiposity, gonadal fat pads and inguinal fat pads were isolated by dissection and the fat pad mass was determined.

6.2.4 Characterizing pulsatile GH secretion in PF MC4RKO mice

For measures of pulsatile GH secretion, blood samples were collected and processed as detailed in section 2.3.1. Pulsatile GH secretion was assessed in PF WT LM and PF MC4RKO mice at 8 and 16 weeks of age. Following collection of blood samples, mice

were returned to their home cage and given 2 days to recover before assessment of metabolite profile.

6.3 Results

6.3.1 Prevention of hyperphagia reversed hyperinsulinemia in MC4RKO mice

To assess the effect of prevention of hyperphagia on insulin secretion and insulin sensitivity in hyperphagic MC4RKO mice, food consumption by MC4RKO mice was restricted to that normally observed in age-matched WT LM. Pair feeding of pubertal MC4RKO mice normalized insulin secretion, thus preventing hyperphagia-associated hyperinsulinemia (Figure 6-1). Compared to the *ad libitum* fed MC4RKO mice, fed and fasting insulin levels were significantly reduced in PF MC4RKO mice at 6 (Figure 6-1A,C) and 12 (Figure 6-1B,D) weeks of age. At 6 weeks of age, fed and fasting insulin levels were not significantly different between PF MC4RKO mice and PF WT LM controls, suggesting that the development of hyperinsulinemia in MC4RKO mice is a consequence of hyperphagia. While remaining lower compared to *ad libitum* fed MC4RKO mice at 12 weeks of age, fed (Figure 6-1B) and fasting insulin (Figure 6-1D) measures in PF MC4RKO mice were significantly elevated compared to PF WT LM controls, irrespective of pair feeding. Fed glucose levels were sustained in MC4RKO mice at 6 and 12 weeks of age, regardless of pair feeding. In contrast, the prevention of hyperphagia resulted in lower fasting glucose in MC4RKO mice at 6 weeks of age when compared to PF WT LM (Figure 6-1G). This may likely be due to the fact that PF MC4RKO mice consumed their food more rapidly, thus leading to the initiation of fasting state following the depletion of food, and consequently decreased fasting glucose levels. Measures of fasting glucose in PF MC4RKO mice remained unchanged at 12 weeks of age when compared to PF WT LM controls (Figure 6-1H). Collectively, observations suggest that the prevention of hyperphagia prevented hyperglycaemia and reduced the severity of hyperinsulinemia in MC4RKO mice.

The reduction in insulin levels in PF MC4RKO mice occurred alongside an improvement in insulin sensitivity. MC4RKO mice remained insulin sensitive at 6 weeks of age when compared to WT LM, regardless of pair feeding (Figure 6-2A,B). Consistent with observations previously described (Chapter 5, Figure 5-5), *ad libitum* fed MC4RKO mice showed a reduction in insulin sensitivity relative to WT LM control at 12 weeks of age (Figure 6-2C,D), regardless of increased insulin secretion (Figure 6-1C,D). This suggests the progressive development of insulin resistance. By contrast, pair feeding significantly improved insulin sensitivity in MC4RKO mice relative to PF WT LM, suggesting that

hyperphagia may be a potential mechanism contributing to decreased insulin sensitivity. Interestingly, PF WT LM control mice appear to develop partial insulin resistance when compared to *ad libitum* fed WT LM. This may in part reflect the adaptive mechanisms in response to altered meal patterning as a consequence of pair feeding intervention.

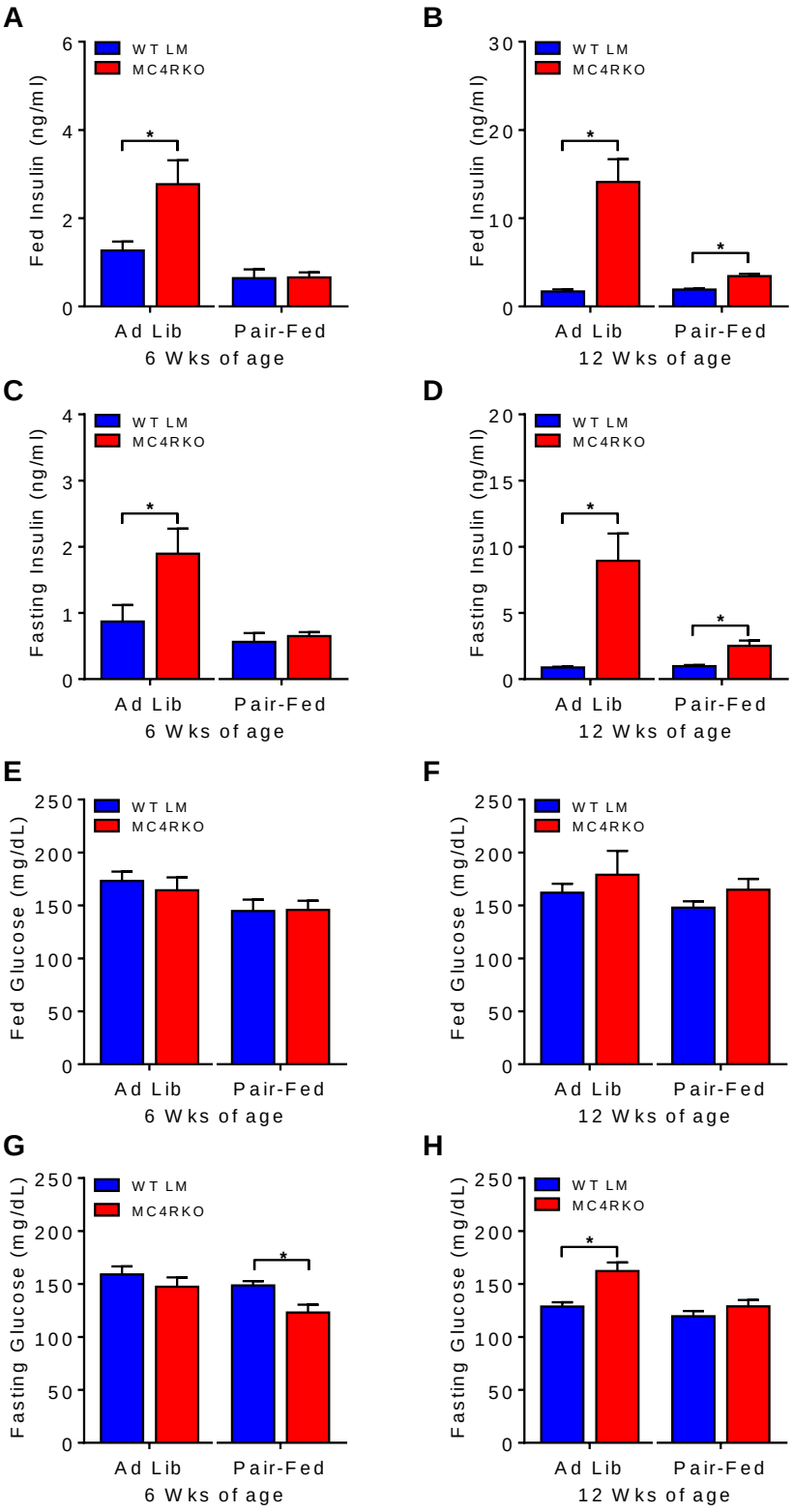

Figure 6-1: Prevention of hyperphagia by pair feeding prevented the development of hyperinsulinemia and normalized fasting glucose in hyperphagic MC4RKO mice relative to pair fed WT LM.

Fed and fasting insulin in *ad libitum* fed and PF WT LM and MC4RKO mice at (A,C) 6 and (B,D) 12 weeks of age. Assessment of fed and fasting glucose during ITT in *ad libitum* fed and pair fed WT LM and MC4RKO mice at (E,G) 6 and (F,H) 12 weeks of age. Data are presented as mean ± SEM. A P value < 0.05 was accepted as significant; WT LM: blue, MC4RKO: red, N=5-8 per group. Abbreviations: Pair fed; PF, melanocortin 4 receptor knockout; MC4RKO, insulin tolerance test; ITT.

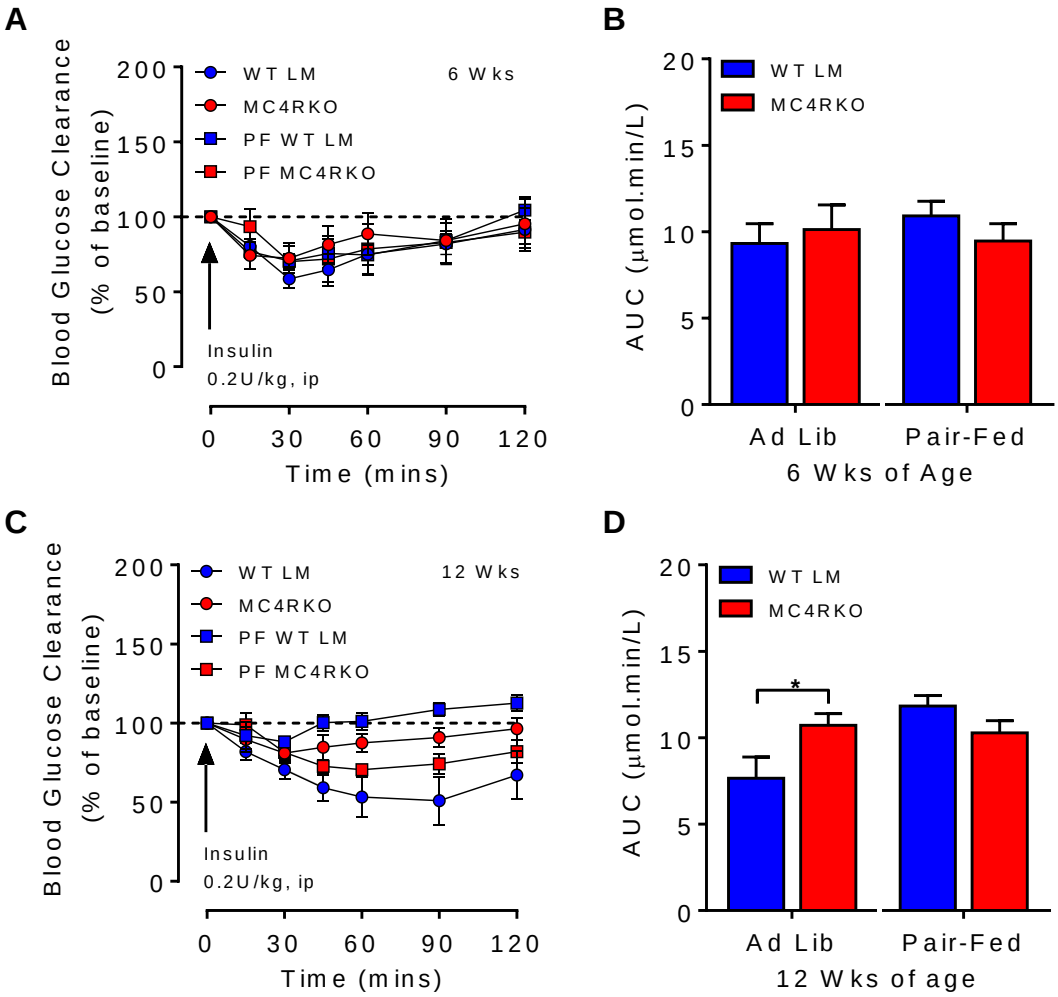

Figure 6-2: Prevention of hyperphagia by pair feeding improved insulin sensitivity in PF MC4RKO mice relative to PF WT LM.

