Validation of FHH-Ghsr^{m1Mcwi} GHSR KO Rat as a Model to Study Ghrelin Biology.

by

Valerie Rachelle Charbonneau

A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in partial fulfillment of the requirements for the degree of Master of Science

in

Neuroscience

Carleton University
Ottawa, Ontario

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-93628-3

Our file Notre référence
ISBN: 978-0-494-93628-3

Abstract

Ghrelin, an orexigenic hormone peptide, is the endogenous ligand for the growth hormone secretagogue receptor (GHSR). Previous research has established ghrelin as a modulator in the regulation of appetitive behaviors and energy balance, and this research has included the use of genetically altered mice with mutations to either the ghrelin or the GHSR gene. Recently a rat model with a mutation to the GHSR gene has become available and the present project attempted to validate this GHSR KO rat model. To this end, GHRS KO rats and their wildtype (WT) littermates (n= 8/group) originally obtained from Transposagen Bio (Lexington, KY) were single housed and monitored for food intake and bodyweight for a period of 4 months. During the first 3 weeks, animals had access to standard chow, whereas the remaining 3 months; animals were exposed to a High fat diet (60% calories from fat). Following several experiments, our results show that, when correcting for body weight, GHSR KO rats ate less and weighed less than WT littermates. Interestingly within the periphery, KO rats exhibited lower triglyceride content relative to WT controls. With regards to the central nervous system, our study was able to replicate previous findings reporting an increase in AgRP (an appetitive regulating protein) mRNA expression. Collectively, our data reinforces the hypothesis that the ghrelin receptor is indeed a potential target to prevent diet-induced obesity, and confirms the GHSR KO rat is a valid model for the study of ghrelin and growth hormone secretagogue receptor biology.

Acknowledgements

This project would not have been possible without the support of many people. First and foremost, I offer my sincere gratitude to my MSc. Thesis supervisor, Dr. Alfonso Abizaid for his continuous support, patience, motivation, enthusiasm, and immense knowledge. I am extremely lucky to have a supervisor who demonstrated he cared about my work by responding to my questions promptly, reading my numerous revisions and providing constructive criticism and excellent advice during the preparation of this thesis. Without him, this would not have been possible.

I am also grateful to other members of my thesis committee: Dr. Hymie Anisman and Dr. Matthew Holahan who offered guidance and support throughout different stages of my research.

I also wish to thank my fellow labmates: Harry MacKay for teaching me lab techniques and great insight; Zack Patterson, Rim Khazall, Martin Wellman, Samantha King, Veronique St-Onges and Trevor Rodrigues for sharing valuable advice and encouragement throughout this process.

The financial support from Natural Sciences and Engineering Council of Canada (NSERC) for this thesis is greatly acknowledged. The Department of Neuroscience at Carleton University provided the equipment needed to produce and complete my thesis.

Last but not least, I owe my loving thanks to my family: my parents Louise and Claude Charbonneau for their loving support and constant belief in me; my older brother Claude Jr. Charbonneau for his knowledgeable guidance, and to my boyfriend Darren Michelutti for his relentless support, understanding and encouragement.

Disclosure summary: The authors have nothing to disclose.

List of abbreviations

ACTH: Adrenocorticotropic hormone

AG: acylated ghrelin

AgRP: Agouti related peptide

Alpha-MSH: alpha menalocyte-stimulating hormone

ANOVA: Analysis of Variance

AP: area postrema

ARC: arcuate nucleus

AUC: Area under the curve

BDNF: brain-derived neurotrophic factor

CA: catecholamine

CART: cocaine-and amphetamine-regulated transcript

CNS: Central nervous system

DA: Dopamine

DIO: Diet-induced obesity

DMH: Dorsomedial nucleus of the hypothalamus

DMNV: dorsal motor nucleus of the vagus

Eliza- Enzyme-linked immunosorbent assay

ENU: ethyl-N-nitrosurea

FHH^{m1Mcwi}: fawn hood hypertensive mutation 1 Medical college of Wisconsin

FHL: fawn hooded low blood pressure

GH: growth hormone

GHRH: growth hormone releasing hormone

Ghrl: ghrelin

GHRL KO: Ghrelin knockout

GHSR KO: Growth hormone secretagogue receptor knockout

GOAT: ghrelin O acyltransferase

ICV: intracerebroventricular

IP: intraperitoneal

LH: lateral hypothalamic area

MC3/4R: melanocortin receptors 3 and 4

mRNA: messenger ribonucleic acid

NA: noradrenaline

Nac: Nucleus Accumbens

NPY: Neuropeptide

NTS: nucleus tractus solitarius

POMC: pro-opiomelanocortin

PVN: paraventricular nucleus

Qrt-PCR: quantitative real-time polymerase chain reaction

SN: substantia nigra

SYBR green:

TH: tyrosine hydroxylase

UAG: unacylated ghrelin

VMH: ventromedial nucleus of the hypothalamus

VTA: ventral tegmental area

WT: Wild type

Table of Contents

List of Illustrations

List of Appendices

1 Introduction

1.1 Obesity Epidemic

Obesity and associated disorders including Type II diabetes and cardiovascular disease represent one the biggest challenges to human health (Wang et al., 2006; Bloom et al., 2008; Lobstein et al., 2004). In addition to posing a worldwide burden upon health care systems (Bloom et al., 2008), the prevalence of obesity in children is also quickly escalating which forecasts an even greater medical threat in the decades to come (Troiano & Flegal, 1999). As a result of this epidemic, a peak of interest in obesity research has emerged in recent decades and these efforts have led to the discovery of a number of hormones regulating appetite, and have generated a certain optimism that drugs for the treatment of obesity will soon follow. However, enthusiasm has been premature and the exact molecular mechanism by which obesity develops remains unclear. Current pharmacological treatments for obesity are either lacking in efficacy of modulating relevant neuropeptides within pathways that control body weight or are burdened with adverse side effects (Shellekens, 2010). Thus, novel strategies and research targets are required for a better understanding of the intricate molecular pathways controlling energy homeostasis (Shellekens et al., 2010).

One particular prospect is the growth hormone secretagogue receptor (GHSR), the only known receptor for the orexigenic hormone ghrelin. A number of studies have identified this receptor as a potential target for the pharmacological treatment of obesity, and a number of experimental models are currently being used to explore the biology of this receptor. Amongst these models, there are a number of genetically altered mice models that have been used during the past decade, and these have yielded useful information. These models, however, are difficult

to test at the behavioral level. Recently, genetically engineered rats have become available for research, including a rat model with a targeted mutation to the GHSR. This rat model remains to be fully characterized to demonstrate that it is a valid model of the GHSR mutation. Therefore, the purpose of this project is to demonstrate that this rat model is indeed a valid model for the study of GHSR and ghrelin biology.

1.2 Energy Homeostasis

Homeostasis is a fundamental process by which a system regulates its internal environment to maintain a stable, constant condition of properties (Bernard; Cannon, 1926). Homeostatic regulation allows an organism to effectively function in a broad range of environmental conditions. Evidence of homeostatic mechanisms within the context of regulating energy balance and appetitive behaviors can be found throughout all cells and system in living organisms. Feeding provides the body's macronutrients (carbohydrates, lipids and proteins) and most micronutrients (minerals and vitamins) that are critical for survival. Therefore feeding behavior is one of the only behaviors we cannot eliminate if we begin to abuse it. For this process to occur, the amount of energy consumed must match precisely the amount of energy expended to respect this law of energy balance. In order to manage and operate the entire organism's energy balance, there are molecular signals that are responsible to modulate food intake while controlling short and long-term energy needs in storage (Woods et al., 1998; Saper et al., 2002).

In vertebrates, a key center regulating energy homeostasis is the hypothalamus. The hypothalamus is a relatively small central region composed of several nuclei and located at the base of the brain where it can access signals from the periphery that send information about water balance, nutrients, minerals, hormones, body temperature, and light/dark cycles.

In the case of energy regulation, several nuclei within the hypothalamus are sensitive to metabolic hormones and metabolic fuels like glucose and free fatty acids, and respond to changes in these signals by altering metabolic rate and producing behaviors aimed at obtaining or avoiding food to achieve homeostasis. In general, when nutrients are low or energy demands are higher than usual, organisms are said to be in a negative energy state (i.e. when the cost of life is higher than the energy available), whereas when there is a surplus of energy stores, the organisms is said to be in a positive energy state. This is an important aspect of energy homeostasis whereby the body will store its fuel in the form of adipose tissue for future on-demand needs (Woods et al., 1998). The hypothalamus is under constant stimulation responding to either of these states to achieve a balance. It acts as a thermostat and regulates our energy homeostasis integrating and interpreting peripheral tissue messages in order to output and delegate the appropriate functions to other areas of the brain. As a result, the body is assigned to act accordingly (i.e., manifest food-seeking behaviors when below homeostasis while lowering energy expenditure for energy conserving purposes until homeostasis is recovered). This concept requires a certain degree of plasticity to allow for constant dynamic changes from stimuli and peripheral signals such as ghrelin and leptin continuously counter-regulating energy balance (Abizaid, 2008). This flexibility develops responses to certain stimuli such that, hedonic or palatable foods and cues are learned and remembered in the future. Proteins are commonly found to rearrange neuronal connections in order for more efficient responses to future stimuli (Abizaid et al., 2008). .

The critical role of the hypothalamus was determined by lesion studies. Of these, classic studies included the destruction of the ventromedial nucleus of the hypothalamus (VMH) using electrolytic type of lesions and subsequently producing animals that became obese and diabetic

(Hetherington and Ranson, 1940). In other lesion studies, obesity and hyperphagia and insulin resistance were also observed after destruction of other nuclei near the midline of the brain including the paraventricular nucleus of the hypothalamus (PVN) and the dorsomedial nucleus of the hypothalamus (DMH) (Brobeck, 1946). In contrast, lesions placed in the lateral hypothalamus produced extreme anorexia (Powley and Keesey, 1970). In studies where they stimulated these regions rather than ablating them, Valenstein et al., (1968) discovered a decrease in food consumption when the VMH was stimulated whereas the opposite occurred while stimulating the LH. This suggests that the medio-basal hypothalamus is important to generate satiety and for metabolic regulation, whereas the lateral hypothalamus was important for more orexigenic hunger mechanisms.

Arcuate Nucleus.

While the VMH, PVN, DMH and lateral hypothalamus are important for the regulation of food intake and energy balance, the hypothalamic arcuate nucleus (ARC) has emerged as a critical first order site where peripheral signals are integrated, and processed and projected to change energy regulation.

First, the ARC resides outside of the blood brain barrier, and as such is in direct contact with circulating peripheral signals like hormones and nutrients. It also contains receptors for almost every known metabolic hormone. There are several circuits within the hypothalamus that contribute to its role in regulating appetite. One such circuit is found in the melanocortin system (Flier et al., 2004). The ARC contains two sub-population of neurons that are critical for the regulation of energy balance seeing as they are counter-regulatory (Flier et al., 2004). The first group coexpresses neuropeptide Y (NPY) and a second orexigenic peptide, agouti-related peptide (AgRP). Together, these neurons project to the PVN where NPY and AgRP have strong

effects on food intake and body temperature (Abizaid et al., 2008). They also stimulate the LH, while sending inhibitory inputs to the VMH (Elmquist, 2001). The second sub neuronal group coexpresses pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), both of which are anorexic peptides (Elias et al., 2001). In fact, POMC is a precursor for several peptides, one of which is the α-menalocyte-stimulating hormone (α-MSH); a neuropeptide that binds to melanocortin receptors 3 and 4 (MC3/4) in the hypothalamus to reduce food intake and energy expenditure (Boston et al., 2007; Cone et al., 2001). Animals with mutations to the POMC gene or to the MC3/4 receptors are leptin insensitive and morbidly obese (Huzsar et al, 1997). Like NPY/AgRP neurons, POMC neurons also project to PVN and α-MSH competes with AGRP for binding sites at MC3/4 receptors. Conversely to AgRP and NPY, POMC cells have stimulatory inputs to the VMH and inhibitory inputs to the LH (Flier et al., 2004).

Peripheral hormones regulating energy balance act on the melanocortin system to modulate food intake and metabolism. For example, ghrelin acts on the ARC to stimulate the activity of NPY/ AgRP neurons, whereas leptin increases the activity of POMC and CART neurons and the release of their peptide products (Morton et al., 2001; Murphy et al., 2006). Therefore, NPY/AgRP neurons activated by ghrelin stimulate feeding and decrease metabolic rate and satiety, while POMC/CART neurons, activated by leptin, stimulate satiety and increase metabolic rate while inhibiting feeding by acting on the NPY/AgRP cell group. Both NPY/AGRP and POMC neurons project to second-order neurons expressing the melanocortin 4 receptor (MC4R). Interestingly, AGRP is a natural antagonist to the MC4R whereas α-melanocyte stimulating hormone (α-MSH) (as previously mentioned to be the peptide derivative of the POMC protein) is a natural agonist to this receptor. Other than responding to leptin and

ghrelin signals, these two opposing cell groups communicate directly. Moreover, AgRP happens to be a direct endogenous antagonist to the MC3/4 receptor (Ollman, et al., 1997). Because targeted genetic disruption of MC4R in mice leads to increased food intake and increased lean mass and linear growth it is thought that AgRP and α-MSH act against each other to maintain homeostasis (Huszar et al., 1997). Hence, this appetite regulating system is very important for constant regulation of energy balance.

1.3 Ghrelin/Arcuate Nucleus/ GHSR expression

Ghrelin and the regulation of food intake and energy balance.

In humans and rodents, ghrelin is a 28 amino acid peptide and is the only peripheral peptide known to increase appetite (Kojima et al., 1999; Delhanty & van der Lely 2011). Ghrelin's highest expression is found within the stomach, where it is syntehsized and secreted from the X/A cells in the oxyntic gastric mucosa of the fundus (Delhanty et al., 2011). In contrast to other gut peptides increasing after food ingestion, ghrelin peaks before food ingestion as part of a mechanism that responds to fasting conditions. In addition, ghrelin secretion is pulsatile and is released in larger quantities before meals suggesting that ghrelin is important for meal initiation (Cummings et al., 2001; Kojima & Kanagawa 2005). Subsequently, ghrelin levels fall as animals begin to eat, a physiological response that may contribute to satiety (Toshinai *et al.* 2001). Ghrelin is composed of two forms. The most predominant form of ghrelin circulating in the blood is unacylated (UAG), representing up to 90%, while acylated ghrelin (AG) composes the remaining of total ghrelin (Delhanty et al., 2011). Originally, ghrelin was discovered as the natural ligand for the growth hormone secretagogue receptor (GHSR), an orphan receptor known to bind drugs that increased the release of growth hormone (GH) from the pituitary gland (Howard et al., 1996; Kojima et al., 2000). As such, ghrelin was found to

increase GH, although soon it was reported that exogenous administration of ghrelin increased food intake and adiposity in animals and humans (Tschop et al., 2000). However, in order for ghrelin to produce any of its characterized effects, a modification in the 3rd serine residue needs to occur. This modification requires that the n-Octanoic acid fatty acid be ligated to the third aminoacid of its molecule (serine) via acylation. This process is mediated by the Ghrelin O-acyltransferase (GOAT) enzyme (Yang, Brown, Liang, Grishin and Goldstein, 2008). Once this modification occurs, ghrelin is able to bind to its receptor. The non-acylated ghrelin also has biological activity although it does not bind to GHSR (Yang et al., 2008).From an evolutionary standpoint, ghrelin's actions are said to help efficiently secure caloric-dense tasty foods to protect during periods of food scarcity (Chuang, Perello, Osborne-Lawrence, Savitt, Lutter & Zigman, 2011; Delhanty et al., 2011). Interestingly, this may also shed light on why levels of AG and GH are elevated within anorexic patients, while basal plasma ghrelin levels are usually lower in obesity; possibly a counter-regulatory response to reestablish ideal body weight (Tschop, Devanarayan, Weywer, Tataranni, Rayussin & Heiman, 2001). Ghrelin also increases the circulating levels of prolactin, ACTH and cortisol (Nakazato, et al., 2001).

The effect of ghrelin on different organs has been reviewed in a number of excellent papers, but for the present paper we will discuss the effects of ghrelin of GHSR located in a number of brain regions, although primarily in the control center of the ARC.

Ghrelin receptor mediates ghrelin's multiple functions.

Ghrelin produces its effects via its actions on the GHSR. This receptor is found in numerous tissues including adipose tissues, liver, pancreas, pituitary gland and brain. GHS-R1a is among the G-protein couple receptor family with the classification of transmembrane receptors. It is composed of 366 amino acids with seven transmembrane alpha helices. This

subset is the functional form of the receptor. The effects of ghrelin on growth hormone release are mediated by ghrelin binding to the third transmembrane domain of the receptor in pituitary gland cells (Congreve M. et al., 2010) although feeding responses may no be mediated by ghrelin binding at this region. Based on affinity and specificity, ghrelin binds to GHSR-1a and this extracellular binding activity coordinates the specific activation of an associated G-protein that in turn, signals downstream cascades. In short, According to Albert et al., (2009), acylated ghrelin signals through G alpha q, i.e. diacylglycerol (DAG) and Inositol trisphosphate (IP3) intracellular messengers to create a calcium influx which is an important element in the process of neurotransmitter activation and release.

The orexigenic and adipogenic effects of ghrelin appear to be mediated by ghrelin receptors in the brain. Evidence for this comes from experiments where ghrelin was delivered chronically into the cerebral ventricles of rats and mice using osmotic minipumps at doses that were too low to produce peripheral effects (Tschop et al., 2000). These animals showed a transient increase in food intake, but showed a sustained increase in weight gain and this weight gain was attributed to an increase of adipose tissue and not in muscle or bone, and independent of the effects of ghrelin on growth hormone (Tschop, 2000). In fact their studies showed that the effects of ghrelin on food intake were mediated in part through the stimulation of both NPY and AgRP cells, because mice with mutations to these genes did not show feeding or adipogenic responses to exogenous ghrelin infusions (Tschop et al 2000; Nakazato et al., 2001). More evidence of ghrelin's role in regulating metabolism via actions on the brain was demonstrated when Theander-Carrillo et al., (2006) reported that chronic infusion of ghrelin into central nervous system increased lipogenesis and inhibited lipid oxidation in white adipose tissue. As a whole, these findings suggest central ghrelin infusion favors partitioning nutrients towards fat

storage instead of an increase in glucose and triglyceride uptake, and as a result, adiposity and weight gain occur. Briefly said, ghrelin's modulation within the hypothalamus is exerting orexigenic effects on neuropeptide Y and melanocortin neurons via binding to GHSR (Cowley et al., 2003).

Theoretical explanations on ghrelin's pathway to communicate with Central Nervous System.

The mechanisms by which ghrelin is transported to the Central Nervous System (CNS) remain largely unknown There are three potential theories that exist to explain the process through which systemic ghrelin is transported across the blood brain barrier. One possibility involves ghrelin molecules infiltrating the blood brain barrier via passive transport given the free fatty acid that is acylated to the serine, and which could facilitate entrance to the brain because it is lipophilic. There are, however, no data as of yet to fully support this hypothesis. In once particular study, it was observed in a mouse model that acylated ghrelin is readily transported across the blood-brain barrier in the brain-to-blood direction, but the quantity of its transport in the blood-to brain direction appears to be negligible (Banks et al., 2002). Nevertheless in 2006, Diano et al., (2006) observed within 10-15 minutes of peripheral infusion, ghrelin crossed the blood brain barrier and reach regions like the hippocampus.