Assessment of ITT in *ad libitum* fed and PF WT LM and MC4RKO mice at (A) 6 and (C) 12 weeks of age, and the corresponding area under curve (AUC) at (B) 6 and (D) 12 weeks of age. Data are presented as mean ± SEM. A P value < 0.05 was accepted as significant; WT LM: blue, MC4RKO: red, N=5-8 per group. Abbreviations: Pair fed; PF, melanocortin 4 receptor knockout; MC4RKO, insulin tolerance test; ITT, area under curve; AUC.

6.3.2 Prevention of hyperphagia in MC4RKO mice is associated with the prevention of aberrant linear growth

Insulin is thought to promote linear growth in GH deficient obese children [339]. Moreover, observations detailed in Chapter 5 suggest that hyperphagia-associated hyperinsulinemia

may facilitate rapid linear growth in GH deficient MC4RKO mice. Assuming this is the case, the prevention of hyperphagia-associated hyperinsulinemia may contribute to the slowing of rapid pubertal growth in MC4RKO mice. Accordingly, the prevention of hyperphagia resulted in the slowing of linear growth rate (Figure 6-3A), and a significant reduction in final adult body length in PF MC4RKO mice (Figure 6-3B). Unlike the rate of rapid linear growth observed in *ad libitum* fed MC4RKO mice (Figure 6-3C), the rate of linear growth between 4 and 8 weeks of age, and between 8 and 16 weeks of age in PF MC4RKO mice was not significantly different to PF WT LM (Figure 6-3A, Table 6-1). This suggests that excess energy intake may potentially contribute to rapid pubertal linear growth. Regardless of the reversal of aberrant linear growth rate, the total body length in MC4RKO mice was significantly longer relative to WT LM from 10 and 16 weeks of age (Figure 6-3B, Figure 6-3D). Presumably, this is associated with the modest increased body length of the pubertal MC4RKO mice at 4 weeks of age prior to pair feeding, thus resulting in an overall longer in adult body length.

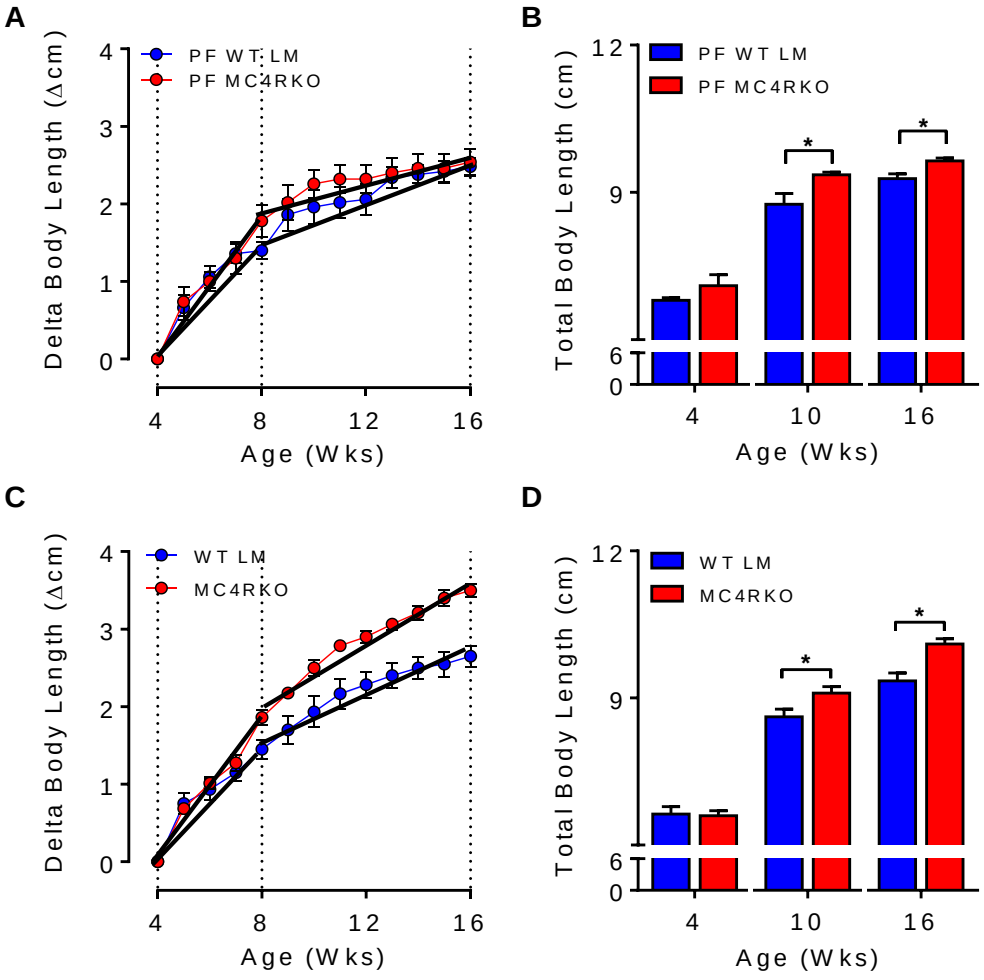

Figure 6-3: Prevention of hyperphagia slows the rate of aberrant linear growth in MC4RKO mice.

Growth curve illustrating cumulative weekly body length gain (A,C) and total body length change (B,D) in PF WT LM and PF MC4RKO mice, and *ad libitum* fed WT LM and MC4RKO mice. Data are presented as mean ± SEM. A P value < 0.05 was accepted as significant; WT LM: blue, MC4RKO: red, N=5-8 per group. Abbreviations: Melanocortin 4 receptor; MC4RKO, wild-type littermates; WT LM, pair fed; PF.

Table 6-1: Rate of growth (slope) relative to cumulative body length in PF WT LM and PF MC4RKO mice, and *ad libitum* fed WT LM and MC4RKO mice.

Body Length	**PF WT LM (slope)**	**PF MC4RKO (slope)**	**P value**
Wks 4 to 8	0.35±0.04	0.41±0.05	0.359
Wks 8 to 16	0.12±0.02	0.08±0.02	0.218
Body Length	**WT LM (slope)**	**MC4RKO (slope)**	**P value**
Wks 4 to 8	0.33±0.04	0.43±0.02	0.027
Wks 8 to 16	0.14±0.03	0.20±0.01	0.016

Data presented as mean±SEM. N=5-8 per group

6.3.3 Prevention of hyperphagia does not prevent weight gained and increased adiposity in MC4RKO mice

Prior observations confirmed that *ad libitum* fed MC4RKO mice gain weight rapidly (Chapter 5, Figure 5-1). While the prevention of hyperphagia slowed the rate of linear growth in PF MC4RKO mice (Figure 6-4A, Table 6-2), PF MC4RKO mice continue to gain a greater amount of body weight relative to PF WT LM (Figure 6-4B). The total body weight gain is similar to that observed in *ad libitum* fed WT LM and MC4RKO mice (Figure 6-4D). Regardless of pair feeding, the rate of body weight gain between 4 and 8 weeks of age, and between 8 and 16 weeks of age was significantly greater in PF MC4RKO mice relative to PF WT LM (Figure 6-4A), similar to that seen in *ad libitum* fed MC4RKO mice when compared to WT LM control (Figure 6-3C). This occurred alongside the accumulation of epididymal and inguinal fat mass in MC4RKO mice at 8 (Figure 6-4E,F) and 16 weeks of age (Figure 6-4G,H). Whereas the amount of adipose mass in PF MC4RKO mice was significantly greater when compared to PF WT LM controls, the amount of epididymal and inguinal fat mass gain was not diff between *ad libitum* fed and PF MC4RKO mice. Presumably, the gain in body weight and increased adiposity in PF MC4RKO mice occur in response to the underlying metabolic defects associated with MC4R dysfunction [359].

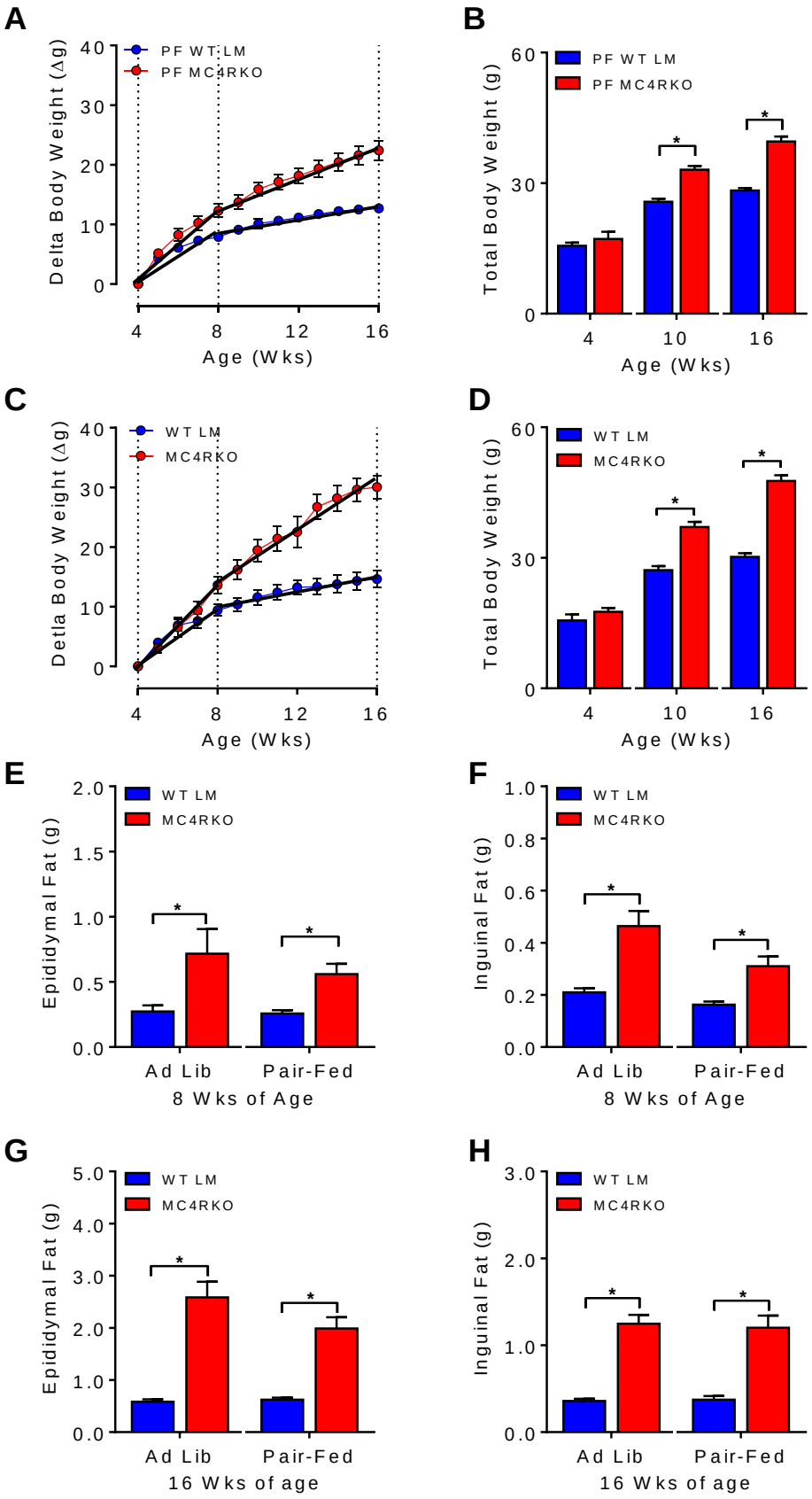

Figure 6-4: Prevention of hyperphagia results in continued weight gain and increased adiposity in MC4RKO mice.

Growth curve illustrating (A,C) cumulative weekly body weight gain and (B,D) total body weight change in PF WT LM and MC4RKO mice, and *ad libitum* fed WT LM and MC4RKO

mice. Epididymal and inguinal fat mass of PF WT LM and MC4RKO mice, and *ad libitum* fed WT LM and MC4RKO mice at (E,F) 8 and (G,H) 16 weeks of age. Data are presented as mean ± SEM. A P value < 0.05 was accepted as significant; WT LM: blue, MC4RKO: red, N=5-8 per group. Abbreviations: Melanocortin 4 receptor; MC4RKO, wild-type littermates; WT LM, pair fed; PF.

Table 6-2: Rate of growth (slope) relative to cumulative body weight in pair fed WT LM and MC4RKO mice, and *ad libitum* fed WT LM and MC4RKO mice.

Body Weight	**PF WT LM (slope)**	**PF MC4RKO (slope)**	**P value**
Wks 4 to 8	1.87±0.20	2.97±0.30	<0.001
Wks 8 to 16	0.58±0.07	1.26±0.16	<0.001
Body Weight	**WT LM (slope)**	**MC4RKO (slope)**	**P value**
Wks 4 to 8	2.25±0.28	3.36±0.37	0.028
Wks 8 to 16	0.64±0.16	2.15±0.25	<0.001

Data presented as mean±SEM. N=5-8 per group

6.3.4 Prevention of hyperphagia restored normal GH/IGF-1 profile in MC4RKO mice

Observations demonstrate that hyperphagia is associated with decreased GH secretion and increased insulin secretion in hyperphagic obese adults. This is preceded by the gain in body weight [14]. Moreover, a progressive rise in insulin levels correlates to an adipose-specific decline in pulsatile GH secretion [98]. Thus, while not directly addressed, this suggests that sustained hyperinsulinemia may contribute to the suppression of GH secretion. Given that prevention of hyperphagia prevented hyperinsulinemia in PF MC4RKO mice (Figure 6-1), it is anticipated that the reversal of hyperphagia-associated hyperinsulinemia would result in the recovery of pulsatile GH release. In agreement with this, the prevention of hyperphagia resulted in the recovery of pulsatile GH secretion in MC4RKO mice at 8 (Figure 6-5A,B, Table 6-3) and 16 weeks of age (Figure 6-5D,E, Table 6-3) relative to PF WT LM. Unlike the *ad libitum* fed MC4RKO mice which developed GH deficiency, characterized by an overall reduction in GH secretory parameters (Chapter 5, Figure 5-2, Table 5-2), the restoration of pulsatile GH release in PF MC4RKO mice at 8 and 16 weeks of age was reflected by a recovery in total (Figure 6-5C) and pulsatile GH secretion (Figure 6-5F), and the mass of GH secreted per burst (Figure 6-5I). Observations suggest that immediate metabolic alterations in response to hyperphagia may play a role in the suppression of GH release normally seen in MC4RKO mice. Furthermore, the restoration of pulsatile GH secretion in PF MC4RKO mice further substantiates observations that altered GH release in *ad libitum* fed MC4RKO mice

(Chapter 5, Figure 5-2) occurred independent of potential direct interactions between the MC4R and key components of the GH axis at the level of pituitary (Chapter 5, Figure 5-8) and hypothalamus (Chapter 5, Figure 5-9). The normalization of pulsatile GH secretion did not impact circulating IGF-1 levels in PF MC4RKO mice (Figure 6-5G). In contrast, muscle-specific IGF-1 content was restored in MC4RKO mice relative to PF WT LM controls (Figure 6-5H). This is presumably in response to the recovery of peak pulsatile GH release. Comparisons of GH secretory parameters from deconvolution analysis are summarized in Table 6-3.

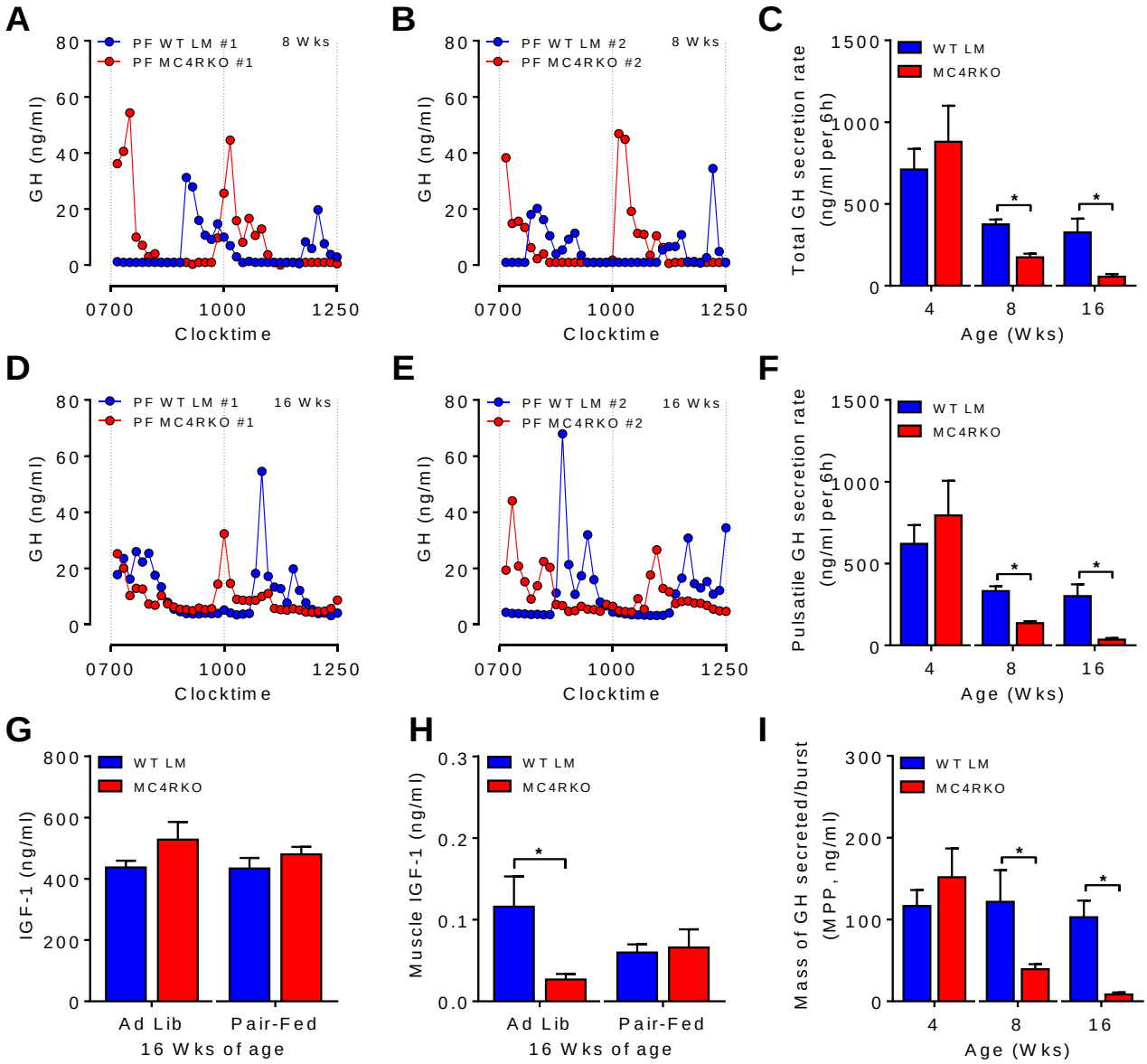

Figure 6-5: Prevention of hyperphagia recovered pulsatile GH secretion in MC4RKO mice.

Representative profiles of pulsatile GH secretion in PF WT LM and PF MC4RKO mice at (A,B) 8 and (D,E) 16 weeks of age. Deconvolution analysis of recovered (C) total, (F) pulsatile GH secretion rate, (I) mass of GH secreted per burst and (F) number of GH secretory events in PF WT LM and PF MC4RKO mice at 8 and 16 weeks of age. (G) Circulating IGF-1 levels remained unchanged in PF WT LM and MC4RKO mice at 16

weeks of age. (H) Muscle IGF-1 was restored in PF MC4RKO mice at 16 weeks of age. Data are presented as mean ± SEM. A P value < 0.05 was accepted as significant; WT LM: blue, MC4RKO: red, N=5 per group. Abbreviations: Melanocortin 4 receptor; MC4RKO, wild-type littermates; WT LM, pair fed; PF, growth hormone; GH, insulin-like growth factor-1; IGF-1.