The second possibility of ghrelin's path to the brain is via its direct actions on the vagus nerve and its brain stem afferents, which project to other brain regions like the hypothalamus. One study revealed that vagotomy prevents peripheral ghrelin's effect on the hypothalamus (Date et al., 2002). However, other findings have suggested no association between ghrelin's effects on the brain via the vagus nerve (Tschop et al., 2000; Nakazato et al., 2001). Evidently, the pathway in which ghrelin is transported to and cross the blood-brain barrier into the CNS remains to be clarified.

Finally the third theory posts ghrelin being produced within the hypothalamus. Indeed the ghrelin peptide has been detected in the hypothalamus of mice, rats and humans, although in much smaller concentrations than in blood (Cowley MA et al. (2003). Ghrelin producing cells have been found in the hypothalamus as well as in the stomach (Kojima et al., 1999). Moreover, intracerebroventricular (ICV) and intraperitoneal (ip) injections of ghrelin produced positive effects for GH secretion and food intake in rats fed standard chow. However, once the diet was replaced with high-fat diet (HFD), ICV or IP injections of ghrelin failed to induce food intake. This behavioral data was supported with a decrease in NPY AgRP mRNA expression and failure to stimulate growth hormone secretion following ghrelin ICV injections. These results suggest that in the context of HFD, this particular diet influences ghrelin's neural targets, by making them less responsive to ghrelin, even if they are possibly produced within the hypothalamus. More research is necessary to fully understand whether or not this theory of hypothalamic – ghrelin-producing cells lies true. Since, the physiological role of endogenous ghrelin produced in the hypothalamus remains undetermined, this topic remains hotly debated. Although if true, brain derived ghrelin would pose for a novel hypothalamic circuit regulating energy homeostasis.

In any case, once ghrelin reaches the hypothalamus, DIO has displayed an impact on the brain's ability to respond to ghrelin, therefore creating a ghrelin resistance, which may possibly act as a mechanism to adapt in positive energy balance.

GHSR central expression and ghrelin's actions within these regions.

Not surprisingly, the hypothalamus and pituitary gland are the main target for circulating ghrelin, given the ability of ghrelin in regulating GH, prolactin and adrenocorticotropin hormone ACTH secretion (Broglio et al., 2002). The ARC arguably contains the highest concentration and density expression of GHSR, coinciding with ghrelin's effects on regulating energy homeostasis,

although other nuclei known for their secondary role in the regulation of energy balance and food intake also express the GHSR (Zigman, Jones, Lee, Saper & Elmquist, 2006). The pituitary gland, second to the ARC, comprises the second highest concentration of GHSR expression. This dense GHSR expression location makes logical sense, since it plays an integral role with GH secretion. Within the ARC, the GHSR is selectively expressed in neurons co-expressing neuropeptide Y (NPY) and agouti-related protein (AgRP). Activation of neurons that co-express NPY and AgRP by centrally administered ghrelin reduces hypothalamic melanocortin tone by increasing the inverse agonist effect of AgRP on hypothalamic melanocortin receptors (Tschop et al., 2000).

As described above, ghrelin increases the activity of orexigenic peptides such as NPY, AGRP and other orexigenic second order neurons such as orexin/hypocretin from the lateral hypothalamus promoting anabolic processes. Ghrelin may also modulate POMC secretion indirectly to achieve the same functions. For example, the inhibitory neurotransmitter GABA is released locally by NPY/AGRP neurons to inhibit POMC secreting cells. Ghrelin binds to GHSR's to increase the activity of local NPY/AGRP axons to increase the rate of secretion of GABA and ultimately reducing the activity of postsynaptic POMC cells (Schwarts et al., 2000; Horvath et al., 2001). Beyond its effects on neuropeptide secretion, ghrelin was also reported to change synaptic plasticity within the ARC. In fact, ghrelin increases the number of stimulatory synapses on neurons that co-express NPY and AgRP and, concurrently increases the number of inhibitory synapses on neurons that secrete pro-opiomelanocortin hormone from neurons that express POMC (Abiziad, 2008; Morton, Ladenheim & Schwartz, 2001; Horvath et al., 2001). The overall result of these changes is that ARC NPY/ AGRP neurons are more likely to be stimulated (i.e. lower threshold) by any given stimulus, whereas POMC neurons are less likely to

be stimulated by any given stimulus when ghrelin levels are elevated (Morton et al., 2001; Pinto et al, 2003).

In addition to the hypothalamus, substantial levels of GHSR mRNA expression have been detected in other regions that are important and work in parallel with the energy homeostasis center. The hippocampus, in particularly the CA2 and CA3 regions of Ammon's horn and the dentate gyrus express high density of GHSR while moderate expressions were found in the CA1 region (Diano, et al., 2006; Guan et al., 1997). The hippocampus has been linked to cognitive processes including regulation of the stress axis, memory formation and consolidation, spatial memory, and navigation (Guan et al., 1997). Peripherally administered ghrelin has been shown to reach the hippocampus within minutes and to produce changes of synaptic plasticity correlating with enhanced spatial learning and memory (Diano et al., 2006). Ghrelin induced plasticity was reflected in increased spine density on hippocampal dendrites as well as in a higher number of synapses in these spines (Diano et al., 2006). This ghrelin function might aid as a potential mechanism to sharpen senses during food deprivation and efficiently remember the localization of food sources and its associated positive or negative experiences.

Another brain region that contains ghrelin receptors outside the hypothalamus and may also contribute to physiological regulating appetite actions of ghrelin is found in the caudal brain stem. Through in situ hybridization, Zigman et al., (2006) validated the presence of GHSR mRNA within all three components of the dorsal vagal complex, including the nucleus of the solitary tract, the area postrema, and the dorsal motor nucleus of the vagus. The presence of GHSR in these structures suggests ghrelin may manifest its actions on energy control through this ascending pathway in parallel or complementary to the hypothalamus. Fittingly, the DVC also contains receptors responsive to other blood-borne signals correlated with the animal' s

metabolic status such as leptin receptors initiating satiety and detection of changes in circulating glucose level (Grill, Schwartz, Kaplan, Foxhall, Breininger & Baskin, 2002).

Two of DVC's component structures, the nucleus tractus solitarius (NTS) and area postrema (AP), are the first to receive visceral afferent (ghrelin, leptin, insulin, cholecystokinin) since they lie outside of the blood brain barrier. These messages subsequently drive a number of autonomic reflexes through communications via efferent projections to hypothalamic structures that are able to orchestrate appropriate responses (Hou et al., 2006; Hintmarch et al., 2011).

The nucleus of the solitary tract is the primary site through which gastrointestinal (GI) afferent information enters the brain (Moran et al., 2001). It is a region that controls sympathetic outputs and that receives information from the vagus nerve, and is also sensitive to ghrelin and satiety signals (Date Y et al. 2002). Intra-cerebral injections of des-acyl ghrelin performed by Tsubota et al., (2005) within the NTS significantly reduced mean arterial pressure and heart rate. The hypotensive activity suggests that des-acyl ghrelin contributes to the regulation of cardiovascular control.

Ghrelin has been shown to also act upon GHSR within the NTS in the brain stem and influence noradrenaline (NA) cell groups and project to alpha1 and beta2 noradrenergic receptors in the ARC to stimulate food intake (Date et al., 2006). Cui, Li and Appleyard, (2011) also recognized the importance of other A2/C2 catecholamine (CA) neurons in the NTS with regards to food intake and ghrelin's modulating effects on this neurons within energy homeostatic function.

The area postrema (AP) also displays moderate expressions of GHSR cells (Zigman et al., 2006) and responds very quickly to changes in glucose, fatty acid concentrations and gut-peptide signals in order to maintain homeostasis. Fry et al., (2008) have examined the ability of

ghrelin to regulate and modulate the electrical activity of area postrema neurons using voltage-clamp electrophysiology recordings and found activation within GHSR cells of the AP, stimulating appetite and regulating pancreatic protein secretion.

The dorsal motor nucleus of the vagus (DMNV) is located adjacent to the fourth ventricle and serves parasympathetic vagal function including the gastrointestinal tract, lungs, smooth muscle and heart (Niedringhaus, Jackson, Evans, Verbalis, Gillis & Sahibzada, 2008). Sometimes called "cardioinhibitory center", one of its roles involves slowing the heart rate. Mediated by efferent vagus nerves, stimulation of the intermediate and rostral DMV increase fundus synthesis of ghrelin while stimulation of the caudal DMV caused a decrease in fundus tone (Niedringhaus et al., 2008). This relationship may shed more light on the fundus's role in secreting ghrelin and its communication with the brain stem.

Faulconbridge et al., (2003) administered ghrelin to the fourth ventricle near the brainstem to explore the functional importance of the brainstem GHSRs and compare feeding responses with those obtained when ghrelin was delivered near hypothalamic sites. Previously Wren et al. discovered the lowest effective ghrelin intracerbroventricular dose to induce hyperphagic behaviors within the third ventricle near the ARC was 30 pmol. Surprisingly, Faulconbridge et al., (2003) obtained a similar response within the fourth ventricle, near the DVC with only 10 pmol. This suggests a possibly more sensitive area to ghrelin administration acting on GHSRs in the DVC. Behavioral hyperphagic measures to support these orexigenic effects included reduction in the latency to feed and an increase in the number of meals taken during the first few hours after treatment (Falconbridge et al., 2003). These results are consistent with a role for central ghrelin signaling in meal initiation. In addition, these findings also draw attention to the importance of DVC as an initial site for ghrelin within the brain. It equally

displays a functional parallel between the DVC and the hypothalamic arcuate nucleus and highlights the importance of the caudal brainstem in regulating food intake by integrating information from visceral organs and in turn conveying them to appropriate higher-order regions. Moreover, these studies also reiterate ghrelin's key role in modulating feeding responses within these regions.

Finally, the mid brain ventral tegmental area (VTA), a brain region well known for its role in the regulation of reward seeking behaviors and motivation for food also contains a relatively high concentration of GHSR. This GHSR expression mediates the direct interaction of ghrelin's effects within a subset of VTA neurons that produce dopamine (DA) and that are associated with reward seeking mechanisms (Guan et al., 1997). Therefore, in addition to moderating normal eating habits, ghrelin acts on the brain's reward centers. This mesolimbic reward circuit is important to consider when discussing energy homeostasis because it targets a motivational reward aspect of feeding that is considered by some; the root of our overeating epidemic.

The VTA reward pathway is separate from the homeostatic circuit found within the hypothalamus. These two regions often communicate whereby the VTA receives information from the primary hypothalamus region. Depending on the energy balance status of the organism, the VTA receives input to modulate motivational aspects of feeding such the "liking " and "wanting" behaviors necessary to seek and obtain food (Abizaid et al 2009; Berridge, 1996). In addition, being sensitive to direct ghrelin stimulation, VTA dopamine cells are innervated by lateral hypothalamic hypocretin/orexin neurons, which are also sensitive to ghrelin (Abizaid, 2009; Toshinai, et al., 2003). Shimbara et al., (2004) reported ghrelin selectively stimulated the intake of foods high in fat. Some research suggests these reward pathways may even override the

hypothalamic homeostatic centers and produce increased appetite (Grill et al., 2001). GHSR mRNA transcript and protein are expressed in relatively high quantities within the VTA and adjacent substantia nigra (SN) regions associated with goal directed behavior. When ghrelin is administered centrally into the VTA and SN or into the Nucleus Accumbens (Nac), food intake increases significantly (Naleid, 2005; Abizaid et al., 2006). The mechanisms underlying these feeding responses involve the direct action of ghrelin on VTA DA cells as DA neurons increase their activity in response to ghrelin as shown in experiments recording from DA cells in slice preparations (Abizaid et al, 2006). Ghrelin does this by binding to DA cells and increasing their firing rate or action potentials. Subsequently, ghrelin increases the release of DA into the nucleus accumbens in association with increased feeding responses and increased motivation (Abizaid et al., 2006; Mathon, et al., 2005). Motivation was measured via goal-oriented behaviors including operant responses or increased locomotor food anticipation (Abizaid et al., 2006; Blum et al., 2009; King et al, 2011). Collectively, these findings suggest ghrelin may act on the VTA to increase food intake by enhancing the incentive value of foods (Abizaid, 2009).

1.4 Previous ghrelin ghrl/GHSR KO mice findings

Understanding the biological effects of ghrelin using animals with single gene mutations to ghrelin related genes.

Before discussing data associated with the use of animals with targeted deletions of the ghrelin gene, one needs to consider the usefulness of genetic approaches to studying obesity. It is clear that there are genes that are directly implicated in the development of obesity. For example, single mutations to the gene encoding the leptin, POMC or the melanocortin 4 receptor (MC4R) proteins lead to morbid obesity and metabolic disorders in rodents and humans (O'Rahilly, Farooqi, Yeo & Challis, 2003). This obese phenotype can be corrected with treatment that

restores the levels of these proteins. Moreover, climbing rates of obesity correlate well with the sedentary lifestyle of human populations around the world and the increase in the availability, amount and affordability of high calorie meals and snacks. Thus, an interaction of genetic make-up, a decrease in physical activity, and an increase in the intake of calories are at the root of the obesity pandemic.

A number of genetic alterations have been shown to predispose some individuals to gain more weight than others given similar lifestyles. Thus, excessive high-caloric food consumption coupled with insufficient physical compensatory activity may impact metabolism in those who are genetically predisposed to maximize metabolic efficiency and fat storage (Delhanty et al., 2011). An example of this are individuals with haplo-insufficiency for Sim 1 (a transcription factor required for normal hypothalamic development) (Holder, Brutte & Zinn, 2000), or for brain-derived neurotrophic factor (BDNF) (Gray et al., 2006) have been shown consistently to become more susceptible to obesity. Similarly, heterozygous loss-of-function missense mutation to the Trk B BDNF receptor are also more vulnerable to become obese compared to non-mutant individuals (Gray et al., 2006).

Finally, a good body of evidence has shown one genetic variant termed Fat mass and obesity-associated protein (FTO) predisposes obesity by affecting the same homeostasis pathway whereby its expression is altered during fasting and feeding (Dina et al, 2007). Individuals who are homozygous for the high-risk allele (AA) of FTO weigh on average 3 kg more than individuals with two low-risk alleles, with heterozygotes having an intermediate risk (Farooqi & O'Rahilly, 2006).

In all, while there are a number of genes that are clearly implicated in the regulation of energy balance, and their mutation can lead to obesity. It is likely that human obesity comes as a

result of the interaction between genes and environment, and that while some may be predisposed to gain weight, they may not because their energy expenditure exceeds their caloric intake (Kanasaki et al., 2010). In contrast, others may gain weight in spite of increased energy expenditure and decreased caloric intake because of their genetic make-up resists a weight-loss phenotype.

Some have suggested that individuals such as these are the product of evolutionary pressures where an energy ``thrifty`` set of genes allowed our ancestors to adapt and survive periods where food was scarce such as winter. This changed with the revolution in our modern society's food industry, and those genes that helped our ancestors survive are now the ones that are decreasing life expectancy because of health complications associated with obesity (Kanasaki et al., 2010).

Diet-Induced Obesity Mice Models

Current animal models of obesity typically include mice that have natural or genetically engineered mutations to single genes (Appendix A). In addition, there are models that include selective breeding for obesity or obesity resistance, both of which are presumed to be the result of alterations in several genes. Finally there are models where animals are exposed to high caloric diets or to varied choices of palatable diets. Both of these experimental manipulations are referred to as diet induced models of obesity.

Animal models representative of Diet-Induced Obesity often include a High-Fat Diet whereby different strains acquire different responses to the HFD. Some inbred strains of mice exhibit significant obesity when fed HFD while other remain lean (West et al., 1992). Among the mice list prone to obesity are the C57BL/6J mice, sand mice and spiny mice because they display similar abnormalities that humans experience during high-fat diets (Collins et al., 2004).

There are many examples of natural monogenetic (single gene) mutations that lead to obesity and metabolic disorders like Type II diabetes. Examples of these are rodents with mutations to the leptin or leptin receptor gene. The first *ob/ob* mouse arose by chance in a colony at the Jackson Laboratory in 1949 (Ingalls, Dickie and Snell, 1950). These mutants were first described in the 1950's in mice colonies breeding the C57BL/J6 mice strain. These mice were originally termed as ob/ob, and their discovery was followed by the discovery of another strain of obese mice that arose spontaneously in the same colony and whose metabolic phenotype was similar to the ob/ob mice strain with an exaggerated diabetic phenotype. These mice were termed as db/db mice (Coleman,1978; Friedman & Halaas, 1998). The genes that were mutated in these animals were the leptin and the leptin receptor genes respectively, and the identification of these mutated genes and the proteins encoded by these genes are considered one of the most significant findings with regards to energy balance regulation (Friedman et al., 1998).

Similar mutations have been identified in rats where the most common models of obesity is the Zucker Fatty Rat (ZFR), a strain of rats that emerged from the Long Evans rat strain and one where the protein encoded by the leptin receptor gene is altered, and therefore has decreased leptin signaling properties (Kurts, Morris and Pershadsingh1989). These rats display an early onset to obesity at 5 weeks of age, although they do not go on to develop diabetes (Bray et al., 1977). Only a sub-strain of ZFR termed Zucker diabetic fatty (ZDF) has been found to develop diabetes (Friedman et al., 1991) and used in diabetes animal models. Another monogenic mouse obesity model is the lethal yellow Agouti mouse, which exhibits a mutation on the gene that encodes the agouti related peptide (AgRP) (Bultman et al., 1992). This was the first obesity gene characterized as the molecular level and is now known to be critical for the regulation of energy balance (Abizaid et al., 2008, Bultman et al., 1992). This agouti gene expression is also found in

human adipose tissue; hence it's relevant nature to human obesity (Kwon, Bultman & Loffler; 1994). In terms of diet-induced obesity rat models, the Sprague Dawley and long Evans rats are commonly used as models for HFD-induced obesity (Srinivasan & Ramarao; 2007) mostly because these strains easily gain weight compared to other rat strains.

Monogenic models provide important biology findings of obesity while polygenic models illustrate the mediation of obesity through the interaction between genes and environment. Indeed, while there are many rodent strains that become obese when exposed to a high-fat diet, not all inbred strains will develop obesity. These findings suggest potential genotypes that predispose animals to develop obesity while others might resist obesity (West et al., 1992). Indeed there are strains of rats originating from the common Long Evans strain that are either prone or resistant to developing obesity when fed a high fat diet. Levin et al., (2003) compared leptin, insulin and ghrelin levels, using radioimmunoassay kits, between rats that were bred to be prone to diet-induced obesity (DIO) and to rats bred to become diet resistant (DR). Levin et al., (2003) discovered a significant increase in body weight between 5 and 7 weeks of age relative to their WT littermates, when fed ad libitum standard chow. During the 5th week period, DIO prone rats consumed 9% more of chow than DR and as a result, gained 19% more body weight. Ghrelin levels also seem to differ during the onset of the dark period between the two groups whereby DIO displayed 29% lower plasma ghrelin levels. Despite these significant changes in behavioral measures and ghrelin, no differences in leptin and insulin were reported. When animals were acutely exposed to a HFD (HE; 31% fat) at 6 weeks, previous behavioral measures on the standard chow were amplified. DIO rats were reported to consume 70% more HFD and contrary to the standard chow diet, exhibited significantly higher insulin and leptin levels than DR rats. These data suggest a critical period in development were rats are vulnerable to develop

abnormal regulation of appetitive hormones such as ghrelin, leptin and insulin. Subsequently, rats prone to DIO are more likely to significantly increase appetite and body weight during this critical period. Some ghrelin deletion studies echo similar results during this critical 5-7 week period of development (Cowley et al., 2003; Sun et al., 2004).