Table 6-3: Deconvolution analysis of pulsatile GH secretion patterns in whole blood tail-tip samples collected from WT LM and MC4RKO mice pair fed on a standard diet, starting at 4 weeks of age. Samples were collected at 10-minute intervals for 6 consecutive hours, starting at 0700 h.

8 Wks of age	**WT LM**	**MC4RKO**	**P value**
Total GH secretion rate (ng/ml per 6h)	406±162	646±95.2	0.246
Pulsatile GH secretion rate (ng/ml per 6h)	363±156	569±74.9	0.280
Mass of GH secreted/burst (MPP, ng/ml)	62.5±25.8	87.8±10.6	0.371
Number of secretory burst/6h	6.00±0.71	6.40±0.60	0.678
16 Wks of age	**WT LM**	**MC4RKO**	**P value**
Total GH secretion rate (ng/ml per 6h)	602±47.9	476±83.1	0.232
Pulsatile GH secretion rate (ng/ml per 6h)	350±36.3	223±45.3	0.093
Mass of GH secreted/burst (MPP, ng/ml)	67.6±10.6	32.4±8.03	0.031
Number of secretory burst/6h	5.60±0.75	7.00±0.45	0.156

Data are presented as mean±SEM. A P value of < 0.05 was accepted as significant. N=5 per group.

6.3.5 Prevention of hyperphagia sustains NEFAs and glucose homeostasis regardless of increased adiposity

Impairment in pulsatile GH release in humans precede dietary-induced weight gain, and the suppression of GH secretion in response to calorie excess is thought to accommodate insulin actions to enhance meal tolerance [14, 71]. In this context, suppressed GH release alongside hyperinsulinemia demonstrates the potential role for the GH/insulin axis to sustain NEFAs and glucose homeostasis in hyperphagic MC4RKO mice (Chapter 5, Figure 5-6). Thus, it was anticipated that the observed recovery of GH release and reduced insulin secretion in PF MC4RKO mice will accommodate sustained storage and clearance of NEFAs and glucose. Accordingly, prevention of hyperphagia did not impact circulating NEFAs (Figure 6-6A,B) or glucose levels (Figure 6-6C,D) in PF MC4RKO mice

at 8 and 16 weeks of age relative to PF WT LM controls. It should be noted that circulating NEFA levels in the ad libitum fed MC4RKO mice at 8 weeks of age and circulating glucose levels at 16 weeks of age in the PF MC4RKO mice appeared to be altered, however these were not significantly different (p values indicated). While demonstrating that circulating fed glucose levels were sustained, regardless of pair feeding, it should be noted that, fasting glucose in PF MC4RKO were decreased at 6 weeks of age when compared to PF WT LM controls (Figure 6-1G). This is presumably due to the onset of fasting following the rapid consumption of first meal (when food pellet was dropped at 0800 h, prior to the start of ITT), and thus resulting in a reduction in fasting glucose. Of interest, pair feeding intervention reduced circulating NEFAs in both PF WT LM controls and PF MC4RKO mice when compared with *ad libitum* fed WT LM and MC4RKO mice. This decrease in NEFAs levels likely reflects an adaptive response to accommodate altered feeding patterns.

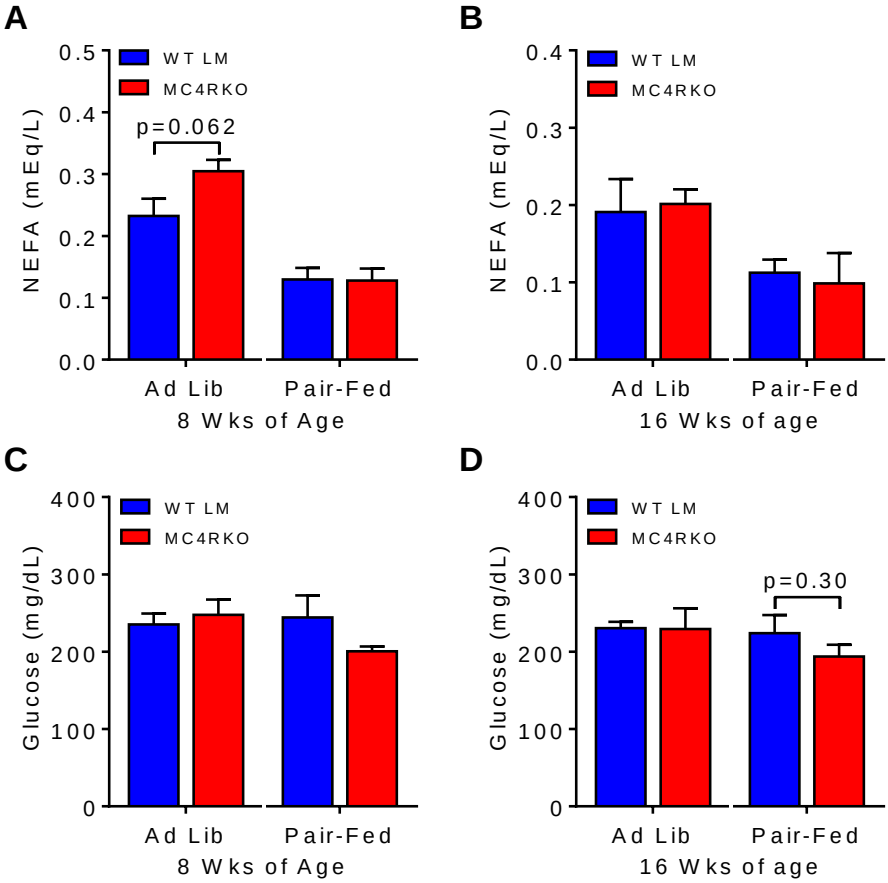

Figure 6-6: Prevention of hyperphagia did not impact plasma NEFAs and glucose levels in PF MC4RKO mice relative to PF WT LM following 4 and 12 weeks of pair feeding intervention.

Pair feeding of MC4RKO mice did not impact circulating levels of (A-B) NEFAs or (C-D) glucose at 8 or 16 weeks of age. Data are presented as mean ± SEM. A P value < 0.05 was accepted as significant; WT LM: blue, MC4RKO: red, N=5-8 per group. Abbreviations: Melanocortin 4 receptor; MC4RKO, wild-type littermates; WT LM, pair fed; PF, non-esterified fatty acids; NEFAs.

6.4 Discussion

Prolonged overeating and consequential weight gain are associated with hyperinsulinemia and diminished insulin sensitivity [14, 284, 360]. This may eventually impact insulin-induced lipogenesis sustained uptake and storage of dietary substrates into insulin-insensitive adipose tissue, resulting in systemic insulin resistance. The suppression of GH release following excess dietary consumption is thought to attenuate insulin resistance, thereby enhancing insulin sensitivity to sustain optimal meal tolerance [14, 71]. Thus, the reciprocal actions of reduced GH and increased insulin release are vital metabolic adaptations that facilitate the homeostatic balance of circulating NEFAs during energy excess. To this extent, adipose tissue may serve as an essential storage site for excess dietary fat and may act as a buffer for fatty acid exchange [361]. Ultimately, altered GH/insulin balance during extended periods of calorie excess reflects a physiologic mechanism to cope with prolonged positive energy balance (including hyperphagia and obesity), thus preventing adverse metabolic risks associated with elevated circulating NEFAs levels [362]. Given the rapid suppression of GH release alongside hyperinsulinemia observed in MC4RKO mice, it was anticipated that a similar compensatory role of GH actions relative to insulin is observed in hyperphagic MC4RKO mice. Second to this, it was proposed that hyperphagia-associated hyperinsulinemia contribute to rapid linear growth in hyperphagic MC4RKO mice.

Observations demonstrate that the prevention of hyperphagia resulted in the normalization in the rate of rapid linear growth of MC4RKO mice following pair feeding. PF MC4RKO mice were growing linearly at a slower rate compared to the *ad libitum* fed MC4RKO mice. In accordance with anticipated outcomes, the slowing in the rate of linear growth in PF MC4RKO mice occurred alongside the reversal of hyperphagia-associated hyperinsulinemia. This was demonstrated by a marked reduction in both fed and fasting circulating insulin measures. In line with this, exercise-induced normalization of insulin levels in MC4RKO mice correlates with a reduction in growth rate and the normalization of body length [363]. Exercise may promote lipid metabolism and enhance hepatic glucose output, thus improving insulin sensitivity and presumably insulin secretion [364]. Consequently, the normalization of linear growth following exercising may occur as a consequence of decreased insulin release. As insulin is thought to promote growth [357], hyperphagia-associated hyperinsulinemia may contribute to rapid pubertal growth in GH deficient MC4RKO mice. Regardless, longitudinal growth is a complex dynamic process influenced by several hormones in which their functions are largely interchangeable, including GH, IGF-1 and insulin. The reversal of hyperphagia-associated hyperinsulinemia

occurred alongside the restoration of pulsatile GH secretion and muscle IGF-1 production in MC4RKO mice (Figure 6-5). These factors may act independently or in concert to modify the adolescence growth spurt. Consequently, insulin may not solely account for the rapid linear growth in MC4RKO mice. Given technical limitations, the effects of insulin-mediated linear growth at the level of bone were not addressed in this study. Thus, the potential role of increased insulin secretion in directly promoting rapid pubertal growth in the absence of GH throughout periods of energy excess warrants further exploration.

As with humans [14, 71, 284], overeating rapidly induces hyperinsulinemia and the resultant insulin resistance in rodents [365]. In agreement with this, the prevention of hyperphagia-associated hyperinsulinemia significantly improved insulin sensitivity in MC4RKO mice, suggesting that enhanced calorie intake is the predominant factor in contributing to the onset of hyperinsulinemia. In humans, hyperinsulinemia precedes and may initiate insulin resistance [287, 360, 366], and thus is likely a dominant influence to the development of T2D during obesity. Moreover, anecdotal evidence from T1D mouse model demonstrates that prolonged insulin exposure mediates the development of insulin resistance [367]. In this context, it is likely that prolonged excess exposure to insulin following excess food consumption may be the primary cause of insulin resistance in MC4R deficiency, and presumably in obesity. Of interest, PF WT LM mice developed insulin resistance as a consequence of pair feeding. Mice consume food throughout the day [368, 369] and, as such, restricted meal frequency following pair feed intervention may alter response to anticipation for diurnal feeding [370], and may impact blood glucose. Thus, unlike PF MC4RKO mice showing normal insulin sensitivity (presumably as a consequence of elevated fasting insulin secretion at 12 weeks of age), PF WT LM may show a different response to insulin-stimulated glucose utilization in response to altered meal frequency, presumably to sustain blood glucose levels. Feeding was restricted to two meals per day in PF WT LM and PF MC4RKO mice. No credible information exists to address the potential role of pattern of meal frequency in regulating insulin sensitivity in mice. Thus, the effects of pair feeding in regulating insulin sensitivity warrant further investigation.