A recent study conducted by Chen, Wang, et al., (2012) examined the long-term effects of different macronutrient diets including both high-fat diet and high-protein diet in Sprague-Dawley rats. Pregnant or lactating females were fed either one of these diets or standard chow and offspring continued these same diets post-weaning. Plasma ghrelin, insulin and glucose measures were investigated at several time points in the off-springs development including the first, third, seventh, and tenth postnatal days (Chen et al., 2012). Also, these same tests were repeated on the same animals towards the end of second, third, fourth, eighth, and twelfth week (Chen et al., 2012). In accordance with previous studies, rats in all three groups exhibited a negative correlation between ghrelin and glucose level. However, only within the high-fat and high-energy group, did rats display a negative correlation between plasma ghrelin and plasma insulin relative to WT rats (Chen et al., 2012). Hence, long-term diets including high fat or high-protein content both negatively affect the relationship between insulin and ghrelin expression within the plasma.

Other models include; the New Zealand Obese (NZO) mouse strain which represents a good model of hyperphagia and reduced energy expenditure, the Tsumura Suzuki Obese Diabetes (TSOD) Mouse which become obese despite not developing diabetes because of their increased beta cell mass and good insulin maintenance secretion (Iiuzuka, et al., 2005), the M16 mouse which exhibit hyperphagia, hyperinsulinemia, and hyperleptinemia compared to controls (Allan et al., 2004) and finally, the Kuo Kondo (KK) mouse which is widely used for testing

experimental therapies in obesity and diabetes research (Okazaki et al., 2002). Finally, Otsuka Long Evans Tokushima Fatty (OLETF) Rats established in Japan became an obesity and diabetes model. Males tend to display hyperplasia of pancreatic islets at 25 weeks of ages and at 60 weeks of age, islets become atrophic (Kawano et al., 1994).

Ghrelin and Ghrelin Receptor mutant mice

A number of genetically engineered rodent models have emerged in the past 10 years to better understand the physiological significance of the ghrl/GHSR system. These models have been tremendously useful, although they have a number of limitations. The first transgenic rodent used for the study of ghrelin was a genetically modified rat with an antisense sequence aimed at reducing GHSR expression that was under the control of the tyrosine hydroxylase promoter (Shuto et al., 2002). Western blot and immunocytochemistry analysis were performed to confirm lower GHSR protein levels in the ARC within transgenic (Tg) rats in comparison to non-Tg littermates. The tyrosine hydroxylase (TH) promoter is usually active within GHRH-containing neurons in the Arc (Niimi et al., 1992). Therefore, the aim of this study was to disrupt the function of GHSR within the Arc and discover the function of the GHSR gene. Shuto et al., (2002) reported that these transgenic rats had in fact, lower body weight and less adipose tissue within relative to WT controls.

Soon after, several reports were published describing the phenotype of mice with genetic mutations to the ghrelin and the GHSR gene (Appendice 1). There were three labs that generated these lines of mice independently (Sun et al 2003; Wortley et al., 2004; Zigman et al., 2005). While these mice were generated in different ways, they all shared a common phenotype under normal conditions (Sun et al., 2008). The phenotype of both GHSR and ghrelin KO mice was surprisingly similar to that of their WT littermates (Sun et al., 2008). However, Ghrl and GHSR

KO mice did show resistance to gaining weight under a high fat diet regimen, and showed less adiposity in body composition under normal conditions (Zigman et al., 2005). Moreover, GHSR and Ghrl KO mice displayed differences in spontaneous locomotor activity compared to their WT littermates (Blum et al., 2009). Both of these genotypes are enhanced in mice with mutations to both the ghrl and the GHSR gene. When animals are exposed to negative energy balance, GHSR KO displayed lower glucose levels suggesting better insulin efficiency and glucose clearance (Sun et al., 2006; Cowley et al., 2005). Interestingly, the offspring of GHSR KO mice mixed with leptin deficient Ob/Ob mice, while still overweight, showed improvements in glucose homeostasis. The fact that the GHSR and ghrelin KO mice failed to produce a clear-cut metabolic profile tended to discourage ghrelin research. Some neuroscientist came to the conclusion that ghrl was a redundant mechanism within appetite regulation (Sun et al., 2003; Wortley et al., 2004). Nevertheless, other scientists such as Zigman et al. (2005) persisted to generate their own GHSR KO mice and discovered otherwise. Perhaps some explanations derive from the various mice strains generated throughout those transgenic mice studies and the ways in which the deletion was manifested. Other supplementary reasons could include the different concentration of fats within the HFD mice were subjected to as well as the critical age period in which they were initially exposed to HFD. Also for these inconsistent results, it is possible that these mice, given their lack of ghrelin throughout their life span, developed compensatory mechanisms that allow them to deal with the lack of ghrelin or its receptor without obvious challenges. In fact this has been found to be the case with mice that mutations to the NPY or the AgRP genes (Higushi, Niki and Shiiya; 2008). When mice were kockouts from birth, reports suggest no particular distinguishable phenotype. However, when mice are manipulated within adulthood, these mice without NPY/AgRP display a strong anorexic phenotype, at times fatal.

Despite these compensatory mechanisms, certain defects have been reported in GHSR and Ghrl KO mice and these include deficits in motivated behaviors for food (Nogueiras, Tschop & Zigman; 2008), manifested through reduced locomotor responses to scheduled meals (Blum et al., 2009; Abizaid; 2006), reduced locomotor responses to psychoactive drugs like cocaine or nicotine, and reduced intake of alcohol (Clifford, Rodriguez, Schul, Hughes, Kniffin, Hart, Etain, Brunel, Fehrentz, Martinez and Wellman; 2011). In addition, these GHSR KO Ghrl KO mice appear to have learning and memory deficits (where ghrelin typically neuroprotects cognitive functions), altered responses will resume once subjected to acute stress. However, they are reported to become prone to depressive like states following chronic stress (Patterson et al., 2010).

1.5 Hypothesis: Validating the GHSR KO DIO Rat Model

GHSR KO rat model.

One of the disadvantages working with mice is that their behavior is more difficult to examine than that of rats. In general most behavioral tasks with mice require substantial amount of training, handling, and in some cases tasks that work well in rats are not ethologically valid in mice. This has posed a number of limitations to the studies of behavior in mice with genetic deletions to the ghrelin and ghrelin receptor gene. Most notably, the ability for rats to learn and perform more complex behavior tasks is significantly greater than that in mice.

Recently, a rat strain with a mutation to the promoter region of the GHSR gene became available commercially. The availability of this strain has been met with enthusiasm by several labs and there are two early reports showing that these rats, like ghrl and GHSR KO mice, have attenuated responses to cocaine and nicotine (Clifford et al., 2011;Wellman, et al., 2011). As

expected these rats do not show feeding responses to ghrelin treatment. Nevertheless, this GHSR KO rat line and its background strain remain poorly characterized.

Given the phenotype of the background Fawn-Hooded Hypertensive (FHH) strain, it is uncertain what kinds of metabolic responses exist in FHH-Ghsr^{m1Mcwi} (see methods for details). The present study was conducted to examine the metabolic profile of FHH-Ghsr^{m1Mcwi} in comparison with that of FHH wild type rats under normal conditions and following exposure to changes in food availability, and after prolonged exposure to a high fat diet. We hypothesized that, like GHSR KO mice, rats with a mutation to the GHSR would eat less and show attenuated feeding responses to a fast than their WT counterparts, and that they would gain less weight and show altered glycemic responses to a glucose challenge. Finally we expected that these GHSR KO rats would show altered hypothalamic expression of peptides of the melanocortin system and circulating metabolic hormones promoting a leaner phenotype than WT rats. To test this hypothesis, we conducted 3 behavioral experiments and analyzed 2 further experiments following sacrifice. The experimental procedure is illustrated in Figure 1.

2 Methods

2.1 Fawn Hooded Rat Background Strain

As mentioned, the background strain that was used to generate this rat model is fairly uncommon. In this case, the strain chosen was the fawn hooded rat, a line established as an outbred stock in Kröning, Göttingen from rats of unknown antecedents (although most likely Wistar and Long-Evans). Following a couple displacements, they finally were established in Hannover, Germany in 1968 (F16). They remained outbred until the mid-1980s when the colony was relocated to Erasmus University in Rotterdam, Netherlands. At this point, Dr. A.P. Provoost generated the standard brother x sister inbred generations. A minimum of 20 brother x sister

generations is necessary to obtain a level of residual heterozygosity that is on average less or equal to 0.01 (Green, 1981). From this process, two strains emerged. One became known as the fawn hooded hypertensive rat (FHH) where the other was labeled fawn hooded low blood pressure (FHL).

It was not until the 1990s that the FHH rat made its way to the Medical College of Wisconsin for future North American studies. Dr. Provoost's strain was used to develop a panel of consomic rats with FHH as the background strain and Brown Norway chromosomes introgressed into the FHH background. From this background, mutant strains were developed primarily using N-ethyl-N-nitrosourea (ENU) mutagenesis where male founders are injected. This chemical is an alkylating agent and is a highly potent mutagen. It essentially manifests its functions by targeting spermatogonial stem cells found in mature sperm. (Ethylnitrosourea-Compound Summary; National Center for Biotechnology Information, 2012). For the GHSR gene, ENU can induce 1 new mutation in every 700 gametes. Once bred with females, the pups are controlled and screened to detect and confirm the proper GHSR gene was in fact targeted to possess ENU-induced mutations. This is conducted using an enzyme-based heteroduplex cleavage assay termed TILLING assay (Medical College of Wisconsin). Both the GHSR KO and wild type FHH rats were developed using ENU mutagenesis. Medical College of Wisconsin happens to be the supplier for Biotransposagen pharmaceuticals service in Kentucky, USA, and the source for the animals in our particular study.

The full sub-strain abbreviation of fawn hooded hypertensive rats begins with the core symbol of the original strain followed by an abbreviation of where the strain is allocated. These two subsets of information are separated by a forward slash (e.g., FHH/EurMcwi; substrain derived at Medical College of Wisconsin Institute (Mcwi) from the colony maintained in Europe.

According to the Rat Genome Database, the symbol representing our particular GHSR KO mutation includes FHH-Ghsr^{m1Mcwi} to represent mutation 1. This mutation generated by ENU occurs in the codon CAG/TAG, which changes the AA q343Stop of the GHSR gene (Rat Genome Database, 2012).

The FHH background strain is known for developing hypertension and kidney complications in late adulthood and generally has a shorter life span than most common laboratory rats strains. These consequences ultimately lead to shorter lifespan and may include focal and segmental glomerular sclerosis, pulmonary and systemic hypertension and proteinuria (excessive serum protein levels in urine) at a young age.

FHH rats also tend to express a platelet storage pool deficiency due to the red-eyed yellow allele r (Suckow et al., 2006). Platelets aid the formation of blood clots and prevent excessive bleeding to occur by coagulating. Platelets also release several growth factors, which play a role in regenerating connective tissues. Therefore, when platelets are forcibly excreted from the circulation quicker than the bone marrow's production, thrombocytopenia occurs. Because FHH rats develop dysfunction in filtering blood to form urine, spontaneous or excessive bleeding arises. Furthermore, Margo et al., (1986) measured the differences in urine secretion between hypertensive fawn hooded rats in comparison to Wistar rats. Margo and colleagues found a significantly greater amount of catecholamines within the urine of FHH rats. With the administration of an antihypertensive (debrisoquin sulfate) compound, FHH rats blood pressure diminished to normal levels and their catecholamine excretion significantly decreased. Altogether, these findings support a link between higher sympathetic output and hypertension within FHH rat strain suggesting a response that acts primarily on the cardiovascular system

(increased heart rate), mediated indirectly via catecholamines secreted from the adrenal medulla. (Silverthorn and Unglaub; 2009).

Furthermore, Tordoff et al., (2010) published an extensive observational set of experiments deciphering tastes preferences among 14 rat strains. Rats were subjected to 17 different taste compounds and of those, FHH rats showed significant preference for water, ethanol, saccharin, sucrose, and monosodium glutamate (MSG) (while food intake remained similar to other groups). However, FHH rats failed to exhibit any significant differences with citric acid, capsaicin, or corn oil consumption in comparison to the other 13 strains. These findings suggest FHH rats desire salty, sweet, alcoholic beverages and are less inclined to consume high fat/high caloric, sour, and bitter compounds. The same study conducted by Tordoff et al., monitored body weights for both male and female rats of all strains and recorded FHH males the 6^{th} lightest rat among 14 strains; weighing less than half the weight of the heavier Long Evans rats. This study occurred over a period of 48 weeks. Consistent with Tordoff's study, low FHH rat body weights were also reported by Wang et al., (1988).

Additionally, Altemus et al., (1994) discovered higher amounts of freezing behavior in response to stress within FHH rats compared to Wistar rats. Through in situ hybridization, quantification of corticotropin releasing hormone (CRH), arginine vasopressin (AVP) and noradrenergic stress response and arousal systems were examined in FFH rats. Subsequently, FHH rats displayed elevated levels of CRH mRNA in the central nucleus of the amygdala and lower levels of CRH mRNA in the paraventricular nucleus of the hypothalamus. In addition to this, FHH exhibited significantly lower adrenal weights compared to Wistar rats despite no differences in corticosterone levels. These results suggest a depressed hypothalamic-pituitary-

adrenal axis activity, which may explain some of the systemic and behavioral traits these FHH rats display.

2.2 Animal Care

Male rats with targeted mutations to the ghrelin receptor gene (GHSR KO) and their WT littermate controls were bred at the Carleton University Neuroscience Institute animal facilities Overall, the GHSR KO rats were seemingly of normal size and their behavior under normal laboratory housing conditions were indistinguishable. All methods followed the guidelines of the Canadian Council on Animal Care and approved by Carleton University's Animal Care Committee. Rats were from breeding pairs originally obtained from Transposagen Bio (Lexington, KY, USA). Animals were kept under a 12-h light/dark cycle with the onset of light set at 08:00 hr. In all experiment conditions, both groups were provided full access to food unless otherwise stated. To verify genotypes and that the GHSR KO group were in fact missing the GHS receptor function, the DNAeasy kit was conducted on adipose tissue samples from both groups following the procedural guidelines provided by the kit and using primers detecting the presence of the GHSR.

2.3 Generation of GHSR KO rats.

Fawn-hooded hypternsive (FHH)-Ghsrm1/Mcwi {[[GHR-R (-/-)] strain were created by the PhysGen Program in Genomic Applications through ENU mutagenesis as described above. This rat line was obtain from Transposagen Biopharmaceuticals, Kentucky, USA –and Medical College of Wisconsin, USA.

2.4 Experimental Design

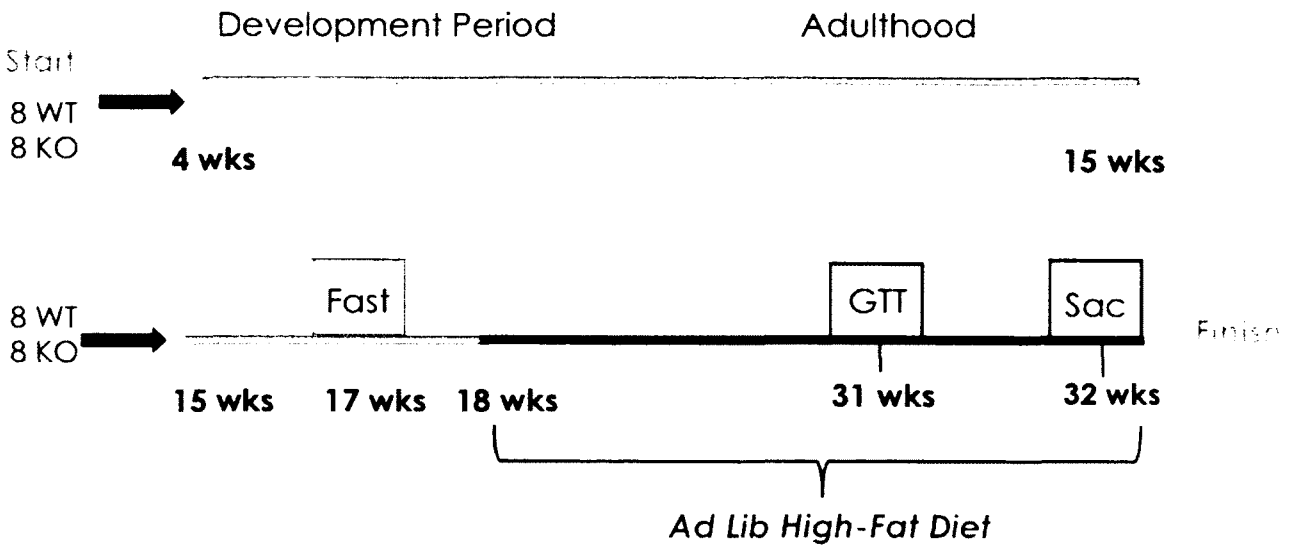

Figure 1. This experimental design encompasses the first three experiments. The last two experiments involve brain and hormonal profile analyses.

Experiment 1

Differences in food intake and body weight between GHSR KO and WT rats in developmental conditions.

In this study, a cohort of age-matched rats including GHSR KO and WT (N=16; WT n=8, KO n=8) were placed on standard chow at 3 weeks post-weaning and remained on this diet for 3 months. We examined food intake and body weight in GHSR KO and WT rats over this long-term period. Both parameters were assessed 5 days/week throughout this time course. Rats were single-housed and monitored daily for standard chow intake (Teklad Global 14% Protein Rodent Maintenance Diet 2014; Harlan Laboratories) and body weight gain. The total caloric content of this diet was 3.4 kc.al/g (Appendix B). These food intake and bodyweight measurements were recorded daily using an electronic scale capable of measuring weights reliably and accurately to the .1gm level (Fisher Scientific) with an error of ± .05 gm.

Experiment 2

Differences in standard chow intake and body weight in adulthood

Before the diet transition to HFD, rodents were given an extended baseline of 3 weeks on continued standard chow where food intake and body weight was continuously measured daily.

Experiment 2.1

Overnight fasting challenge.

On the evening of day 13 of this baseline, an overnight 16 hr fast was conducted. All animals had their food removed overnight until 8:30am on Day 14. At this time, food was returned to their cage and the rebound feeding responses of all rats were measured for the next six hours (30 min, 1hr, 2hr, 4hr and 6hr) to determine differences in rebound feeding between WT and GHSR KO rats.

Experiment 3

Differences in food intake and body weight between GHSR KO and WT rats under chronic high-fat diet exposure in adulthood.

Following experiment 1, GHSR KO and WT FHH rats had their normal chow removed after 3 weeks and were now exposed to a high fat diet (60%cals from fat; Open Source Diets). In order to examine the role of GHSR on long-term body weight homeostasis, we challenged GHSR KO rats to a western HFD for 3 months. This diet contained a total of 5.24 kcal/g of energy density (Appendix C). As in experiment 1, daily body weight and food intake measurements were recorded. In addition to these parameters, on one week before sacrifice, both GHSR KO and WT rats were subjected to a Glucose Tolerance Test.

Following the study, body weight composition was assessed through carcass analysis. This enabled to determine if body weight gain was attributed to excess body fat within their body composition.

Experiment 3.1

Glucose Tolerance Test (GTT).

As previously mentioned, a week before sacrifice, GHSR KO and WT were fasted overnight and administered an intraperitoneal (ip) bolus injection of glucose (2g/kg) mixed in saline to test for glucose clearance. Blood glucose was measured at intervals of 15 min, 30 min, 60 min, and 120 min following the injection, using a Bayer glucose meter on samples obtained from the tails of the animals using conventional needles included in the glucose testing apparatus. Glucose tolerance was assessed by calculating the area under the curve of the glucose excursion curves. The body weights were also measured at 24 and 48 hr after refeeding.
A week later, animals were again fasted overnight and sacrificed by rapid decapitation. At this time, glucose was also measured to determine fasting glucose levels at time of decapitation.

Sacrifice.

At the time of sacrifice, various tissues were obtained including brain (fast freezed), liver, white adipose tissue, and muscle. Additional blood samples were also collected to measure blood concentrations of free fatty acids and metabolic hormones like leptin, insulin, growth hormone, PYY, and active ghrelin. For the purpose of this thesis we will only discuss the processing of brain tissues and blood samples.

Experiment 4

Differences between GHSR KO rats and WT rats in the expression of genes encoding for hypothalamic metabolic peptides.

Immediately after sacrifice, the brains from all rats were removed from the skull and the hypothalamic area were rapidly dissected and processed for RT-qPCR to observe any differences between GHSR KO rats and WT rats within appetite regulating mRNA expression in the ARC. Transcripts for the GAPDH and beta actin protein were used as controls. Total RNA of cells was isolated using TRIzol Reagent (Invitrogen, Carlsbad, ca, USA), following the manufacturer's instructions. The cDNA was synthesized from 1ug RNA using the SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time RT-PCR was performed on an ABI 7900 using the SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). After amplification, the PCR product was subjected to 2% agarose gel electrophoresis. GAPDH and beta-actin were used as internal controls. All primer and probe information are depicted in (Appendix D). RNA was treated with DNAse and ran on the Nano drop to quantify the concentration of RNA in each solution sample. The ratio of sample purity (260/280) and spectrophotometer absorbances between 1.8 and 2.0 nm wavelengths are the accepted standards we used for measuring purity of RNA extractions in these samples.

Experiment 5

Differences between GHSR KO rats and WT rats in and in circulating metabolic hormonal profile.

Blood was collected after an overnight fast using EDTA-coated Microvette tubes (Becton, Dickinson and Company (BD) and immediately chilled on ice. After 15 minutes of centrifugation at 3000g and 4°C, plasma pipetted and stored at -80°C.

Rat Gut Hormones.

To determine the levels of metabolic hormones at the time of death, we used Millipore's Milliplex Rat Gut hormone MAP Panel (Billerica, MA), a kit that allowed us to quantify many

gut hormones simultaneously. Our hormones of interest were insulin, leptin, pancreatic polypeptide and total PYY found within rat plasma. Milliplex MAP is based on the Luminex Map technology where bioassays occur on the surface of the fluorescent-coded beads termed microspheres. After being processed by the method provided by the kit and based on the intensity of the fluorescent dye on the reporter molecule, the program is able to identify and quantify the amount of insulin, leptin and PYY found within the rat plasma. All samples were measured in duplicate.

Active Ghrelin.

To selectively quantify active ghrelin in our (WT n=5, KO n=5) plasma samples, the Millipore Rat/Mouse Ghrelin (active) ELISA kit (Billerica, MA) was conducted by the method provided with the kit. This assay is characterized by a Sandwich ELISA type, which initially catches the active form of ghrelin within the plasma sample with anti-human ghrelin IgG. From the standard curve measures, we were able to calculate and identify the concentration of active ghrelin (pg/mL) in our unknown samples. Within this particular assay, the typical amount of active ghrelin should range between 25pg/mL to 2,000 pg/mL. All samples used in this assay were tested in duplicate.

Plasma Triglycerides.

In order to examine differences in plasma levels of triglycerides we used the Millipore Adipolysis Assay kit (Billerica, MA). This is a standard method to determine triglyceride concentrations, and it involves enzymatic hydrolysis by lipase of the triglycerides to glycerol and free fatty acids. The glycerol produced is then measured by coupled enzyme reactions. To quantify the amount of triglycerides in the sample, a colorimetric or fluorometric measurement of the glycerol released is used. A higher absorbance at 540 nm is directly proportional to

triglyceride concentration of the sample. Samples were assayed in duplicates.

Growth Hormone.

To detect and quantify Growth Hormone in our rat plasma samples (N=10), we used the Rat/Mouse Growth Hormone ELISA kit from Millipore (Billerica, MA) by the method provided with the kit. We expect to see GHSR KO with less Growth Hormone compared to WT littermates. Growth Hormone concentrations in our plasma samples can be interpolated onto the standard curve and analyzed.

3 Results

Experiment 1 Behavioral characteristic of the FHH rats during Developmental Period.

Food intake.

A 2X11 one-between-one-within ANOVA on rats cumulative food intake was conducted with Genotype (GHSR KO, WT rats) as the between subjects factor and Weeks (Week 5 to Week 15) as the within subjects factor. The results showed no main effect for genotype F (1, 14) = .168, $p > .05$. There was a main effect for weeks, F (2.210, 30.936) =38.989 $p < .0001$. There was no genotype X week interaction, F (2. 210, 30.936) = 1.295, $p > .05$ (figure 2a).

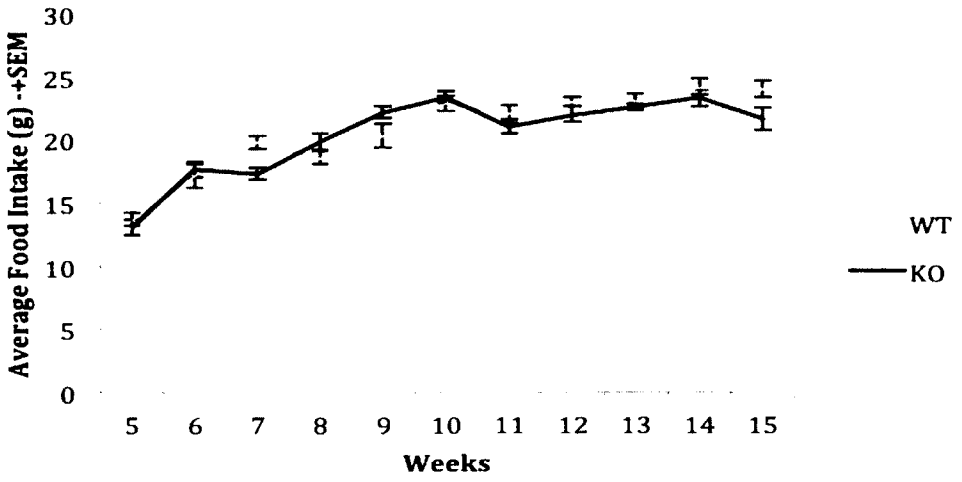

Figure 2a. Food intake measure from 4 weeks to 15 weeks of age with ad libitum standard chow conditions.

Body Weight.

A 2X12 one-between-one-within ANOVA on rats cumulative body weight was conducted with Genotype (GHSR KO, WT rats) as the between subjects and Weeks (Weeks 4-15) as the within subjects factor. The results showed a significant main effect for genotype, F (1,14) = 9.465, p = .008, and a significant main effect for weeks, F (2.412, 33.769) =2727.389, p < .05. GHSR KO rats accumulated significantly less weight over the course of the 11 weeks. There was no significant genotype X week interaction, F (2.412, 33.769) =1.416, p > .05 (Figure 2b)

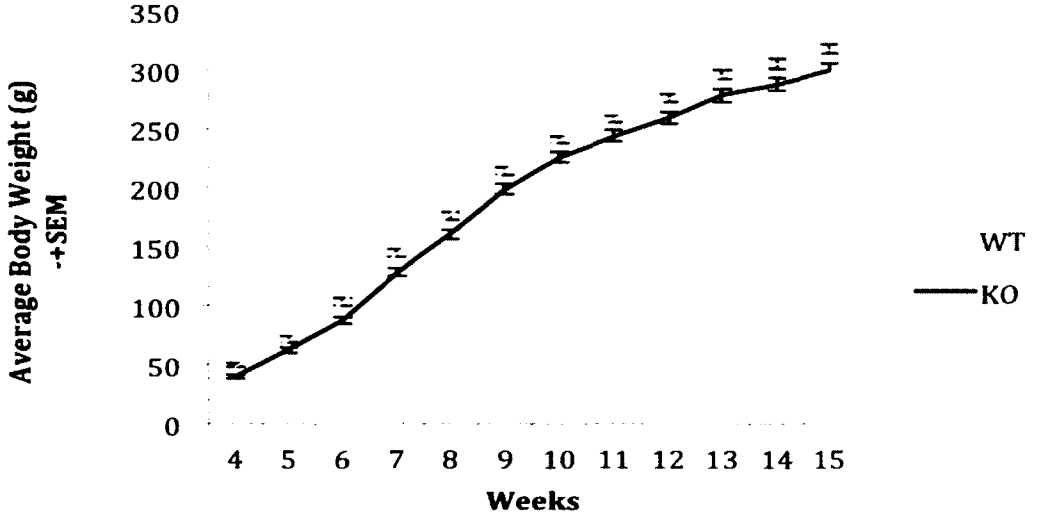

Figure 2b. Average body weight of GHSR KO and WT rats between weeks 4 and weeks 15 old with ad libitum standard chow diet.

Body Weight.

Following the first 4 months, WT control FHH rats (M=204.20 SD=7.80) weighed significantly more diet than the KO (M=189.51 SD=10.80), t (14) = 3.12 p > .05 (Figure 3) with ad libitum standard chow (Figure 2b).

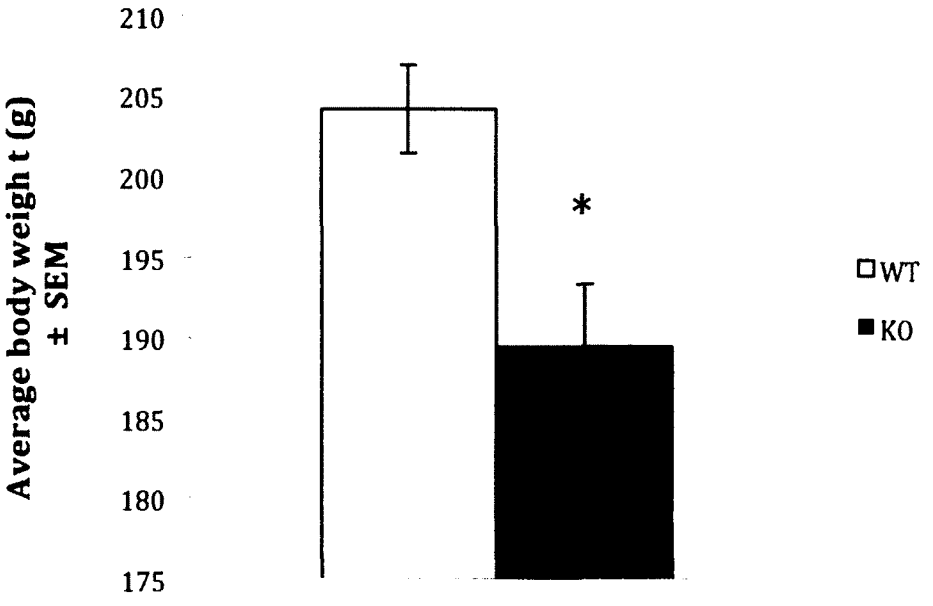

Figure 2c. Body weight following 4 months post-natal with ad libitum standard chow conditions.

Experiment 2 Behavioral characteristics of the FHH rats after 4 months of age.

Reduced chow intake in GHSR KO rats.

Food intake was analyzed after once it was normalized to account for body weight differences between the groups at the onset of 4 months of age. When normalized to body weight, an independent groups t-test revealed that GHSR KO rats (M = 0.082, SD = 0.005) did

not significantly consume less chow than WT rats (M = 0.087 SD = 0.005), (Figure 3) t (14) = 1.5663, p = .11.

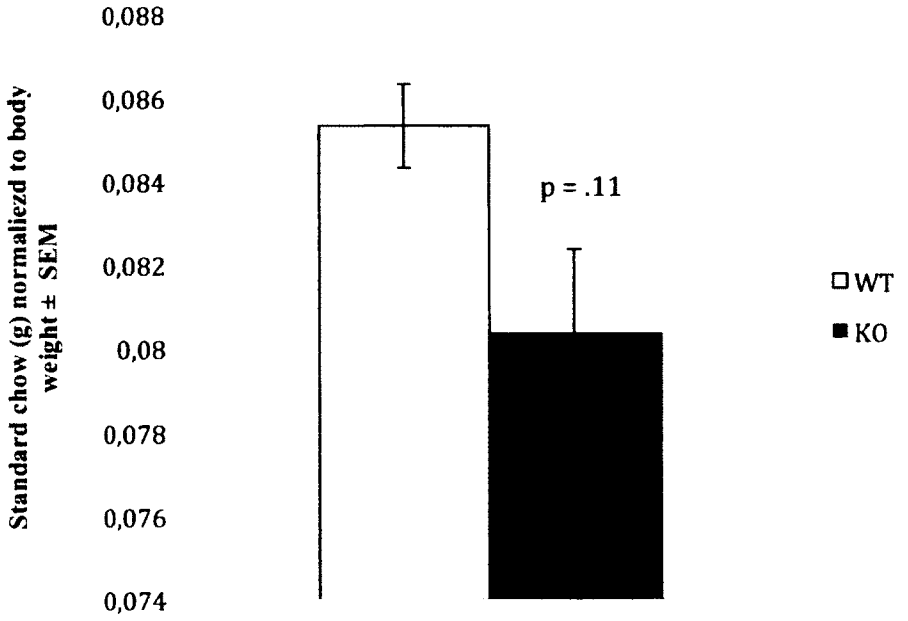

Figure 3. GHSR KO consumed the same amount of chow as WT littermates in adulthood.

Reduced body weight of GHSR KO rats when subjected to chow in adulthood.

In adulthood, GHSR KO demonstrated significant changes in terms of body weight. GHSR KO (M=289 SD=16.97) weight significantly less than WT (M=307.63 SD=12.32); (Figure 4a-4b) t (14) = 2.51 p < .05.

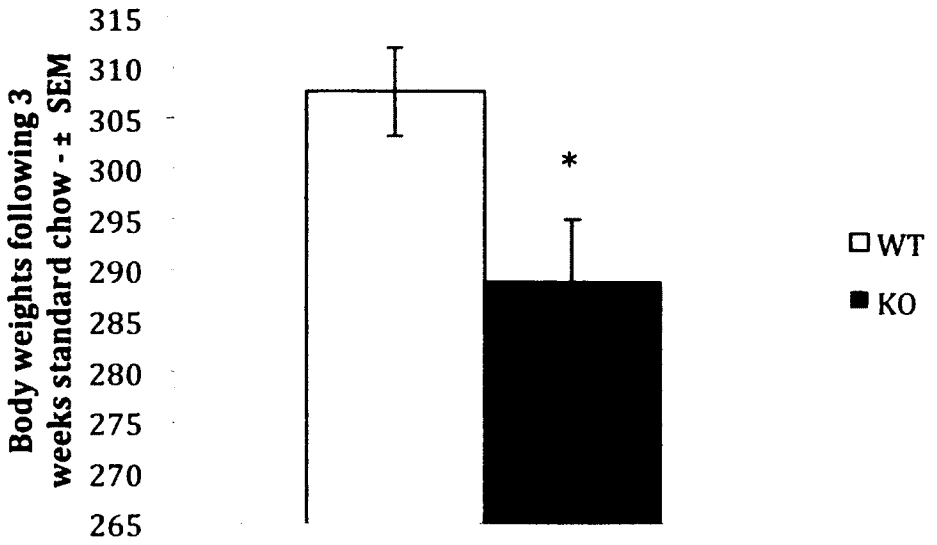

Figure 4a. Average body weight following 3 weeks of standard chow.

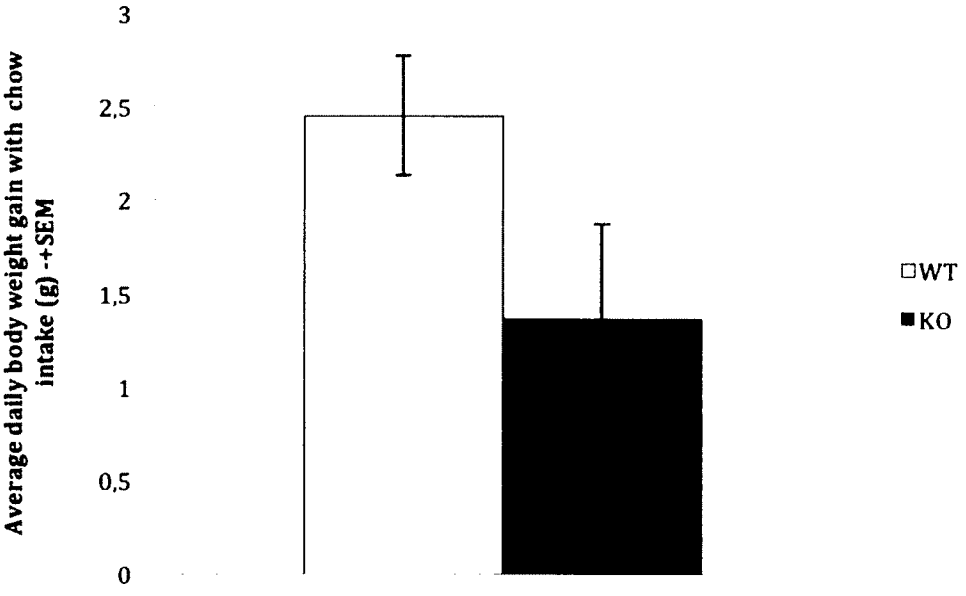

Figure 4b. Daily body weight gain following 3 -week standard chow diet in adulthood.

While on standard chow, the average daily body weight gain of these 16 rats (n=8/group) did not differ significantly between groups although a lower body weight seems to be displayed in the GHSR KO compared to the WT.

Experiment 2.1.GHSR KO rats eat less following an overnight fast.

A repeated measures ANOVA was conducted to examine differences in the intake of food shown by GHSR KO and WT controls after the animals were fasted overnight and subsequently given free access to standard chow diet the following morning. As can be seen in Figure 5, the periodic measurements of food intake following the morning representation revealed a constant lower rebound feeding within GHSR KO rats. Cumulative food intake also suggests GHSR KO display a rebound feeding to a lesser of extent.

As shown in figure 5, the repeated measures was conducted with genotype (WT or GHSR KO) as the between subjects factor and time (30 min, 60 min, 120 min, 4 hrs, 6 hrs) as the within subjects factor. The results showed a significant main effect for genotype, $F(1, 14) = 4.92$, $p < .05$, $\eta^2 = .26$ and a significant main effect for time, $F(2.81, 39.36) = 45.02$, $p < .05$, $\eta^2 = .76$. The results of the unpaired t-tests indicated that GHSR KO ($M = .02$, $SD = .004$) consumed significantly less g/per body weight than WT littermates ($M = .03$ $SD = .009$) at 4 hrs, $t(14) = 1.93$, $p < .05$. In terms of the genotype, GHSR KO rats ate significantly less during rebound feeding than WT littermates. In both main effects, the null hypothesis is rejected. However, there was no significant genotype X time interaction, $F(2.81, 39.36) = .26$, $p > .05$, $\eta^2 = .019$. Thus, the impact of the genotype did not depend on time.