Current observations provide substantial evidence suggesting that hyperphagic MC4RKO mice developed GH deficiency (Chapter 5, Figure 5-2) regardless of sustained rapid linear growth (Chapter 5, Figure 5-1). This reduction in GH secretion occurs alongside hyperphagia-associated hyperinsulinemia (Chapter 5, Figure 5-5). Of interest, observations confirmed that MC4Rs are not expressed on somatotrophs and GHRH neurons, and thus may not directly modulate pulsatile GH release through these cell types.

This suggests that the observed suppression of GH release in MC4RKO mice occurs secondary to metabolic alterations that develop alongside hyperphagia. Given the proposed suppression of GH release by an elevation in insulin secretion [14], hyperphagia-associated hyperinsulinemia may contribute to the progressive loss of GH release in MC4RKO mice. Observations in this study provide compelling evidence demonstrating that the reversal of hyperphagia-associated hyperinsulinemia occurred alongside the complete restoration of pulsatile GH secretion in MC4RKO mice, and the recovery of muscle-specific IGF-1 levels. To this extent, hyperphagia-induced hyperinsulinemia may be a potential mechanism that contributes to the suppression of GH release throughout periods of energy excess. The mechanisms by which insulin directly regulates GH release are not completely understood. Previous studies demonstrate that insulin inhibits GH release from pituitary somatotrophs via InsR [170, 280-282]. Thus, elevated insulin may modulate GH release directly at the anterior pituitary gland in MC4RKO mice.

Consistent with prior observations [359], PF MC4RKO mice were significantly heavier and accumulated more adipose mass in comparison to PF WT LM. Moreover, the amount of adipose mass was not significantly impacted by the consequence of pair feeding. Thus, observations suggest that a functional MC4R is critical in the regulation of metabolism and energy expenditure. Consequently, impairments in metabolic defects and reduced activity as a consequence of MC4R dysfunction is predominantly the principal cause for weight gain in MC4RKO mice. Importantly, the prevention of hyperphagia-associated hyperinsulinemia occurred alongside the recovery of pulsatile GH release in PF MC4RKO mice, regardless of increased adiposity. Based on the premise that pulsatile GH secretion declines with increased adiposity [12], observations suggest that altered adipose mass may not predominantly account for reduced GH release in obesity. In addition, altered GH release in MC4RKO mice did not coincide with changes in circulating IGF-1, NEFAs, or glucose that may regulate GH release. Rather, observations demonstrate that the progressive suppression of GH secretion relative to an elevation in circulating measures of insulin in hyperphagic MC4RKO mice. This physiological adaptation is essential to sustain fatty acid flux. In agreement with this, circulating levels of NEFAs and glucose in PF MC4RKO mice were maintained within the same range to that seen in PF WT LM. This again highlights the concept that altered GH/insulin balance in hyperphagic and rapidly growing MC4RKO mice is a metabolic adaptation to sustain fatty acid and glucose flux. Of interest, circulating NEFAs levels appears to be reduced in response to pair feeding. Accordingly, mice eat intermittently throughout the day, consuming most of their calories during the dark phase [368, 369]. Thus, mice may manifest food anticipatory

responses prior to their expected meal times [371]. Consequently, WT LM and MC4RKO mice may exhibit reprogrammed diurnal food anticipatory activity to restricted schedules of pair feeding [370]. In this regard, changes in circulating NEFAs levels may be confounded by timing of meal frequency when pair feeding was initiated or the time of sampling relative to the meal. No observations exist to demonstrate the anticipatory response relative to the circadian release of NEFAs, and thus observations warrant further investigation.

In conclusion, prevention of hyperphagia-associated hyperinsulinemia by pair feeding normalized the rate of linear growth and recovered pulsatile GH release in MC4RKO mice. This occurred alongside sustained circulating NEFAs and glucose measures. Thus, altered GH and insulin balance in hyperphagic and rapidly growing MC4KRO mice reflect metabolic requirements that sustain fatty acid and glucose flux during periods of excess energy consumption. Second to this, metabolic adaptations for suppressed GH release may override the traditional role of GH in promoting pubertal growth associated with MC4R deficiency, and thus linear growth may occur independent of the normal anabolic actions of GH in promoting growth. This may occur in response to hyperinsulinemia, or co-secreted factors that facilitate insulin action to maintain optimal fatty acid and glucose homeostasis.

CHAPTER SEVEN: GENERAL DISCUSSION AND CONCLUSIONS

Excess calorie intake during childhood is often associated with tall stature [372, 373], whereas malnutrition results in stunted growth [374]. The dynamic changes in longitudinal growth coupled with changes in energy balance suggest that mechanisms that regulate linear growth are tightly controlled relative to food intake. Under normal circumstances, GH is the predominant regulator of linear growth during puberty, resulting in the attainment of final adult height [1]. This is supported by observations in children with idiopathic short stature as a consequence of childhood onset GHD, whereby the administration of GH results in the normalization of adult height [9, 375]. Following the attainment of final adult height, the anabolic role of GH in driving pubertal linear growth shifts to that of a metabolic hormone to primarily regulate body composition and adiposity throughout adulthood. In adults, GH deficiency results in reduced bone density and muscle mass and increased adipose mass [120, 376]. Early cessation of GH therapy in childhood onset GHD results in reduced bone and muscle mass, and a predisposition to the development of obesity as adults [377]. In this regard, GH is an important determinant of somatic maturation throughout early adulthood.

The secretion of GH declines with increased adiposity, culminating in GH deficiency in obesity [12, 13]. Importantly, the decline in GH secretion in humans precede dietary-induced weight gain [14]. This suppression of GH secretion following excess calorie intake enhances insulin resistance and improves meal tolerance [14, 71]. Thus, the suppression of GH secretion during extended periods of food consumption in humans is pivotal to accommodate the uptake of dietary fat via insulin-mediated lipogenesis [14, 71]. Insulin may modulate GH release relative to food intake including short-term and prolonged hyperphagia, and weight gain including dietary-induced weight gain and obesity. Of interest, rapid linear growth velocity is observed in pubertal obese children [339], regardless of GH deficiency [153]. At this time, the loss of GH release likely reflects the conservation of insulin function as seen in adults. Accordingly, rapid linear growth in obese children is unlikely attributed to GH-mediated effects, suggesting that factors other than GH may facilitate and promote linear growth at this time.

Dysfunction of MC4R signaling results in hyperphagia, rapid weight gain, and obesity [58, 68]. As with pubertal obese children, defects in MC4R signaling results in accelerated linear growth, however, the cause for rapid growth may differ considerably. Assessment of GH secretion in MC4R deficient adults confirms a partial recovery of pulsatile GH secretion relative to obese individuals [68]. Consequently, it is thought that activation of MC4R signaling contributes to the suppression of GH release in obesity, and

that GH hypersecretion following the loss of MC4R promotes rapid linear growth in MC4R deficient children. As discussed in Chapter 5, pharmacological activation or disruption of MC4R signaling does not impact spontaneous GH release in rodents [20-22]. Thus, it is unlikely that the MC4R directly modulate pulsatile GH release. Given the proposed actions of suppressed GH secretion to accommodate insulin actions in response to calorie excess, the proposed recovery of endogenous GH release to promote rapid linear growth in hyperphagic obese MC4R deficient children seems physiologically contradictory. Importantly, GH secretion during linear growth in pubertal MC4R deficient children has not been described. Thus, it remains unknown whether pubertal GH hypersecretion contributes to rapid pubertal linear growth in hyperphagic MC4R deficient children. Therefore, the demonstration of GH/IGF-1 mediated growth in a transgenic mouse model of MC4R deficiency will provide valuable insights regarding the role of MC4R in mediating GH release and promoting rapid pubertal linear growth during energy excess.

This thesis sought to investigate whether defective MC4R directly contributes to altered GH release during hyperphagia-induced weight gain. Second to this, this thesis addressed whether GH hypersecretion following the loss of MC4R signaling contributes to enhanced pubertal linear growth. In addressing these notions, 4 major findings were observed: 1) MC4Rs do not colocalize with somatotrophs and GHRH neurons, confirming that MC4Rs do not directly modulate GH release through interactions with somatotrophs or GHRH neurons; 2) rapid pubertal growth in hyperphagic MC4RKO mice does not occur in response to an elevation in GH/IGF-1 secretion; 3) MC4RKO mice develop GH deficiency during periods of rapid linear growth; and 4) prevention of hyperphagia in MC4RKO mice restores normal GH/insulin balance. Collectively, these observations clearly demonstrate that hyperphagia is the prevailing factor underlying the metabolic disruptions observed in MC4RKO mice that results in the suppression of GH release and enhanced linear growth. The progressive suppression of GH secretion following energy excess occurs with extended periods of overeating. This reduction in GH secretion that corresponds to the rise in insulin secretion is likely a consequence of the inhibitory effects of hyperphagia-associated hyperinsulinemia that occurs to accommodate the continual clearance of dietary fat from circulation into storage. Moreover, the physiological adaptations that contribute to the suppression in GH release during periods of pubertal hyperphagia likely sustain rapid linear growth in the absence of GH. These observations are discussed below and summarized in Figure 7-1.