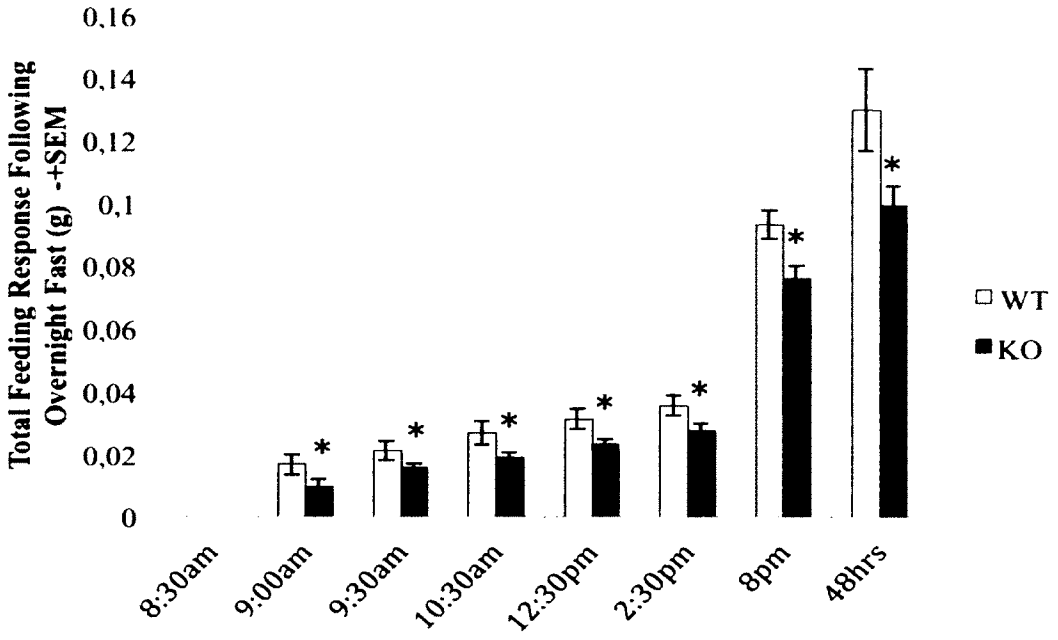

Figure 5. Rebound feeding measures between GHSR KO and WT.

Experiment 3. Behvioral characteristics of adult WT and GHSR KO rats when chronically subjected to HFD.

Reduced HFD intake in GHSR KO rats.

In order to examine the physiological significance of GHSR on long-term body weight homeostasis, we challenged GHSR KO rats and wild-type littermates to a Western-type HFD for 3 months. As done when the animals were fed regular chow, caloric intake was calculated in proportion to body weight. When accounting for body weight, GHSR KO rats (M=0.043 SD=0.006) exposed to the HFD exhibited consumed significantly less calories than their WT controls (M=0.039 SD=0,0008), t (14) =3,024; $p < .05$; (Figure 6-7).

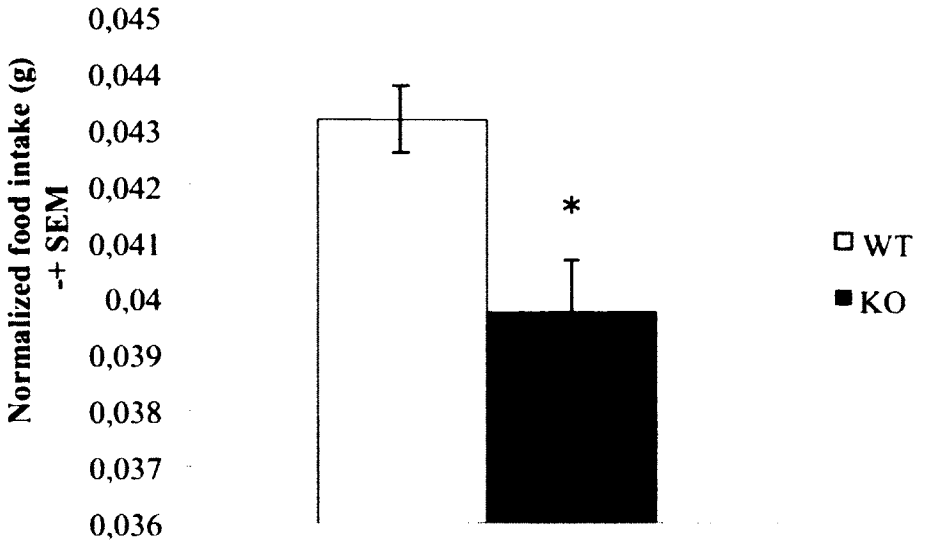

Figure 6. Accounting for body weight, GHSR KO consumed significantly less HFD.

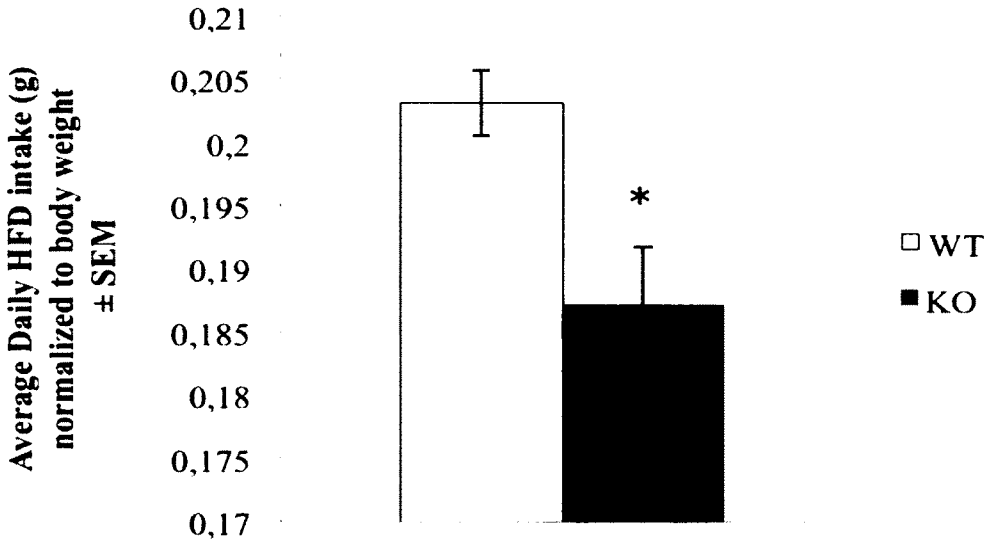

Figure 7. Total Daily average HFD intake.

Differences between groups in body weight gain when subjected to HFD.

GHSR KO (M=64.94 SD=19.84) displayed a slight decrease in resisting body weight gain within the chow context compared to WT (M= 79.63 SD=19.91); (Figure 8) t (14)= 0.9901, p = .1440.

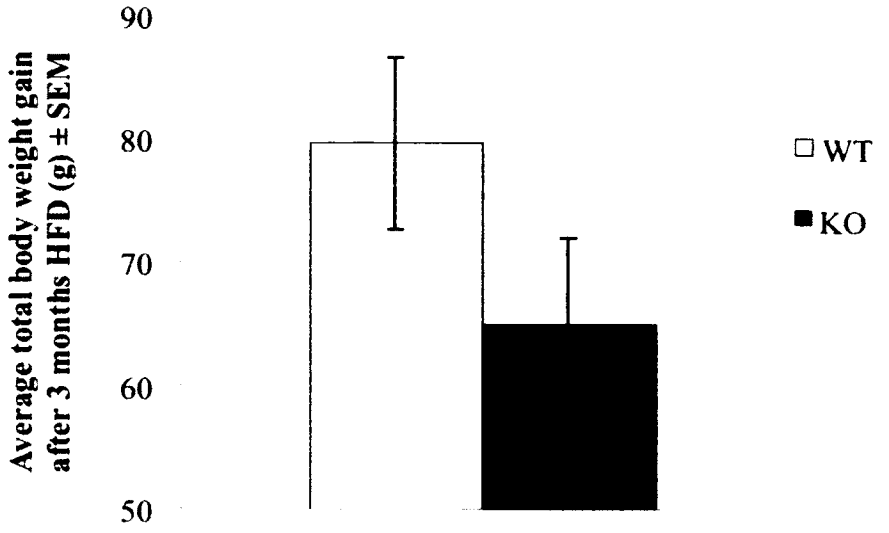

Figure 8. Total average body weight gain following 3 months exposure to HFD.

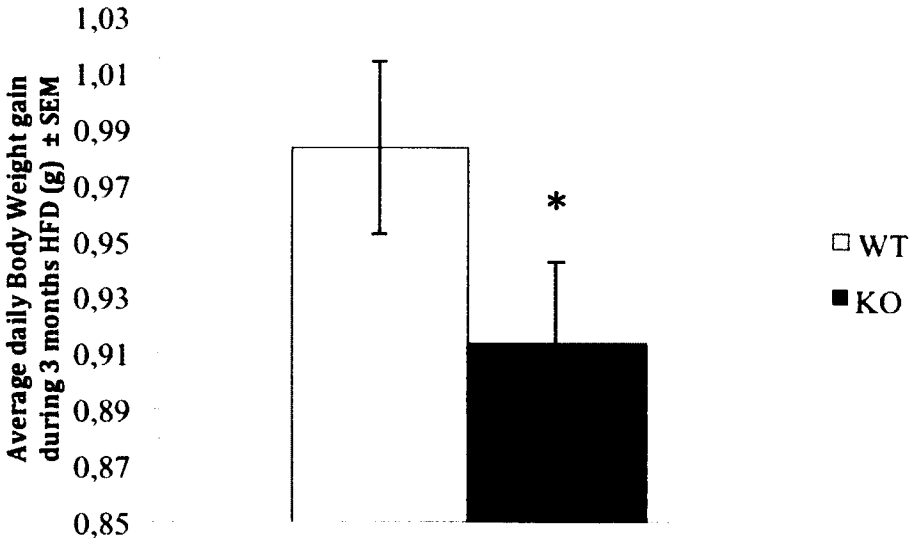

Figure 9. Average daily body weight gain over the course of three months of HFD.

Differences in body composition.

No significant differences were observed between groups in body composition comparing fat mass between groups. As shown in figure 10, Visceral fat: WT (M= 18.50 SD= 1.67) KO (M= 19.52 SD= 2.20) t (13) = 1.02 p > .05; Retroperitoneal fat: WT (M= 12.82 SD= 2.34) KO (M= 12.71 SD= 1.28) t(13) = 0.11 p > .05; Subcutaneous fat: WT (M= 23.18 SD= 3.13) KO (M= 26.30 SD= 3.16) t (13) = 1.92 p > .05; Brown fat: WT (M=0.54 SD= 0.13) KO (M= 0.57 SD= 0.15) t (14) = 0.47 p >. 05; Total fat: WT (M=55.04 SD= 6.34) KO (M= 59.09 SD= 6.30) t (13) = 1.24 p > .05.

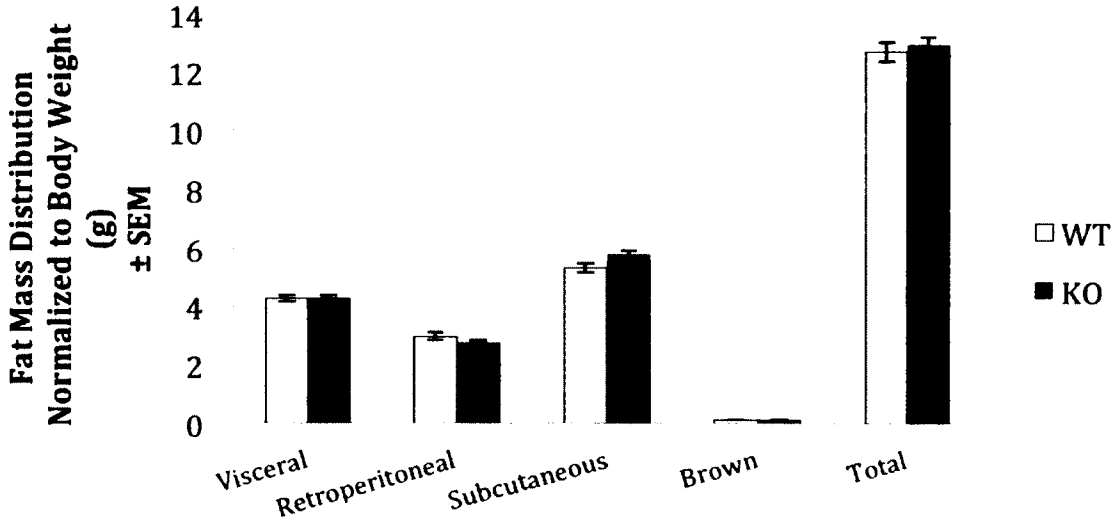

Figure 10. Fat mass distribution across all regions withinWT and GHSR rats.

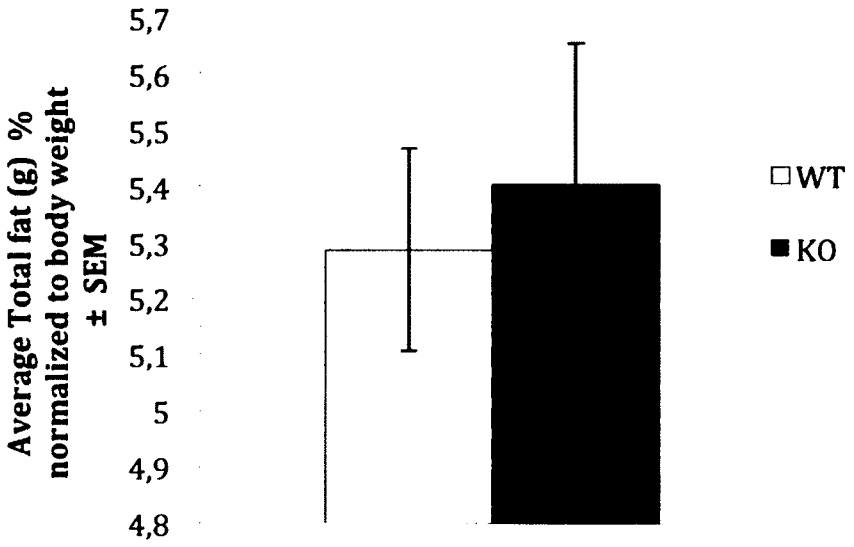

Figure 11 . Average Total fat (g) mass % normalized to body weight (g) -+SEM.

Experiment 3.1 Metabolic characteristics of the adult WT rats and GHSR KO rats when exposed to a GTT.

A repeated measures ANOVA on a particular genotype rat model and GTT was conducted with genotype (WT and GHSR KO) as the between subject factor and AUC time (AUC 0-15, AUC 15-30, AUC 30-60, AUC 60-120, AUC SUM) as the within subjects factor. Results revealed a significant main effect for time, $F (1.092, 15.283) = 394.917$, $p < .05$, $\eta^2 = .96$, while there was no significant main effect for genotype, $F (1, 14) = 1.82$, $p > .05$, $\eta^2 = .12$. Furthermore, there was no significant genotype X time interaction, $F (1.092, 15.283) = 1.53$, $p > .05$, $\eta^2 = .10$ (Figure 12-13).

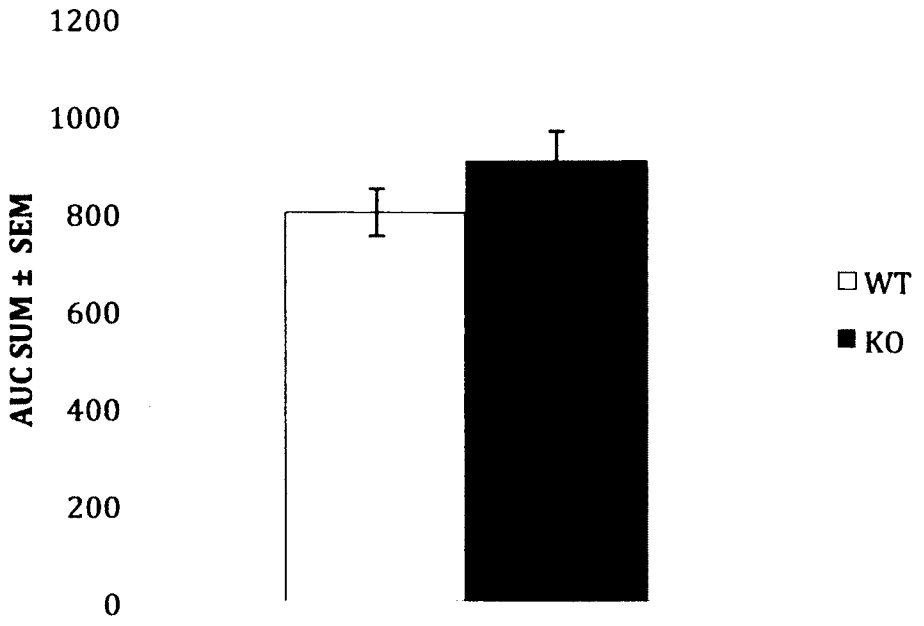

Figure 12. No significant differences found in glucose tolerance test.

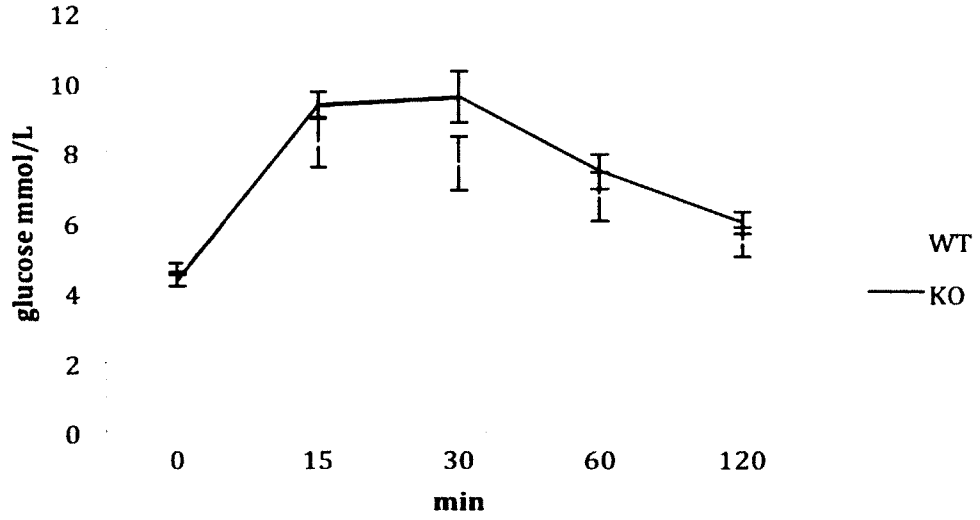

Figure 13. AUC curve following bolus of glucose injection in WT and GHSR KO rats.

Experiment 4 Arcuate Nucleus orexigenic peptide mRNA differences between WT rats and GHSR KO rats

Genetic mutation of ghrelin receptor alters basal expression of hypothalamic neuropeptides AgRP mRNA expression.

Gene expression was examined with qRT-PCR, and results from the assay were examined with independent group t-tests. These analyses showed that brain tissues within the ARC in GHSR KO group displayed a significant 6 fold increase in AGRP mRNA expression in comparison to AgRP mRNA expression (Figure 14) in the hyporthalami of WT controls t (7)= 1,831 , P < 0.05; see Figure 14). The expression of POMC (Figure 15), leptin receptors and NPY mRNA, however, was not different between the groups (p. > .05).

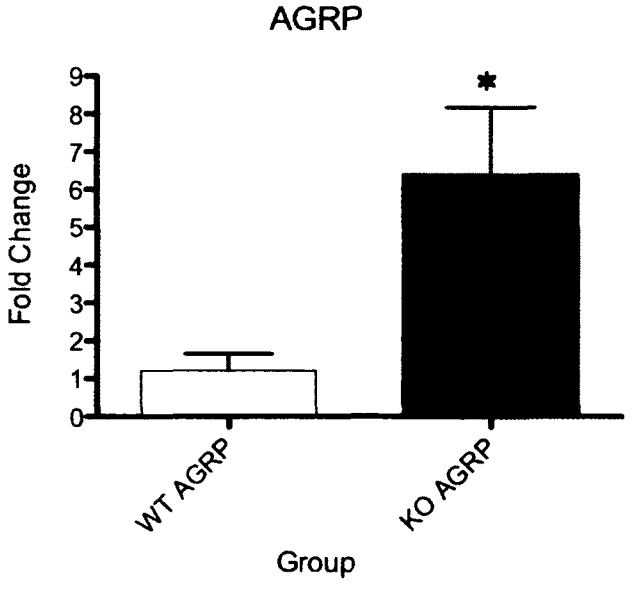

Figure 14. Hypothalamic neuropeptide AgRP mRNA expression in ARC between WT rats and GHSR KO rats.

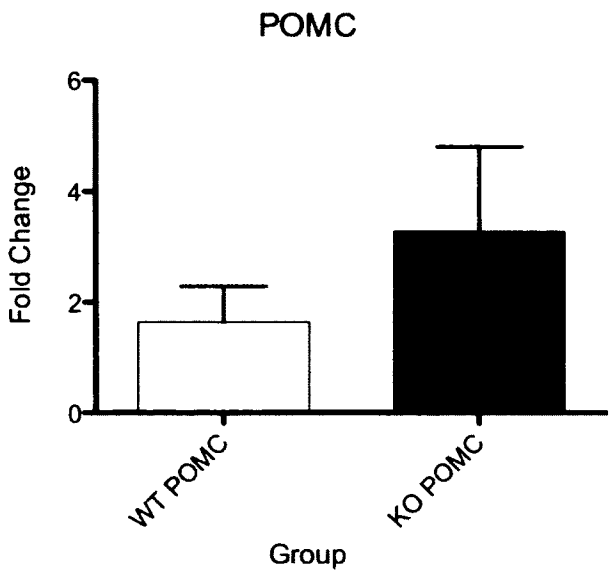

Figure 15. Hypothalamic neuropeptide POMC mRNA expression between WT and GHSR KO.

Experiment 5. Hormonal differences between plasma WT rats and GHSR KO rats.

Fasting Active Ghrelin concentration.

GHSR KO displayed trends of reduced active ghrelin within plasma; WT (M= 130,5 SD=136,1) and GHSR KO (M= 40, 27, SD= 16,35) conditions; t (8)= 1,471, P = 0.0897 (Figure 16).

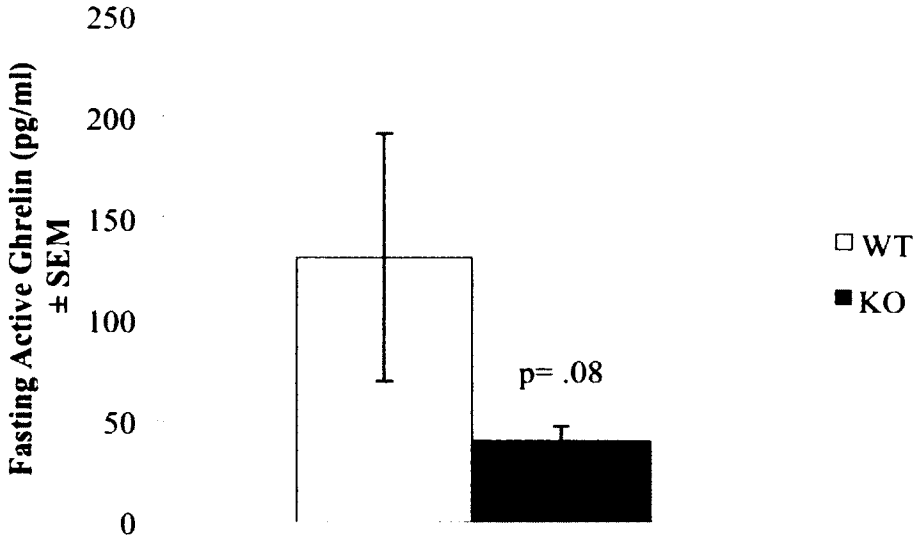

Figure 16. Fasting active plasma ghrelin content between GHSR KO rats and WT rats.

Fasting Glucose concentrations.

No significant differences were found in fasting glucose measures between. WT rats (M =4.53 SD = 0.37) and GHSR KO rats (M=4.16 SD=0.53) (Figure 17); t (14) = 1.58 p > .05.

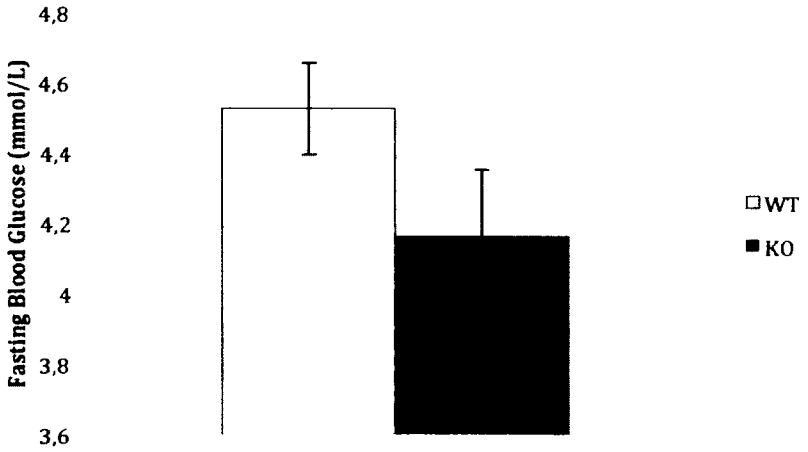

Figure 17. Fasting glucose concentrations between GHSR KO rats and WT rats.

Fasting insulin concentration.

Correspondingly, after 3 months of treatment with the high fat diet intake, the fasting plasma insulin concentration (Figure 18) of GHSR KO rats (M= 2885 SD=393.5) was similar to WT rats (M= 2491 SD=1505) and) conditions; t (12)= 0,6690 , p > 0.05.

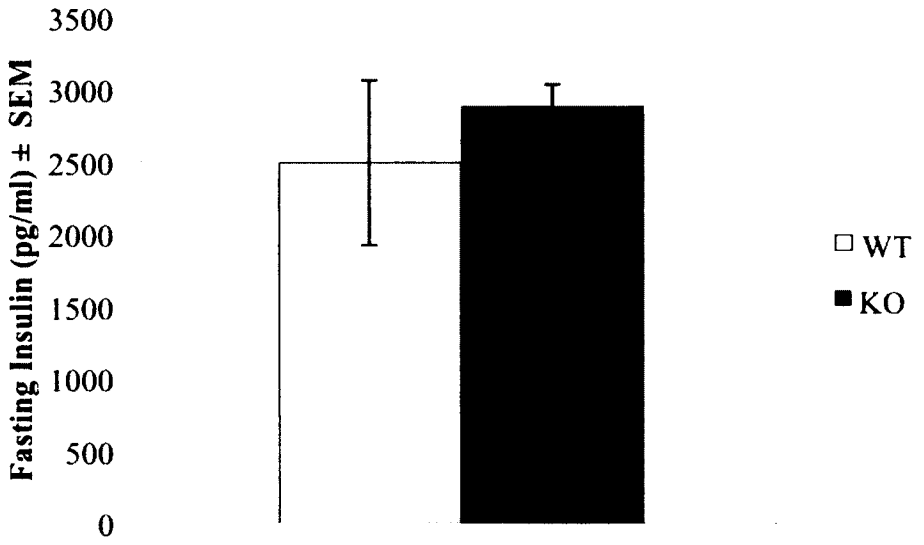

Figure 18. Fasting plasma insulin concentration between GHSR KO and WT.

Fasting leptin concentrations.

Fasting plasma leptin concentrations were similar between WT rats (M= 4974 SD=3147) and GHSR KO rats (M= 3298, SD= 1569); t (12)= 1,261, p = .11 (Figure 21).

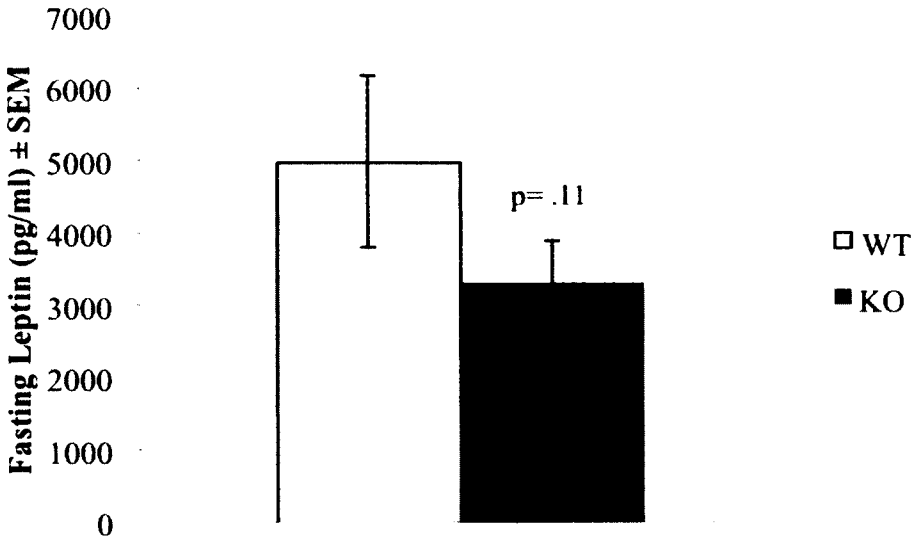

Figure 19. Fasting plasma leptin concentrations between GHSR KO rats and WT rats.

Fasting triglyceride content.

An altered lipid profile was displayed within GHSR KO rats. GHSR KO rats GHSR KO (M= 40,27 SD= 16,35 exhibited a reduction in fasting levels of triglycerides relative to WT rats; WT (M= 156,6 SD= 142); t (8)= 1.486, $p < .05$ (Figure 20).

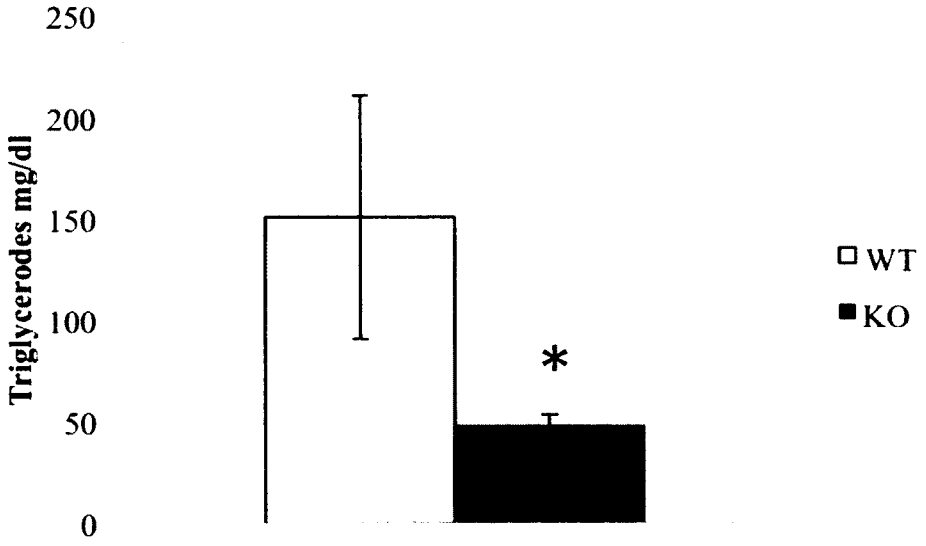

Figure 20. Fasting plasma triglyceride content was decreased in GHSR KO rats relative to WT rats.

Fasting PYY levels.

GHSRK KO rats exhibited no differences in PYY plasma levels relative to WT littermates; WT (M= 84.73 SD=100.7) and GHSR KO (M= 47.04 SD=25.93) conditions; t (12)= 0.9591, P = 0.1782.

Fasting Growth Hormone concentration.

No significnat differences were observed between GHSRK KO and WT rats's plasma GH levels; WT (M= 2.606 SD= 2.342) and GHSR KO (M= 4.255 SD=4.058) conditions; t(12)= 0.9591, $p > 0.05$.

4 Discussion

Experiment 1: Behavioral characteristic of the FHH rats during Developmental Period.

Effect of GHSR genetic mutation on food intake in GHSR KO rats.

GHSR KO rats ate the same amount of food as the WT rats while they were developing. This relationship remained true both in development as well in adulthood. Our results are in accordance with previous Ghrl/GHSR KO mice models previously reported (Zigman et al., 2005; Wortley et al., 2004). Thus, this genetic mutation on the ghrelin receptor did not affect food intake since GHSR KO rats did not eat any less than WT rats.

Effect of GHSR KO genetic mutation on body weight in GHSR KO rats.

When looking at body weight, our results show GHSR KO rats were smaller from weaning until the age of 4 months. When matched for aged, GHSR KO rats weighed less throughout the post weaning periods between 4weeks up until 4 months. As early as the first day of weaning, they were different by approximately 9 g and that difference persisted throughout their developmental period and adulthood.

A second cohort was examined for regular behavioral measures such as food intake and body weight following 4 months for an extended 3 weeks period. In this cohort of rats, GHSR KO rats still gained less weight and consumed less food, when correcting for body weight.

Initial reports of mice with targeted deletions to the ghrelin gene exhibited no changes in feeding behavior or body composition when fed standard chow diet, which was somewhat surprising (Sun et al., 2003; and Wortley et al., 2004). However, additional studies were able to further analyze the metabolic phenotype of mice lacking ghrelin (Wortley et al., 2005b) or GHSR (Zigman et al., 2005; Sun et al., 2004) and discovered that both are resistant to diet-induced obesity when fed a high-fat diet, eating less and preferentially utilizing more stored fat as an energy substrate than wild-type mice. These data lend support to the notion that ghrelin-responsive pathways are an important component of coordinating body-weight control.

Experiment 2-2.1 Behavioral characteristics of the FHH rats during Adulthood Period including an overnight fast.

Despite no differences in chow food intake between GHSR KO rat and WT rats under normal conditions, when challenged with an overnight fast, GHSR KO rats ate less than WT rats. This supports the idea that ghrelin is important for meal initiation and perhaps meal duration. It is possible that satiety signals have a larger effect on meal duration because ghrelin is not present in these animals. Future analysis would benefit from investigating other meal factors that might support this resistance to over eating such as meal rate, and meal duration, or the number and size of meals in one sitting.

Experiment 3. Behvioral characteristics of adult WT and GHSR KO rats when chronically subjected to HFD.

When fed HFD in adulthood, GHSR KO ate less than WT rats. Previous studies have demonstrated ghrelin injections, both peripheral and central, increased adiposity (Tschop et al., 2000; Pierez-Tilve et al., 2011). This is due to ghrelin's preference for glucose as a metabolic fuel (Wortley et al., 2004). For instance, Ghrl KO mice exhibit a lower respiratory quotient than WT mice after only 6 weeks on the HFD diet (Wortley et al., 2004). This suggests that animals without ghrelin, preferentially use fat as fuel. Perhaps this switch in fuel is coordinated through stimulating orexigenic neurons and promoting preferential use of glucose as its fuel with ghrelin intact. When put on a HFD and with a mutation to the ghrelin receptor gene, perhaps a dysfuntion occurs in the activatIon of the hypothalmaic pathway in GHSR KO rats or mice (Briggs et al., 2010). Moreover, another speculation could refer to reports regarding ghrelin's stimulatory actions on the reward-VTA pathway to increase the preference and motivation to obtain palatable food through a Fixed Ratio 1 (FR1) paradigm (King et al., 2011). Nonetheless, these are speculatory explanations and more studies would be necessary for a better undesrtanding on why GHSR KO eat less HFD.

The effect of GHSR KO on body weight and body composition.

Our second cohort of GHSR KO rats gained less weight when fed both standard chow and HFD. Our results support previous studies measuring the intake of regular and HFD in GHSR and ghrelin KO mice (Zigman et al., 2005; Wortley et al., 2004; Wortley et al., 2005; Lin et al., 2011). GHRS KO rats, however, were not different in amount of body fat accumulated than WT rats, even following months of HFD. This similarity between groups could also be due

to the background strain in itself being resistant to body weight gain, as well as failing to show a preference to HFD (Reed et al., 2011). In one study, FHH rats fed a diet similar to the one used in our study gained less weight than rats from a number of other rat strains (Reed et al., 2011). Since they are a normally quite light in comparison to other strains, this may be a confounding variable. In other words, the background strain may mask the mutation to the ghrelin receptor gene and prevent us from observing differences between GHSR KO and WT rats.

Experiment 3.1 Metabolic characteristics of the adult WT and GHSR KO rats when exposed to a GTT.

Interestingly, we found no major differences between GHSR KO and WT rats in the responses to a glucose tolerance challenge. Previous studies have shown that GHSR KO mice exhibit lower glucose levels following a glucose challenge suggesting GHSR KO mice display more efficient glucose clearance and insulin utilization (Sun et al., 2006; Cowley et al., 2005). In addition, we found no differences in fasting plasma content of glucose and insulin. Perhaps, this similarity between GHSR KO and WT rats is due to FHH rats consuming large amounts of HFD and as a result, fail to become obese. Futhermore, FHH rats may be very efficient at metabolizing and managing their glucose levels. Future studies would be necessary to gain a better understanding of their glucose tolerance properties under both conditions of standard chow and HFD.

Experiment 4 Arcuate Nucleus orexigenic peptide mRNA differences between WT and GHSR KO

The effect of the GHSR mutation within the ARC.

Following prolonged exposure to a high fat diet, GHSR KO rats showed increase mRNA expression of AgRP in the ARC compared to WT rats also exposed to a high fat diet. Our results

are similar to previous data reported by Ligen et al., (2012) noting an increase in AgRP and POMC within the ARC of GHSR KO rats. We also examined other gene primers including the leptin receptor (OBRB) and NPY and no significant results were observed. These results have also been reported by Ligen Li et al., (2012). This could be due to compensatory mechanisms, which are explained in detail further along this discussion. Thus, our rat model mimics what occurs in ARC of GHSR KO mice, confirming our rat model is valid to the GHSR KO phenotype in mice previously characterized.

Experiment 5. Hormonal differences between plasma WT and GHSR KO.

The effect of GHSR mutation on fasting plasma active ghrelin.

GHSR KO rats demonstrated ghrelin concentrations that tended to be lower than WT rats. This could be because ghrelin levels are reduced in animals exposed to a high fat diet (Perreault et al., 2003). It is possible that GHSR KO rats are slightly more affected by this than WT rats. A former study observed that both ghrelin secretion and the sensitivity to the orexigenic effects of exogenous ghrelin were reduced in obese mice when compared to lean mice fed standard chow (Briggs et al., 2010). Conversely, the sensitivity to ghrelin is reduced in obesity but improves after weight loss (Perreault et al., 2004). Therefore, our findings coincide with previous studies demonstrating that HFD decreases ghrelin levels in rodents and that plasma ghrelin levels also are lower in obese humans (Handjieva-Darlenska & Boyadjieva 2009; Cummings et al., 2001).

Effect of GHSR KO on fasting plasma insulin content.

It is well known that obesity or in particular, a chronic HFD, often leads to insulin resistance, which can further lead to pancreatic b-cell failure and type 2 diabetes (Kahn et al., 2006). Past studies have reported the deletion of ghrelin increases insulin secretion in response to glucose challenge and subsequently increases peripheral insulin sensitivity (Sun et al., 2006).

Some studies have shown that there is insulin resistance shown within WT mice but not in GHSR KO mice. One study from Sun et al., (2011) examined rats at different age periods. WT rats (4 months old) became severely insulin resistant in response to a bolus of insulin, while old GHSR KO mice (18, 20, 24 and 26 months old) were significantly more responsive to an insulin challenge when compared to age-matched WT mice. Moreover, GHSR KO rats maintained a response curve similar to that of insulin-sensitive young mice (Ligen Li et al., 2011). Together these functional tests suggest that GHSR KO mice have improved peripheral insulin sensitivity and this GHSR KO effect has been shown to improve as a function of aging (Sun et al., 2007). It would be interesting if future studies could investigate Insulin Tolerance Tests in these FHH GHSR KO and WT rats. This way, a more complete picture could be uncovered regarding ghrelin's role within glucose homeostasis of this background strain. Lastly, a comparison between strains with these measures would aid in better understanding the strain effect.