7.1 Hyperphagia-associated GH suppression is a metabolic adaptation that facilitates insulin action

Under normal physiological circumstances, circulating GH and insulin act in concert to maintain the storage or use of circulating NEFAs relative to metabolic demand [378]. GH antagonizes the effects of insulin to regulate lipolysis and glucose oxidation, resulting in the rise in circulating NEFAs (Figure 1-9) [124]. In contrast, an elevation in insulin secretion following food consumption promotes glucose disposal and lipoprotein metabolism by stimulating lipogenesis, glycogen and protein synthesis, and by inhibiting lipolysis, glycogenolysis and protein breakdown (Figure 1-8) [270]. Much of the anti-lipolytic effects of insulin have been attributed to actions of insulin-induced adenosine monophosphate-activated protein kinase (AMPK)-mediated phosphorylation of the hormone sensitive lipase (HSL) [378-380], resulting in the regulation of triacylglyceride synthesis. In contrast, GH replacement therapy did not alter *Hsl* mRNA expression in adipose tissue in GHD adults [381], thus suggesting that GH may increase whole-body lipolytic rate independent of HSL. More recently, lipolysis appears to be primarily initiated by adipose triacylglyceride lipase (ATGL) [378], however, the role of GH-mediated ATGL activation of lipolysis has not been verified. Thus, it remains unknown whether the suppression of GH release relative to energy excess contributes to altered ATGL-mediated fatty acid flux. In view of this, excess dietary consumption appears to be associated with increased circulating NEFA flux [362]. However, a recent comprehensive assessment of NEFA kinetics in obese humans demonstrated that circulating measures of NEFAs are maintained [382]. Subsequent meta-analysis further revealed that the rate of NEFA exchange in circulation is sustained, regardless of the severity of obesity [285]. Given the reciprocal actions of GH and insulin in maintaining NEFAs storage, one may assume the inverse relationship between GH and insulin secretion promotes the clearance and storage of surplus of dietary fat. Thus, suppressed GH secretion that occurs with increased adiposity and alongside the rise in insulin secretion, following excess dietary consumption is likely a physiological adaptation that facilitates the homeostatic balance of use or storage of circulating NEFAs. Consequently, an increased in circulating NEFAs levels should only be observed following a disruption to the GH/insulin balance that sustain NEFAs balance. Indeed, recent measures demonstrate that impaired pulsatile GH secretion in healthy adults precede dietary-induced weight gain, and occurs alongside an elevation in insulin secretion [14, 71]. This suppression of GH release following excess food consumption ameliorates insulin resistance, thereby sustaining optimal meal tolerance [71]. These observations demonstrate that the suppression of GH secretion

alongside enhanced insulin secretion is an essential physiological adaptation to ameliorate insulin resistance. An inverse relationship between circulating GH and insulin is further illustrated in high-fat diet induced mouse models, whereby a progressive decline in GH secretion relative to an adipose-specific elevation in circulating insulin measures was demonstrated following dietary-induced weight gain [97, 98]. Observations from these studies were limited to relatively short periods of high fat feeding (8 weeks of dietary intervention), and thus the relationship between GH and insulin as long-term mediators of NEFAs flux remains to be determined.

Given the proposed interactions between GH and insulin in sustaining NEFAs flux, it is anticipated that the shift between circulating GH and insulin secretion is sustained throughout extended periods of hyperphagia. As detailed in Chapter 5, impaired pulsatile GH release in hyperphagic MC4RKO mice occurred alongside the early development of chronic hyperinsulinemia. Moreover, observations demonstrate that circulating NEFAs in MC4RKO mice throughout hyperphagia-induced weight gain is maintained within a normal range, regardless of increased adiposity. Given that GH may induce insulin resistance [383, 384], the removal of GH release during overeating may act to preserve insulin action, by reducing insulin resistance induced by lipolysis and GH signaling. Studies in humans demonstrate that the infusion of GH decreased the rate of glucose infusion during the euglycemic-insulinemic clamp [384-386], suggesting the impairment of insulin actions. However, given that GH treatment in humans results in increased rate of lipolysis and elevated circulating NEFAs [125], the observed effects of GH on compromised insulin actions may, at least in part, be attributed to the rise in circulating NEFAs in response to the lipolytic actions of GH, thereby interfering with insulin signaling to induce insulin resistance [387]. Consequently, suppressed GH secretion that accompanies increased adiposity may attenuate the severity of insulin resistance. To this extent, pharmacological reversal of suppressed GH release by GH replacement therapy following hyperphagia induced a greater increase in circulating insulin, fasting insulin, and insulin response to a meal in hyperphagic adults [71]. Regardless of sustained glucose clearance in response to elevated insulin, assessment of insulin function by homeostasis model assessment-estimated insulin resistance (HOMA-IR) results in reduced insulin sensitivity. Importantly, preventing the suppression of GH secretion in hyperphagic adults increased circulating NEFAs concentrations that would normally be driven into storage by insulin [71]. In line with this, excess GH in circulation severely impaired actions of insulin and lipid metabolism despite sustained glucose disposal [388, 389]. Under a normal diet, bGH overexpressing transgenic mice develop hyperphagia, insulin resistance and show the accumulation of

plasma triglycerides [388, 390]. Due to the inability to sustain the storage and clearance of dietary fat, hyperphagic bGH mice finally develop hyperlipidemia and diabetes, indicating the complete disruption of insulin function [388, 390]. In support of this interpretation, defective GH signaling in GHRKO mice is associated with altered visceral adipose distribution and enhanced insulin sensitivity, regardless of increased adiposity [391, 392]. Accordingly, the metabolic profiles of visceral adipose may be lipolytically active to facilitate the actions of insulin in rechanneling the availability of circulating fatty acid into storage [393]. To this extent, it appears that the suppression in GH secretion that normally occurs during overeating may protect against insulin resistance and hyperlipidemia that typically occurs with overeating. Ultimately, this may prevent adverse pathophysiologic sequelae associated with insulin resistance including cardiovascular risk and the eventual development of T2D following prolonged periods of excess food consumption [394]. Given that MC4RKO mice are hyperphagia, prolonged sustained positive energy intake may eventually disrupt the protective actions of suppressed GH secretion following the complete removal of GH. Ultimately, this will culminate in insulin resistance, disrupting mechanisms to sustain circulating NEFAs exchange. Thus, an extension to assess changes in NEFAs balance in MC4RKO mice remains to be determined. The findings by Cornford *et al* (2012) provide compelling evidence that an elevation in circulating NEFAs may not contribute to early development of obesity [395]. Rather, altered GH/insulin balance may represent an essential metabolic adaptation that sustains NEFAs and glucose homeostasis in response to excess dietary intake, regardless of age. Mechanistic studies to demonstrate disruption between GH and insulin balance resulting in disrupted NEFA flux do not exist. Furthermore, observations in hyperphagic MC4RKO mice were limited to 20 weeks of age. Considering that the release of GH declines with age, a further extension to confirm GH and insulin as long-term mediators of NEFAs homeostasis warrants further investigation. Given that MC4RKO mice developed hyperphagia between 4 and 5 weeks of age, the physiological requirement for suppressed GH release that accommodates the rise in insulin secretion during periods of sustained pubertal hyperphagia may in turn favour linear growth.

7.2 Rapid linear growth in pubertal hyperphagic MC4RKO mice occurred in the absence of increased GH/IGF-1 synthesis

Loss of MC4R signaling results in disrupted satiety signals, resulting in hyperphagia and rapid weight [58, 68]. Clinical observations demonstrate that pulsatile GH secretion is restored in obese hyperphagic MC4R deficient adults, regardless of increased adiposity

[68]. Moreover, defects in MC4R signaling results in an increase in adult height/body length in humans and rodents [58, 68]. While not directly assessed, observations suggest that increased pubertal GH release following the loss of MC4R signaling promotes accelerated linear growth, and that MC4R mediates the suppression of GH release in obesity. Observations from Chapter 5 demonstrate that rapid linear growth in MC4RKO mice occurs between 5 and 8 weeks of age, and while slowing, the rate of linear growth exceeds that seen in WT LM between 8 and 16 weeks of age. Therefore, as seen in humans with defective MC4R signaling [68], deletion of the MC4R resulted in rapid linear growth in MC4RKO mice that occurs predominantly during pubertal maturation leading into adulthood. Given that the GH/IGF-1 system is critical for normal growth and development during puberty [1], and that the loss of MC4R signaling in humans is thought to contribute to GH hypersecretion, it was anticipated that GH hypersecretion would coincide with periods of rapid linear growth in MC4RKO mice. However, observations clearly demonstrate impaired pulsatile GH secretion occurring in MC4RKO mice by 8 weeks of age. At this time, MC4RKO mice are still growing at a greater rate compared to WT LM. As detailed in Chapter 3, the slowing of rapid pubertal linear growth is accompanied by a decline in total GH secretion throughout adulthood. While this may infer that requirements for GH actions is suffice towards the attainment of somatic growth during transition into adulthood, the magnitude of GH reduction in MC4RKO mice was greatly exaggerated compared to that normally occurring in WT mice (first characterized in Chapter 3). In fact, the rapid suppression of GH release in MC4RKO mice by 8 weeks of age preceded the slowing of rapid linear growth, which does not occur until 16 weeks of age. Thus, observations suggest that the suppression of GH secretion is unlikely to promote rapid linear growth in MC4RKO mice relative to WT LM. Based on the premise that GH-deficient obese children demonstrate increased growth velocity [327, 339], it was proposed that factors other than GH may contribute to rapid linear growth in MC4RKO mice at this time.

While rapid linear growth in prepubertal obese children [339] occurs alongside the development of GH deficiency [153], circulating levels of IGF-1 appear to remain unchanged [153, 192] or are elevated [195]. Given that IGF-1 is a major determinant of postnatal growth, sustained IGF-1 actions may therefore exert a GH-independent growth stimulating effect to promote rapid linear growth in these children. Of interest, observations in liver-specific IGF-I deficient mice demonstrate an approximate 80% reduction in circulating IGF-1 levels. The reduction in circulating IGF-I is associated with elevated circulating GH (based on single GH measurements) [109, 110] and sustained free IGF-1 levels [189]. Importantly, the reduction in circulating IGF-1 levels did not impact postnatal

growth in liver-specific IGF-I deficient mice [109, 110], suggesting that free IGF-I may sustain the normal growth and development in these mice. As discussed above, systemic IGF-1 administration in humans and rodents displaying GH deficiency or disrupted GHR signaling stimulates linear growth [181-184]. Deletion or disruption of IGF-1 or IGF-1R by homologous gene recombinant in mice results in severe intrauterine growth retardation and postnatal growth failure [185-188] (Chapter 1.7.3). These studies demonstrate that IGF-1 may play a critical role in promoting normal longitudinal growth. However, observations do not directly clarify the physiological roles between circulating and local production of IGF-1 in promoting linear growth. Thus, it remains unclear how local production of IGF-1 may contribute to normal linear growth. In addition, IGF-1 is known to exert negative feedback on GH secretion by decreasing GH mRNA and GH release [396-398]. Thus, while liver-derived IGF-1 appears to play a central role in the feedback regulation of GH release, circulating IGF-I levels may not be of critical importance in regulating postnatal growth. Rather, normal linear growth in liver-specific IGF-I deficient mice may in part be related to sustained autocrine/paracrine actions of IGF-1. As with obese MC4R deficient or obese adults [68, 153, 192], circulating IGF-1 levels were normal in obese MC4RKO mice compared to WT LM. Because the abundance of circulating IGF-1 is associated with changes in basal GH secretion, rather than GH pulse amplitude [353], it is not unexpected that circulating IGF-1 levels are maintained in hyperphagic MC4RKO mice regardless of suppressed peak GH release. Furthermore, insulin may increase hepatic GHR expression and enhance post-receptor signaling of GHR to increase IGF-1 production [356]. Consequently, sustained circulating IGF-1 synthesis may, in part, be attributed to the observed chronic hyperinsulinemia in MC4RKO mice. This likely occurs via insulin-mediated activation of the hepatic GHR in MC4RKO mice [356], and thus warrants further investigation. Regardless, observations suggest that sustained circulating IGF-1 concentrations may not contribute to rapid linear growth in MC4RKO mice. Total circulating IGF-1 may not adequately reflect IGF-1 bioactivity [192, 399]. In view of this, changes in circulating IGFBPs may modify and interfere with interactions between IGF-1 and IGF-1R, thereby modulating IGF-1 bioavailability [192, 400]. Amongst all IGFBPs identified, IGFBP1 and IGFBP2 are proposed to be pivotal in the inhibition of IGF-1 actions [401, 402]. IGFBP3 is recognized as the major carrier protein, since approximate 90% of circulating IGF-1 is bound to IGFBP3 [180]. Given that IGFBP3 is dependent of the release of GH [111], the reduction in circulating IGFBP3 in MC4RKO mice likely occurred as a consequence of suppressed pulsatile GH release. Circulating IGFBP1 and IGFBP2 levels are primarily mediated by insulin. In agreement with previous findings [14, 192, 341, 342,