The effect of GHSR KO on fasting plasma leptin profile.

Leptin is one of several hormones secreted by adipose tissue and is often correlated with the amount of lipids stored in WAT. Our results reveal no significant differences in plasma leptin content between GHSR KO and WT rats. This may be not surprising given that GHSR KO and WT rats had similar body fat content after a 15-week exposure to the HFD. Moreover, our results are also consistent with previous literature stating leptin level values were unchanged in GHSR KO mice following 19 weeks of standard chow and 3 weeks of HFD exhibited (Zigman et al., 2005).

Lipid profile

White adipose tissue stores energy in the form of triglycerides and supplies energy to the body as ATP through lipolysis/B oxidation (Amati et al., 2009; Khan et al., 2006). To determine whether

GHSR KO rats maintained a healthier lipid profile during the chronic HFD exposure, the fasting plasma lipid profiles of WT and GHSR KO rats were analyzed. Several levels of the hormones secreted by adipose tissue often correlate with the amount of lipids stored in WAT; specifically visceral adiposity (Amati et al., 2009). Despite no significant differences in fat mass distribution, an altered lipid profile was manifested in GHSR KO rats. In our study, WT rats displayed more plasma triglyceride content than GHSR KO rats after exposure to the HFD. Therefore, our results support the idea that deletion of GHSR improves lipid profiles.

The effect of GHSR gene mutation on fasting plasma peptide YY profile.

Our results reveal GHSR KO rats do not show any differences in satiety hormones relative to WT littermates. These findings are consistent with the other satiety hormone such as leptin. Therefore, we cannot attribute their food consumption to differences in satiety hormones. Other studies have reported PYY is reduced in GHSR KO mice when subjected to HFD. PYY's role in energy homeostasis mainly occurs when an energy restriction exist. While ghrelin increases in these negative energy balances, PYY decreases in the circulation and the reverse occurs in cases of positive energy balance (Stanley et al., 2005). This however, was not the case in the current study and both WT and GHSR KO rats had similar plasma concentrations of leptin and PYY.

The effect of GHSR gene mutation on fasting plasma growth hormone.

Our studies show no effects in GH concentrations between GHSR KO and WT rats. Presumably, we would have expected a difference in growth hormone given that ghrelin increases the release of GH. However, GH is not only secreted in response to ghrelin but other GH secreting factors as well including growth hormone releasing hormone (GHRH). Therefore,

GHSR KO rats may have normal concentrations of GH because other factors may maintain GH concentrations normal.

4.6 Compensatory Mechanisms

It has been speculated for quite some time that compensatory mechanisms may develop from birth when organisms are born with a gene mutation early in life. This could serve as and adaptation for survival because this loss of function is a global mutation found in every tissue.

Therefore the lack of effects in our study is assumed to be attributed to a compensatory mechanism(s). The only ways to discover if this is the case would be to eliminate this confound and induce the KO of the ghrelin receptor gene in adulthood. Possibilities to delete genes encoding for the ghrelin receptor during adulthood include more advanced conditional gene knockouts such in-vivo Cre-Lox recombination. Cre-Lox recombination is commonly used to carry out deletions for tissue-specific knockouts and also time-specific knockouts (Guo, Deshmukh & Van Duyne, 1997). This is not possible in conventional gene knockouts since the gene is knocked out in early embryonic cells. Briefly, cre-recombinase is an enzyme that recombines in a particular LoxP sequence the genomic DNA in order for and endogenous gene such as the ghrelin recetpor gene to be deleted (Guo et al., 1997; Zigman et al., 2005).

Viral Vectors can also manipulate DNA in adulthood. They are used to deliver genetic material into cells (Robbins & Ghivizzani, 1998). That is how protein-coding genes are expressed, such as marker genes like Green Fluorescent Protein in order to label and track cells of interest. All viruses will attack their hosts and introduce their genetic material into the host cell as part of their replication cycle (Robbins et al., 1998). This genetic material contains basic instructions of how to reproduce more copies of these viruses. Once these are carried out, more cells become infected (Robbins et al., 1998).

Recently an anti-ghrelin vaccine carrying a viral vector was designed to trigger the production of antibodies to neutralize ghrelin and was administered to DIO and wildtypes mice (Andrade, Couselo Carreira, Ribeiro, Texeria et al., 2011). The goal was to prevent ghrelin from communicating with the brain. This would help in maintaining weight loss since circulating ghrelin levels rise following weight loss.

Together, along with ghrelin antagonist studies, these in vivo techniques could overcome the confound of compensatory mechanism and be able to bypass this background strain effect in order to detect the gene mutation effect on the phenotype.

Resistance to diet-induced obesity.

All together, data shows rat with GHSR mutation eat less on a HFD than WT littermates and as a result, weight less. Possibly, this greater response is due to a meal preparatory factor in which ghrelin signals and prepares the body for the fuel it's about to consume. Emerging evidence reports ghrelin's ability to interpret energy reserve amounts and in response, build fat reserves in times of need (Wortley et al., 2005). Without this GHSR signaling pathway, the process is compromised and animals are less able to interpret and signal when energy stores are low. Not having GHSR receptors has potential beneficial effects on preventing the reward-pathway to become overly stimulated and override homeostastic signals. This is the case when high calorically dense food is easily accessible in large quantities such as our western environment today. Therefore, in this context, ghrelin receptor antagonists could prove useful in controlling adiposity in human obesity associated with a HFD.

Despite male GHSR KO rats displaying comparable body compositions to those of their wild-type littermates following 3 months of exposure to a HFD, this condition resulted in significantly less daily accumulation of both body weight and triglyceride content in GHSR KO

rats as compared with littermate controls. Our future studies would benefit from untangling where this weight loss and lower triglyceride content could be attributed. Comparing the percentage of fat in this particular background strain when fed a HFD to the percentage of lean body mass including bones and muscles and other fat free tissues such as organs could provide a better idea to pursue other mechanism which underlie their body weight loss. Other speculations could include increased locomotor activity or increased sympathetic tone due to their hypertensive nature.

4.7 Strengths

The significance of our study is that it can provide a valid GHSR KO rat model that displays a similar phenotype as that seen in mice with similar mutations. To our knowledge, there is only one other report using the GHSR KO rat model examining ghrelin's role in drug addiction behaviors (Clifford, Rodrigues, Schul, Hughes, Kniffin et al., 2011). However, the characterization of this rat model is yet to be determined. Therefore, this thesis presents data that validates this particular rat model. Future studies may ensue with more background strain phenotype available.

4.8 Limitations

The background strain used to generate the FHH rat, as shown by our studies, is resistant to gaining weight and does not overeat when given a high fat diet. This may pose a major limitation to this rat model, and it would be advisable to cross breed this strain onto a background that is more prone to obesity like the Long Evans Rat. Nevertheless, and in spite of this limitation, our studies show clear metabolic and behavioral differences between the GHSR KO and WT FHH rats that match those seen in GHSR mutant mice.

4.9 Validation of DIO Rat Model

In summary, our GHSR FHH KO rat models many of the features of the obesity syndrome and our results are similar to previous work (Zigman et al., 2005; Wortley et al., 2004, Lin et al., 2011). The mechanisms by which ghrelin promotes food intake are multifaceted and include to a great extent, enhancing the rewarding properties of certain foods (Abizaid et al., 2009; Chuang et al., 2011). Therefore, targeting the gene encoding for ghrelin would presumably affect feeding behaviors. As it would be expected, GHSR KO ate less food and gained less body weight under HFD conditions. As a result, GHSR KO displayed a significant decrease in triglyceride content as well as reduction trends in ghrelin suggesting a dysregulation in theis appetite-regulatory hormone. Moreover, GHSR KO rats displayed an increase in AgRP mRNA expression. This finding also supports the notion of a compensatory mechanism.

Overall, this model would become a pivotal model for understanding the interplay between genetic background and environmental challenges such as high-fat/high-calorie diets that predispose to the development of metabolic syndrome (Collins et al., 2004). Also, this animal model could be used to expand diet conditions and exposure animals to other diets such as high fructose or high protein in order to depict and untangle more of the diets influence on the overall energy balance of the FHH GHSR KO rat.

5 Conclusions

We conclude to have demonstrated that the genetic disruption of the gene encoding for ghrelin receptors system with FHH rats leads to a similar phenotype found within GHSR KO mice studies. In particular, a series of mice studies show that the deletion of the ghrelin/GHSR gene reduces food intake, body weight and adiposity, through reduction of appetite and

augmentation of energy expenditure and fat catabolism (Wortley et al., 2004; Zigman et al., 2005).

Our results indicate that when accounting for body weight differences, GHSR KO rats consume significantly less food than their WT littermates. This was observed regardless of the diet they consumed. Furthermore, when challenged with an overnight fast (to observe rebound feeding responses), GHSR KO rats consumed significantly less than WT suggesting ghrelin's short-term effects of initiating meals and regulating food intake remained intact. Within the GHSR KO hormonal profiles, our results support previous findings demonstrating reduced triglyceride content relative to WT, which would presumably reflect their lower levels of white adipose tissue and lower body weights. Furthermore, ARC mRNA expression reveals a dysregulation in orexigenic neuropeptides stimulated by ghrelin.

Based on lower body weight accumulation exhibited in both GHSR KO and WT rats (after HFD), our data suggests the possibility that the resistance to diet-induced obesity may be further influenced by the FHH background strain. As a result, this background strain may potentially mask differences produced by the GHSR gene mutation. However more characterization is needed in order to confirm this speculation. Possibly even a comparison analysis with other strains previously established to be more prone to obesity such as the Long Evans or Wistar rat would be beneficial.

Together, our data suggests that endogenous ghrelin is essential for the maintenance of normal levels or patterns of food intake, or increases in food intake after a fast. Thus, the absence of ghrelin receptors, due to genetic deletion leads to the inability for ghrelin to bind to their receptors and as a result, exert its functions. Thus, our data validates utilization of the GHSR KO rat as a model to target diet-induced obesity since rats deficient of ghrelin's receptor seem to

exhibit a protective phenotype resisting high fat diet induced-obesity. This type of research is important and relevant because of the high prevalence and associated metabolic diseases when ghrelin-signaling pathway is dysfunctional including obesity and Type 2 Diabetes. For individuals at highest risk of the complications of severe obesity, such findings provide a starting point for attaining more rational mechanism-based therapies. Clinical implications could prove useful in controlling adiposity through ghrelin receptor antagonists in human obesity associated with HFD.

References

Abizaid, A., Gao, Q., Horvath, T.L. (2006). Thoughts for food: brain mechanisms and peripheral energy balance. *Neuron*; 51: 691–702.

Abizaid, A., Liu, Z. W., Andrews, Z.B.., Shanabrough, M., Borok, E., Elsworth, J., Roth, R., Sleeman, M., Picciotto, M., Tschop, M., Gao XB., Horvath, TL. (2006). "Ghrelin modulates the activity and synaptic input organization of midbrain dopamine neurons while promoting appetite." *J Clin Invest* 116(12): 3229-39.

Abizaid A, Horvath TL. (2008). Brain circuits regulating energy homeostasis. *Regul Pept.* 149:3–10.

Abizaid A. (2009). Ghrelin and dopamine: new insights on the peripheral regulation of appetite. *J Neuroendocrinol*;21(9):787-93.

American Psychological Association. (2010). Publication manual of the American Psychological Association (6th ed.). Washington, DC.

Andrade, S., Couselo Carreira, M., Ribeiro, A., Texeria, L., Monteiro, D., Lage, M., Casanueva, F., Monteiro, M. (2011). Development of an Anti-Ghrelin Vaccine for Obesity Treatment. Endocr Rev, 32;P2-305.

Andrikopoulos S. (2010). Obesity and type 2 diabetes: slow down!--Can metabolic deceleration protect the islet beta cell from excess nutrient-induced damage? Mol*Cell Endocrinol.* Mar 25;316(2):140-6.

Arosio M, Ronchi C.L., Gebbia, C., Pizzinelli, S., Conte, D., Cappiello, V., Epaminonda, P., Cesana, B.M., Beck-Peccoz, P., Peracchi, M. (2004). Ghrelin administration affects circulating pituitary and gastro-entero-pancreatic hormones in acromegaly. *Eur J Endocrinol* 150:27.

Aydin, S., Sahin, I., Ozkan, Y., Dag, E., Gunay, A., Guzel, S.P., Catak, Z., Ozercan, M.R., (2012).Examination of the tissue ghrelin expression of rats with diet-induced obesity using radioimmunoassay and immunohistochemical methods. *Mol Cell Biochem.*;365(1-2):165-73.

Banks, W.A., Tschop, M., Robinson, S.M., Heiman, ML. (2002). Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. *J Pharmacol Exp Ther* 302:822–827.

Berridge, K.C & Robinson, T.E. (1995). The mind of an addicted brain: Neural sensitization of "wanting" versus "liking". *Current Directions in Psychological Science,* 4, 71-76.

Berridge, K.C., (2004). Food reward: brain substrates of wanting and liking. *Neurosci. Biobeha*;81:179-209.

Blum ID., Patterson, Z., Khazall R., Lamont EW., Sleeman M.W., Horvath T.L. & Abizaid A. (2009). Reduced anticipatory locomotor responses to scheduled meals in ghrelin receptor deficient mice. *Neuroscience*, 164 (2): 351-35.

Boston, B.A., Blaydon, K.M., Varnerin, J., Cone, R.D. (1997). Independent and additive effects of central POMC and leptin pathways on murine obesity. *Science*;278:1641–1644.

Bray, G. A. (1977) "The Zucker fatty rat: a review," *Federation Proceedings*, vol. 36, no. 2, pp. 148–153.

Briggs, D.I., Enriori, P.J., Lemus, M.B., Cowley, M.A., and Andrews, Z.B. (2010). Diet-induced obesity causes ghrelin resistance in arcuate NPY/AgRP neurons. Endocrinology 151, 4745–4755.

Brobeck, J.R., (1946). Mechanism of the development of obesity in animals with hypothalamic lesions. *Physio Rev* 26:541-559.

Broglio, F., Arvat, E., Benso, A., Gottero, C., Prodam, .F, Granata, R., et al. (2002). Ghrelin: much more than a natural growth hormone secretagogue. *Isr Med Assoc J*;4:607-13.

Broglio, F., Gottero, C., Benso, A., Prodam, .F, Volante, M., Destefanis, S. et al., (2003). Ghrelin and the endocrine pancreas. Endocrine; 22:19-24.

Bultman, S. J., Michaud, E. J., Woychik, R. P. (1992). "Molecular characterization of the mouse agouti locus," *Cell*, vol. 71, no. 7, pp. 1195–1204.

Cannon, W.B., (1926) "Physiological regulation of normal states: some tentative postulates concerning biological homeostatics." *In: A. Pettit (ed.). A Charles Richet : ses amis, ses collègues, ses élèves, Paris: Éditions Médicales*. p. 91.

Chen, H., Wang, Y., Ma, L., Zhao, J., Li, Y., Li, M. (2012). Long-term high animal protein diet reduces body weight gain and insulin secretion in diet-induced obese rats. *J Sci Food Agric*. doi: 10.1002/jsfa.5679.

Chuang, J.C., Perello, M., Sakata, I., Osborne-Lawrence, S., Savitt, J.M., Lutter, M., Zigman, J.M. (2011). Ghrelin mediates stress-induced food-reward behavior in mice. *Journal of Clinical Investigation*; DOI: 10.1172/JCI57660.

Clifford, P.S., Rodriguez, J., Schul D., Hughes, S., Kniffin, T., Hart, N., Etain, S., Brunel, L., Fehrentz, JA., Martinez, J., Wellman, PJ. (2011). Attenuation of cocaine-induced locomotor sensitization in rats sustaining genetic or pharmacologic antagonism of ghrelin receptors. Addiction Biology. DOI: 10.1111/j.1369-1600.2011.00339.x.

Coleman, D. L. (1978) Obese and Diabetes: two mutant genes causing diabetes-obesity syndromes in mice. *Diabetologia* 14, 141–148.

Collins S., Martin T. L.,. Surwit R. S, Robidoux J., (2004). "Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics," *Physiology and*

Behavior, vol. 81, no. 2, pp. 243–248.

Cone, R.D., Cowley, .MA., Butler, A.A., Fan, W., Marks, D.L., Low, M.J., (2001). The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. Int. *J. Obes.Relat. Metab.* Disor;25:S63-S67.

Cowley, M.A., Smith, R.G., Diano, S., Tschop, M., Pronchuk, N., Grove, K.L., Strasburger, C.J., Bidlingmaier, M., Esterman, M., Heiman, M.L., et al. (2003). The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* 37:649–661.

Cummings, D.E., Purnell, J.Q., Frayo, R.S., Schmidova, K., Wisse, B.E., Weigle, D.S., A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes.* 2001 Aug;50(8):1714-9.

Cummings, D.E., Foster-Schubert, K.E., Overduin, J. (2005). Ghrelin and energy balance: focus on current controversies. *Curr Drug Targets*;6:153-69.

Cui, C., Ohnuma, H., Daimon, M., Susa, S., Yamaguchi, H., Kameda, W., Jimbu, Y., Oizumi, T., Kato, T. (2008). Ghrelin infused into the portal vein inhibits glucose- stimulated insulin secretion in Wistar rats. *Peptides* 29:1241–1246.

Cui RJ., Appleyard, S.M. (2009). Ghrelin inhibits visceral afferent activation of catecholamine neurons in the solitary tract nucleus. *Appetite* 52:824.

Date, Y., Kojima, M, Hosoda, H., Sawaguchi, A., Mondal, M.S., Suganuma, T., Matsukura, S., Kangawa, K., Nakazato, M. (2000). Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 141:4255–4261.

Date. Y., Murakami. N., Toshinai, K., Matsukura. S., Niijima, A., Matsuo. H., Kangawa. K., Nakazato. M. (2002). The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology* 123:1120–1128.

Delhanty, P.J., van der Lely. A.J. (2011). Ghrelin and glucose homeostasis. *Peptides*. 32: 2309-18.

Diano, S., Farr, S.A., Benoit, S.C., McNay, E.C., da Silva, I., Horvath, B., Gaskin, F.S., Nonaka, N., Jaeger, L.B., Banks, W.A., Morley, J.E., Pinto, S., Sherwin, R.S., Xu, L., Yamada, K.A., Sleeman, M.W., Tschop, M.H., Horvath, T.L. (2006). Ghrelin controls hippocampal spine synapse density and memory performance. *Nature Neurosci* 9: 381–388.

Dina, C., Meyre, D., Gallina, S., Durand, E., Korner, A., et al (2007). Variation in FTO contributes to childhood obesity and severe adult obesity. *Nat Genet.*;39(6):724–726.

Drucker, D.J. (2007). The role of gut hormones in glucose homeostasis. *JClinInvest.*;117:24–32.

Elias, C.F., Lee, C.E., Kelly, J.F., Ahima, R.S., Kuhar, M., Saper, C.B., Elmquist, J.K. (2001). Characterization of CART neurons in the rat and human hypothalamus. *J. Comp. Neurol*;432:1–19.

Elmquist, J.K. (2001). Hypothalamic pathways underlying the endocrine, autonomic, and behavioral effects of leptin. *Physiol. Behav;*74:703–708.