403], the rapid decline in plasma IGFBP1 and IGFBP2 in obese MC4RKO mice occurred alongside the development of hyperinsulinemia. Thus, elevated circulating insulin levels may modify IGF-I bioavailability as a result of insulin-mediated changes in IGFBP concentrations [192, 341]. In turn, this may indirectly promote IGF-1 mediated growth via common insulin/IGF-1 converging pathways [328]. Consequently, observed changes in circulating IGFBPs are thought to contribute to altered IGF-I/IGFBP3 molar ratio (marker of bioactive IGF-I) and finally an increase in IGF bioactivity to drive rapid linear growth in MC4RKO mice. Irrespective of this, a change in IGF-1/IGFBP3 molar ratio that reflects increased IGF-1 bioactivity was not apparent in MC4RKO mice, suggesting that bioactive IGF-1 may not contribute to rapid linear growth in MC4RKO mice. Changes in IGF-1/IGFBP3 molar ratio may not accurately reflect IGF-1 bioactivity since direct assessment of IGF-1 bioavailability was not performed in this study. Consequently, it remains unknown whether free IGF-1 levels promotes rapid growth rate in MC4RKO mice. Of importance, increased linear growth in obese adults is associated with elevated circulating free IGF-1 levels [191, 192, 197]. Moreover, liver-specific deletion of IGF-1 in mice sustained normal free IGF-1 levels [189]. Thus, the local production of IGF-1 may account for normal growth and development.

Collectively, observations demonstrate that rapid linear growth velocity in MC4RKO mice does not occur as a direct consequence of increased GH/IGF-1 secretion. Given that the rapid suppression of GH secretion preceded rapid pubertal linear growth in hyperphagic MC4RKO mice, physiological adaptations needed to cope with prolonged positive energy excess would presumably ensure sustained rapid pubertal linear growth, independent of GH. This may occur in response to hyperinsulinemia.

7.3 Altered GH/insulin balance in hyperphagic MC4RKO mice contributes to rapid pubertal linear growth

Increased metabolic intake is essential to cope with rapid childhood growth during pubertal maturation [404]. Thus, an increase in GH actions at this time may accommodate the attainment of adolescence growth spurt, while facilitating the exchange of dietary substrates relative to metabolic demand. As discussed above, alterations in GH/insulin balance during periods of excess energy intake represent an essential physiological adaptation to prevent hyperlipidemia. This is demonstrated by observations of suppressed GH/increase insulin secretion that sustained NEFAs/glucose balance in hyperphagic MC4RKO mice (detailed in Chapter 5). Consequently, it was thought that altered GH/insulin balance during periods of energy excess to promote fat storage in hyperphagic

adolescents would result in decreased pubertal linear growth as a consequence of reduced GH secretion. Nonetheless, childhood obesity is often associated with rapid pubertal linear growth and consequently tall stature [339], regardless of GH deficiency [15]. Traditionally, the pubertal increase in linear growth associated with GH has been attributed to androgens or estrogens secretion in pubertal children [405]. Irrespective of this, rapid linear growth velocity in obese children was achieved prior to the onset of pubarche, suggesting that rapid pubertal linear growth may be mediated independent of pubertal maturation, and therefore the production and release of gonadal steroids actions [339]. Consequently, the physiological balance between GH/insulin that occurs to encourage sustained NEFAs and glucose clearance during excess energy consumption may surpass the role of GH and steroid hormones, and therefore may represent significant changes in the evolution of mechanisms underlying continued pubertal linear growth during excessive energy availability. Hyperphagia is a core feature of many obesity models, and the fact that current observations in hyperphagic MC4RKO mice merely reflected changes secondary hyperphagia associated hyperinsulinemia. Thus, further assessment to validate the relationship between GH and insulin balance in other altered somatic growth of hyperphagic models per se must be considered. Of importance, decreased NEFA concentrations and glucose oxidation and reduced insulin sensitivity is often seen in pubertal children. Given the reciprocal actions of GH and insulin in promoting fat storage, altered insulin sensitivity and thus perturbed NEFA/glucose exchange in pubertal children are likely mediated by elevated GH secretion that coincides with pubertal development [406]. To this extent, it seems that the shift between GH/insulin that favors physiologic pubertal insulin resistance influence insulin-sensitive fuel metabolism during puberty. Presumably, this counters insulin action in reducing glucose oxidation and decreasing lipid oxidation to provide energy substrates necessary for growth in pubertal children. This pattern of substrate utilization may change relative to energy excess during adulthood. To achieve this, the shift between GH and insulin balance must be altered accordingly to promote the deposition of dietary fat. This explanation is substantiated by compelling evidence demonstrating the disruption in GH/insulin balance and the debilitating consequences on NEFA exchange, resulting in profound hyperlipidemia and risks of cardiovascular disorders (detailed in Chapter 7.1) [71, 388, 390-392]. Therefore, the inverse relationship between GH and insulin in the maintenance of sustained NEFAs and glucose flux appears to be conserved, regardless of age. Consequently, one may assume the need to suppress GH secretion that sustains NEFAs and glucose homeostasis

supersedes the role of GH as a regulator of pubertal linear growth. This may occur in response to an elevation insulin secretion.

7.4 Hyperphagia-associated hyperinsulinemia mediates rapid pubertal linear growth in MC4RKO mice

Observations in humans and rodents proposed the potential role of insulin in suppressing GH release [14, 97, 98, 283], and this may occur independent of IGF-1 [283]. Moreover, insulin is thought to promote growth via common insulin/IGF-1 mediated pathways [358]. Thus, hyperphagia-induced hyperinsulinemia may contribute to rapid linear growth in MC4RKO mice. Of interest, the slowing in the rate of linear growth in MC4KRO mice coincided with the development of systemic insulin resistance, and a reduction in muscle specific InsRβ expression. It is thus tempting to speculate that systemic insulin resistance contributed to a slowing in growth rate. It should be noted, however, that muscle specific IGF-1Rβ expression in MC4RKO mice was preserved throughout all ages assessed (Chapter 5, Figure 5-10). Whilst signaling at a reduced binding affinity [351], insulin may act via sustained IGF-1R to promote rapid growth in the absence of GH in MC4RKO mice. Nonetheless, direct evidence to suggest activation of insulin-stimulated IGF-1R-mediated growth does not exist. Given the complexity of the insulin/IGF-1 system (detailed in Chapter 1.7.3), it remains unknown how actions of insulin directly facilitate rapid linear growth. Considering that circulating insulin rapidly changes with overeating (discussed above), observations suggest that hyperphagia-associated hyperinsulinemia is likely responsible for the rapid linear growth and the suppression of GH release. Based on the premise that obesity and hyperphagia is associated with hyperinsulinemia and GH deficiency, the relationship between pulsatile GH secretion and insulin in promoting linear growth was further assessed by restricting food intake in pubertal MC4KRO mice to that normally consumed by WT LM.

Following calorie restriction by pair feeding, the rate of growth in MC4RKO mice slowed in parallel to that seen in PF WT LM. This occurred alongside the normalization of hyperinsulinemia, the restoration of pulsatile GH secretion and a recovery in muscle-specific IGF-1 expression (Chapter 6, Figure 6-5; presumably due to the recovery in peak GH release [407]). PF MC4RKO mice continued to gain weight and accumulated increased adiposity. Activation of the MC4R results in increased energy expenditure mediated through upregulated diet-induced thermogenesis [41]. Thus, weight gain and increased adiposity in PF MC4RKO mice likely occur in response to metabolic deficits and hypometabolism as a consequence of defective MC4R actions [359]. The recovery of GH

release in PF MC4RKO mice occurred regardless of increased adiposity, suggesting that altered adipose mass may not be the principle cause for impaired GH secretion in obesity. This restoration of pulsatile GH release was reflected by a recovery in total, pulsatile GH secretion and mass of GH secreted per pulse at 8 and 16 weeks of age. The extent of mass of GH pulse recovered in PF MC4RKO mice was significantly lower at 16 weeks of age compared to PF WT LM. This may be due, in part, to persistent metabolic effect of a modest rise in insulin secretion in PF MC4RKO mice at 12 weeks of age (Chapter 6, Figure 6-1). Regardless, prevention of hyperphagia-associated hyperinsulinemia results in the restoration of pulsatile GH profiles in MC4RKO mice. In support of these findings, weight loss in humans reversed obesity-associated hyperinsulinemia [155, 408], and consequently restored impaired GH secretion, [153-155, 171]. Thus, enhanced calorie consumption appears to be a key factor that underlies the onset of hyperinsulinemia. Given the interactions between GH and insulin in sustaining meal tolerance [71], one may speculate that insulin directly contributes to the reduction in GH release in hyperphagic MC4RKO mice. Insulin inhibits GH release from isolated somatotrophs via InsR [170, 280-282]. These effects dominate regardless of the development of systemic insulin resistance [170], suggesting that somatotrophs remains insulin responsive during periods of energy excess. In this context, it is likely that an elevation in insulin in response to excess dietary intake promotes the progressive and sustained suppression in GH release, even in the presence of systemic insulin resistance. Furthermore, the inverse relationship between GH and insulin is demonstrated in T1D patients, whereby GH secretion is markedly elevated in these patients [274, 275]. Intensive insulin treatment in T1D patients results in the reduction in GH secretion. Importantly, the suppression of GH release following intensive insulin treatment in this population occurred alongside the recovery of circulating IGF-1 to within a normal range [274, 275]. This may be explained by insulin-mediated hepatic GHR activation to stimulate IGF-1 secretion [356], and thus may feed back to inhibit GH release. Therefore, further studies focusing on the pharmacological prevention of hyperphagia-associated hyperinsulinemia may potentially address the role of insulin in regulating GH release during periods of energy excess. Based on the premise that the somatotrophs may remain insulin responsive [170], elevated insulin secretion may act via somatotrophs-specific InsR to inhibit GH secretion in hyperphagic MC4RKO mice. This warrants further investigation.

7.5 Altered GH release in MC4RKO mice occur in response to hyperphagia-associated hyperinsulinemia rather than direct MC4R-mediated actions

Based on the premise of recovered GH pulsatility in obese MC4R deficient adults [68], it appears that the suppression of GH secretion in obesity is mediated via activation of the MC4R. Despite this association, the restoration of pulsatile GH release in MC4RKO mice in the absence of hyperphagia confirmed that the suppression of GH release occurs entirely independent of direct effects of the loss of MC4R signaling. As detailed in Chapter 5, the MC4R is not expressed on somatotrophs or GHRH neurons. This observation provides further evidence to suggest that the MC4R does not directly modulate GH release. As seen in DIO rodent models [170, 172], hypothalamic gene expression for GHRH and SRIF does not change in obese MC4RKO mice. This is in agreement with pharmacological observations that exclude MC4R as a direct intermediate regulator of GH secretion, whereby hypothalamic GHRH expression or pulsatile GH release remained unchanged in response to the activation or disruption of the MC4R signaling by MC4R agonist (MT-II) or antagonist (SHU9119), respectively [20-22]. Thus, it is unlikely that suppressed GH release would have occurred as a consequence of loss of MC4R signaling in MC4RKO mice. Rather, observations suggest that the suppression of GH secretion following dietary-induced weight gain and obesity is most likely attributed to direct inhibition of hyperphagia-induced hyperinsulinemia. The mechanisms by which insulin directly inhibits GH release are yet to be confirmed, with current observations primarily limited to direct somatotroph-mediated interactions [190, 283]. As described in Chapter 1.8.1.3, insulin may act centrally to stimulate the release of hypothalamic catecholamines [277] to activate SRIF neurons, thereby inhibiting GHRH-induced GH secretion [278]. However, hypothalamic gene expression of GHRH and SRIF does not change in DIO rodents [170, 172] or in MC4RKO mice (Chapter 5). Furthermore, pharmacological inhibition of SRIF activity did not seem to recover GHRH-induced GH secretion in obese adults [279]. Therefore, impaired GH secretion following hyperphagia and dietary-induced weight gain may not occur in response to alterations in hypothalamic control of GH secretion. Accordingly, assessment of *Ghrh* and *Srif* mRNA expression may not necessarily reflect GHRH and SRIF output, and thus measures of gene expression may not accurately reflect hypothalamic control of GH release. Furthermore, assessment of GHRH and SRIF output from mice do not exist. Thus, the premise that altered hypothalamic GHRH and SRIF activity directly contribute to suppression of GH release in MC4RKO mice requires further investigation. Regardless, given observations discussed above, it is likely that the suppression of GH secretion occur

predominantly in response to elevated insulin as a consequence of hyperphagia-and presumably obesity-associated hyperinsulinemia, acting directly on the somatotrophs.

7.6 Future Directions

Current observations provide a comprehensive overview of the physiological role of the MC4R in modulating GH release, and demonstrate an inverse relationship between GH and insulin secretion relative to hyperphagia. This metabolic shift in GH/insulin balance that sustain fatty acid and glucose exchange appears to be a preferential physiologic adaptation to protect against metabolic consequences during prolonged periods of energy excess, and thus appears to be conserved regardless of pubertal requirements that normally favors growth. Consequently, the metabolic requirements for sustained fatty acid and glucose clearance following periods of energy excess may override the role of GH in sustaining continued pubertal growth in humans, and this is thought to occur independent of GH actions (Figure 7-1). In this context, findings presented herein provide valuable insights underlying altered somatic growth in mouse models of pubertal hyperphagia, and may clarify the dichotomy of GH-dependent growth in obese-hyperinsulinemic adolescence. Furthermore, identification of the metabolic events that maintains optimal NEFAs and glucose homeostasis may expand current knowledge regarding the metabolic and endocrine consequences preceding the early stages of weight gain. This may in turn shed light on the development of obesity-related diseases, and thus offer intervention strategies to prevent adverse pathophysiological consequences associated with obesity.

Based on compelling evidence provided, the suppression of GH secretion in response to elevated insulin secretion may be natural occurring metabolic event that follows periods of excess energy consumption to facilitate the storage of excess dietary substrates. Moreover, observations suggest that hyperphagia-associated hyperinsulinemia is mostly responsible for the suppression of GH release, presumably to alleviate potential metabolic risks detrimental to health. In contrast, relatively little is known about how metabolic adaptations in response to energy excess directly contribute to rapid linear growth. A major caveat of the current study is the lack of data to illustrate the interactions of GH and insulin secretion with measures of bone-specific IGF-1 levels. Given that excess energy consumption may alter linear growth, a chronic elevation in insulin levels following overeating may influence IGF-1 bioactivity by insulin-stimulated IGF-1 production and suppression of hepatic IGFBPs. This may in turn increase bioavailability of IGF-1 actions directly on the bone. Accordingly, increased bone mineral density has been demonstrated in obese individuals [409], and in humans [60] and rodents [410] with

defects in MC4R signaling. This suggests that elevated bone mass may occur alongside rapid longitudinal growth. To this extent, future studies to define the mechanisms of rapid linear growth following progressive weight gain is of critical importance, and this requires the careful consideration of the potential overlapping direct and indirect actions of GH, insulin and IGF-1, since mice retaining either function may not deviate from normalcy to a great extent.

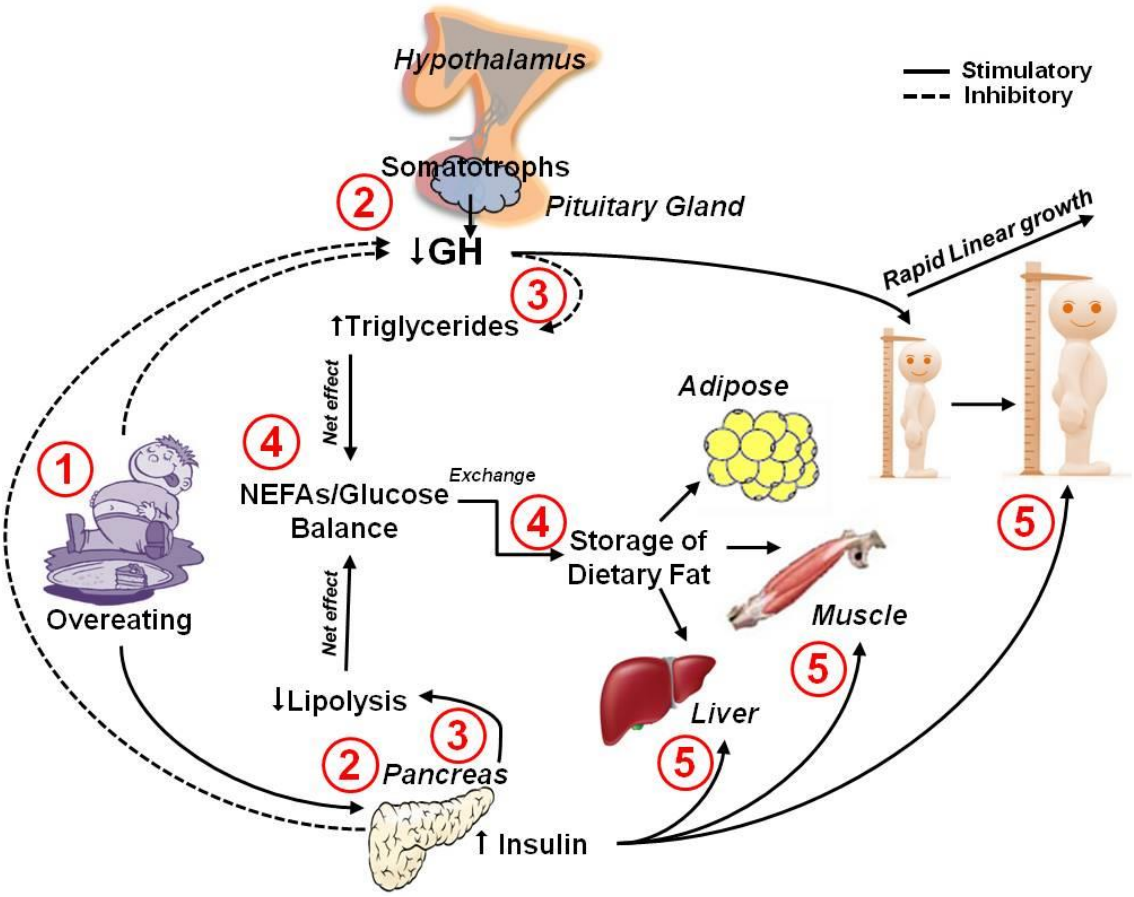

Figure 7-1: Schematic illustrating the physiological interactions between GH and insulin that occurs during energy excess.

Prolonged hyperphagia underlies major metabolic responses (1). Altered GH/insulin balance (decreased GH and increased insulin) following excess energy consumption (2) results in altered lipid metabolism and decreased lipolysis (3) to promote the storage and exchange of dietary fat into adipose, muscle and the liver (4), thereby sustaining NEFAs/glucose homeostasis. To maintain this homeostatic balance, a chronic elevation of insulin release suppresses GH secretion, presumably by acting directly on the somatotrophs located within the anterior pituitary gland (2). Elevated insulin as a consequence of excess dietary intake may also act on the liver to indirectly increase IGF-1 bioavailability, leading to reduced hepatic IGF-1/IGFBPs (5). Thus, during pubertal obesity, elevated insulin and IGF-1/IGFBPs may exert direct actions in the muscle as well as growth plate via specific receptors to sustain linear growth (5) in the absence of GH. Abbreviations; growth hormone; GH, non-esterified fatty acids; NEFAs, insulin-like growth factor-1; IGF-1, IGF-1 binding proteins; IGFBPs.

8. References

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