Farooqi, I.S., Drop, S., Clements, A., Keogh, J.M., Biernacka, J., Lowenbein, S., Challis B.G, O'Rahilly. S. (2006). Heterozygosity for a POMC-null mutation and increased obesity risk in humans. *Diabetes.* 55(9):2549–2553.

Farooqi, S., O'Rahilly, S. (2006). Genetics of obesity in humans. *Endocr Rev.*;27(7):710–18.

Faulconbridge, L.F, Cummings, D.E., Kaplan, J.M., Grill, H.J. (2003). Hyperphagic effects of brainstem ghrelin administration. *Diabetes* 52:2260–2265.

Flier, J.S. (2004). "Obesity wars: Molecular progress confronts an expanding epidemic". *Cell* 116 (2): 337–50).

Frayling, T.M., Timpson, N.J., Weedon, M.N., Zeggini, E., Freathy, R.M., Lindgren, C.M., Perry, J.R., Elliott, K.S., Lango, H., Rayner, N.W., Shields, B., Harries, L.W., Barrett, J.C., Ellard, S., Groves, C.J., Knight, B., Patch, A.M., Ness, A.R., Ebrahim, S., Lawlor, D.A., Ring, S.M., Ben-Shlomo, Y., Jarvelin, M.R., Sovio, U., Bennett, A.J., Melzer, D., Ferrucci, L., Loos, R.J., Barroso, I., Wareham, N.J., Karpe, F., Owen, K.R., Cardon, L.R., Walker, M., Hitman, G.A., Palmer, C.N., Doney A.S, Morris, A.D, Smith, G.D., Hattersley, A.T., McCarthy, M.I. (2007). A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. *Science*316 :889 –894.

Friedman, J.M., Halaas, J.L. (1998). Leptin and the regulation of body weight in mammals. *Nature* 395:763–770.

Fry, M., Ferguson, A.V. (2009). Ghrelin modulates electrical activity of area postrema neurons. *Am J Physiol Regul Integr Comp Physiol 296*: R485–R492.

Gnanapavan, S., Lokla, B., Bustin, S.A., Morris, D.G., McGee, P., Fairclough, P. (2002). The tissue distribution of the mRNA of ghrelin and subtype of its receptor, GHS-R, in humans. *J Cline NDOCRINOL metab*;87:2988.

Gray, J., Yeo, G.S., Cox, J.J., Morton, J., Adlam, A.L., Keogh, J.M., Yanovski, J.A., E, l.

Handjieva-Darlenska T, Boyadjieva N. The effect of high-fat diet on plasma ghrelin and leptin levels in rats. J Physiol Biochem. 2009 Jun;65(2):157-64.

Grill, H.J., Kaplan, J.M. (2001). Interoceptive and integrative contributions of forebrain and brainstem to energy balance control. *Int. J. Obes. Relat. Metab. Disord*;25:S73-S77.

Grill, H.J., Schwartz, M.W., Kaplan, J.M., Foxhall, J.S., Breininger, J., Baskin, D.G. (2002). Evidence that the caudal brainstem is a target for the inhibitory effect of leptin on food intake. *Endocrinology* 143 :239 –246.

Guan, X.M. Yu, H., Palyha,O.C. et al., (1997). Distribution of mRNA encoding the growth hormone secretagogue receptorin brain and peripheral tissues. *Brain Res. Mol. Brain Res.* 48:23-29.

Guo, F., Desmukh, N. Van Duune, G. (1997). Structure of Cre recombinase complexed with DNA in a site-specific recombination synapse. Nature. 389, 40-46.

Hetherington, A.W., Ranson, S.W. (1940). Hypothalamic legions and adiposity in rat.*Anat Rec* 78:149–172.

Higuchi, H., Niki, T., Shiiya, T. (2008). Feeding behavior and gene expression of appetite-related neuropeptides in mice lacking for neuropeptide Y Y5 receptor subclass. *World J Gastroenterol* 14:6312–6317.

Holder, J.L. Jr, Butte, N.F., Zinn, A.R. (2000). Profound obesity associated with a balanced translocation that disrupts the SIM1 gene. *Hum Mol Genet* 9 :101 –108.

Horvath, T.L., Diano, S., Sotonyi, P., Heiman, M., Tschop, M.: Minireview: (2001). Ghrelin and the regulation of energy balance: a hypothalamic perspective. *Endocrinology* 142:4163–4169.

Hou, Z., Miao, Y., Gao, L., Pan, H., Zhu, S. (2006) Ghrelin-containing neuron in cerebral cortex and hypothalamus linked with the DVC of brainstem in rat. *Regul Pept* 134:126.

Howard, A.D., Feighner, S.D., Cully, D.F., Arena, J.P., Liberator, P.A., Rosenblum, C.I., Hamelin, M., Hreniuk, D.L., Palyha, O.C., Anderson, J., et al. (1996). A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* 273:974–977.

Huszar, D., Lynch, C.A., Fairchild-Huntress, V., Dunmore, J.H., Fang, Q., Berkemeier, L.R.,Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F.(1997). Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell*; 88(1):131-41.

Inhoff, T., Monnikes, H., Noetzel, S., Stengel, A., Goebel, M., Dinh, Q.T. (2008). Desacyl ghrelin inhibits the orexigenic effect of peripherally injected ghrelin in rats. *Peptides;* 29:2159-68.

Kahn, S.E., Hull, R.L., Utzschneider, K.M. (2006) Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 444: 840–846.

Kanasaki., K., Koya. D., (2010) Biology of Obesity: Lessons from Animal Models of Obesity. *J Biomed Biotechnol.* 2011:197636. Review.

Kawano K., Hirashima T., Mori S., Natori T., (1994.) OLETF (Otsuka Long-Evans Tokushima fatty) rat: a new NIDDM rat strain, *Diabetes Research and Clinical Practice*, vol. 24, pp. S317–S320.

Kawano, K., Hirashima, T., Mori, S., Saitoh, Y., Kurosumi, M., Natori, T., (1992). Spontaneous long-term hyperglycemic rat with diabetic complications: Otsuka Long-Evans Tokushima Fatty (OLETF) strain, *Diabetes*, vol. 41, no. 11, pp. 1422–1428.

King SJ, Isaacs AM, O'Farrell E, Abizaid A. (2011). Motivation to obtain preferred foods is enhanced by ghrelin in the ventral tegmental area. Horm Behav.;60(5):572-80.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo H., Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402, 656—660.

Kojima, M., Kangawa, K. (2005). Ghrelin: structure and function. *Physiol. Rev.* 85, 495—522.

Korbonits, M., Goldstone, A.P., Gueorguiev, M., Grossman, A.B. (2004.) Ghrelin–a hormone with multiple functions. Front Neuroendocrinol 25: 27–68.

Kurtz, T.W., Morris, R.C., Pershadsingh, H.A. (1989). "The Zucker fatty rat as a genetic model of obesity and hypertension". *Hypertension* (Dallas, Texas: American Heart Association) 13 (6): 896–901.

Kwon, H. Y., Bultman S. J., Loffler. C. (1994). "Molecular structure and chromosomal mapping of the human homolog of the agouti gene," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 21, pp. 9760–9764.

Levin, B.E., Dunn-Meynell, A.A., McMinn, J.E., Cunningham-Bussel, A., and Chua, S.C. Jr. (2003). A new obesity-prone, glucose intolerant rat strain (F. DIO). *Am J Physiol Regul Integr Comp Physiol* 285: R1184-R1191.

Lin, Y., K. Matsumura, et al. (2004). "Ghrelin acts at the nucleus of the solitary tract to decrease arterial pressure in rats." *Hypertension* 43(5): 977-82.

Lin L, Saha PK, Ma X, Henshaw IO, Shao L, Chang BH, Buras ED, Tong Q, Chan L, McGuinness OP, Sun Y. (2011). Ablation of ghrelin receptor reduces adiposity and improves insulin sensitivity during aging by regulating fat metabolism in white and brown adipose tissues. Aging Cell. (6):996-1010.

Malik, S., McGlone, F., Bedrossian, D., Dagher, A. (2007). Ghrelin modulates brain activity in

areas that control appetitive behavior. *Cell Metabolism* 7 (5): 400–9.

Mathon, D.S., et al. (2005). Increased gabaergic input to ventral tegmental area dopaminergic neurons associated with drecreased cocaine reinforcement in mu-opiod receptor knockout mice. *Neuroscience*. 130:359-367.

McNay, E.C. (2007). Insulin and ghrelin: peripheral hormones modulating memory and hippocampal function. *Curr Opin Pharmacol.*7:628–632.

Moran, T.H., Ladenheim, E.E., Schwartz, G.J. (2001). Within-meal gut feedback signaling. *Int J Obes Relat Metab Disord* 25 [Suppl 5]: S39-S41.

Morton, G. J., Cummings, D. E., Baskin, D. G., Barsh, G. S. & Schwartz, M. W. Central nervous system control of food intake and body weight. *Nature* 443, 289–295 (2006).

Murphy, K.G., Bloom, S.R., (2004). Gut hormones in the control of appetite. *Exp Physiol*; 89: 507–516.

Perez-Tilve D, Heppner K, Kirchner H, Lockie SH, Woods SC, Smiley DL, Tschöp M, Pfluger P. (2011). Ghrelin-induced adiposity is independent of orexigenic effects. FASEB J. Aug;25(8):2814-22.

Perreault M, Istrate N, Wang L, Nichols AJ, Tozzo E, Stricker-Krongrad A. (2004). Resistance to the orexigenic effect of ghrelin in dietary-induced obesity in mice: reversal upon weight loss. *Int J Obes Relat Metab Disord*; 28: 879–885.

Pinto, S., Roseberry, A.G., Liu, H., Diano, S., Shanabrough, M., Cai, X., Friedman, J.M., and Horvath, T.L. (2004). Rapid rewiring of arcuate nucleus feeding circuits by leptin. *Science* 304, 110–115.

Pfluger, P. T., H. Kirchner, et al. (2008). Simultaneous deletion of ghrelin and its receptor increases motor activity and energy expenditure.*Am J Physiol Gastrointest Liver Physiol*

294(3): G610-8.

Powley, T.L., Keesey, R.E. (1970). Relationship of body weight to the lateral hypothalamic feeding syndrome. *J Comp Physiol Psychol* 70:25-36.

Reed DR, Duke FF, Ellis HK, Rosazza MR, Lawler MP, Alarcon LK, Tordoff MG. Body fat distribution and organ weights of 14 common strains and a 22-strain consomic panel of rats. Physiol Behav.

Robbins, P., Ghivizzani, S. (1998). Viral Vectors for Gene Therapy. Phamacology & Therapeutics: 80(1);25-47.

Saper, C.B., Chou, T.C., Elmquist, J.K. (2002). The need to feed: homeostatic and hedonic control of eating. *Neuron*, 36:199-211.

Schellekens, H., Dinan, T.G., Cryan, J.F. (2010). Lean Mean Fat Reducing "Ghrelin" Machine: Hypothalamic Ghrelin and Ghrelin Receptors as Therapeutic Targets in Obesity. *Neuropharmacology.*;58:2–16.

Shimbara, T., Mondal, M.S., Kawagoe, T., Toshinai, K., Koda, S., Yamaguchi, H., Date, Y., Nakazato, M. (2004). Central administration of ghrelin preferentially enhances fat ingestion. *Neurosci Lett;* 369: 75–79.

Shuto, Y., Shibasaki, T., Otagiri, A, et al. (2002). Hypothalamic growth hormone secretagogue receptor regulates growth hormone secretion, feeding, and adiposity. *J. Clin. Invest.* 109 (11): 1429–36.

Suckow, M.A., ,Weisbroth, S.H., Franklin, C.L. (2005) . *The laboratory rat.* (2nd Edition):American College of Laboratory Animal Medicine Series (pp. 26, 80-92,). Burlington, MA: Elsevier Academic Press publications.

Sun, Y., Ahmed, S., Smith, R.G. (2003). Deletion of ghrelin impairs neither growth nor appetite.

Mol Cell Biol 23:7973–7981.

Sun, Y., Asnicar M, Saha, PK, Chan L, Smith, R.G. (2006). Ablation of ghrelin improves the diabetic but not obese phenotype of ob/ob mice. *Cell Metab*;3:379.

Sun Y, Asnicar M, Smith RG. (2007). Central and peripheral roles of ghrelin on glucose homeostasis. Neuroendocrinology 86:215–228.

Sun, Y., Butte, N.F., Garcia, J.M., Smith, R.G. (2008). Characterization of adult ghrelin and ghrelin receptor knockout mice under positive and negative energy balance. *Endocrinology*;149:843-50.

Srinivasan K., Ramarao P. (2007). Animal models in type 2 diabetes research: an overview, *Indian Journal of Medical Research*, vol. 125, no. 3, pp. 451–472.

Theander-Carrillo C., Wiedmer, P., Cettour-Rose, P., Noguerias, R., Perez-Tilve, D., Nogeuiras, R., Perez-Tilve, D., Pfluger, P., Castaneda, T., Muzzin, P., Schurmann, A., Szanto, I., Tschop, M. (2006). Ghrelin action in the brain controls adipocyte metabolism. J. Clin. Invest. 116:1983–1993. doi: 10.1172/JCI25811.

Tordoff, M.G. (2010). Taste solution consumption by FHH-Chr nBN consomic rats. *Chem Senses*;35:473-489.

Toshinai K, Date Y, Murakami N, Shimada M, Mondal MS, Shimbara T, Guan JL, Wang QP, Funahashi H, Sakurai T, Shioda S, Matsukura S, Kangawa K, Nakazato M. (2003). Ghrelin-induced food intake is mediated via the orexin pathway. *Endocrinology*; 144: 1506–1512.

Troiano R.P., Flegal K.M., Kuczmarski R.J., Campbell S.M., Johnson C.L.. (1995). Overweight prevalence and trends for children and adolescents: the National Health and Nutrition Examination Surveys, 1963 to 1991. *Arch Pediatr Adolesc Med*;149:1085-1091.

Tschop, M., Devanarayan, V., Weyer, C., Tataranni, P.A, Ravussin, E., Heiman, M.L. (2001). Circulating ghrelin levels are decreased in human obesity. *Diabetes* 50:707–709.

Tschop, M., Smiley, D.L., Heiman, M.L.., (2000). Ghrelin induces adiposity in rodents. *Nature* 407:908–913.

Tsubota, Y., Owada-Makabe, K., Yukawa, K., Maeda, M. (2005). Hypotensive effect of des-acyl ghrelin at nucleus tractus solitarii of rat. *Neuroreport* 16:163–166.

Valenstein, E.S., Cox V.C., Kakolewski J.W.(1968). Modification of motivated behavior elicited by electrical stiulation of the hypothalamus.*Science* 159: 1119-1120.

Vestergaard, E.T., Hansen, T.K., Gormsen, L.C., Jakobsen, P., Moller, N., Christiansen JS et al. (2008). Ghrelin infusion in humans induces acute insulin resistance and lipolysis independent of growth hormone signaling. *Diabetes*;57:3205-10.

Wang, H. J., F. Geller, et al. (2004). Ghrelin receptor gene: identification of several sequence variants in extremely obese children and adolescents, healthy normal-weight and underweight students, and children with short normal stature. J Clin Endocrinol Metab 89(1): 157-62.

Wellman, P.J.., Clifford, S., Rodriguez, J., Hughes, S., Francesco, C., Melotto, S., Tessari, M., Corsi., M., Bifone, A., Gozzi, A., (2011). Brain reinforcement system function is ghrelin dependent: studies in the rat using pharmacological fmRI and Intracranial self-stimulation, *Addiction Biology*, DOI: 10.1111/j.1369-1600.2011.00392.

West, D. B., Boozer, C. N., Moody, D. L., Atkinson, R. L., (1992). Dietary obesity in nine inbred

mouse strains," *American Journal of Physiology*, vol. 262, no. 6, pp. R1025–R1032.

Wise, R.A. (2004) Dopamine, learning and motivation. *Nat Rev Neurosci* 5: 483–494.

Woods, S.C., Seeley, R.J., Porte, Jr. D., Schwartz, M.W. (1998). Signals that regulate food intake and energy homeostasis. *Science* 280:1378– 1383.

Wortley, K.E., Anderson, K.D., Garcia, K., Murray, J.D., Malinova, L., Liu, R., Moncrieffe, M., Thabet, K., Cox, H.J., Yancopoulos, G.D., Wiegand, S.J., Sleeman, M.W. (2004). Genetic deletion of ghrelin does not decrease food intake but influences metabolic fuel preference. *Proc Natl Acad Sci USA* 101:8227–8232.

Wren, A.M., Small, C.J., Abbott, C.R., et al. (2001). Ghrelin causes hyperphagia and obesity in rats. *Diabetes*;50:2540-7.

Yang, Y., Atasoy, D., Su, H., Sternson, S. (2011). Hunger States Switch a Flip-Flop Memory Circuit via a Synaptic AMPK-Dependent Positive Feedback Loop *Cell,* Volume 146, Issue 6:16; 992-1003.

Zhang, W., Lin, T.R., Hu, Y., Fan, Y., Zhao, L., Stuenkel, E.L., Mulholland, M.W. (2004). Ghrelin stimulates neurogenesis in the dorsal motor nucleus of vagus. *J Physiology* 559(3):729-737.

Zigman, J.M., Jones, J.E., Lee, C.E., Saper, C.B., and Elmquist, J.K. (2006). Expression of ghrelin receptor mRNA in the rat and the mouse brain. J. Comp. Neurol. 494:528-548.

Zigman, J.M., Nakano, Y., Coppari, R., Balthasar, N., Marcus, J.N., Lee, C.E., Jones, J.E., Deysher, A.E., Waxman, A.R., White, R.D., et al. (2005). Mice lacking ghrelin receptors resist the development of diet-induced obesity. *J. Clin. Invest. 115,* 3564–3572.

Ligen L., Sun, Y., (2012). Presented at the Endocrine's Society 94th Annual Meeting and EXPO Houston, Tx.

Medical College of Wisconsin: http://www.mcw.edu/mcw/home.htm#.T70X5lHcjoA.

Millipore Headquarters, Billerica, MA. http://www.millipore.com/index.do.

Rat Genome Database: http://rgd.mcw.edu/rgdweb/search/strains.html?term=FHH&obj=strain

Appendix A.

Different phenotypes of inbred mouse strains with diet or genetically induced obesity.

Strain	*Characteristics*	*Crossed with obese mice, and so forth*
C57BL/6J	*HFD-induced obesity and diabetes*	*Lep $^{ob/ob}$ mice exhibit obesity but not diabetes*
C57BLKS/J	*HFD-induced obesity and diabetes Weaker than C57BL/6J*	*Lep $^{ob/ob}$ mice exhibit obesity, severe diabetes*
DBA/2	*More glucose tolerance than C57BLK/6 on a HFD*	*Lep $^{ob/ob}$ mice exhibit obesity, severe diabetes*
129sv	*Low insulin; more glucose tolerance than other strains on a HFD*	*Homozygous for db allele db mice display mild hyperglycemia with marked hyperinsulinemia and develop hypoglycemia leading to sudden death*
BTBR	*Abdominal obesity with peripheral but not hepatic insulin-resistance.*	*Lep $^{ob/ob}$ mice exhibit obesity with severe diabetes*
A/J	*Low glucose level even on a HFD; Obesity and diabetes-resistant*	*Not reported*
BALB/c	*Similar to A/J; glucose tolerance*	*Lep $^{ob/ob}$ mice exhibit reduced adiposity and increased thermogenesis and are fertile.*
C3H	*High glucose tolerance with robust Insulin secretion.*	*Not reported*
AKR	*Sensitive to DIO with hyperinsulinemia and insulin resistance.*	*Not reported*
CAST/ei	*Lean at 12 weeks on HFD*	*Not reported*
Nonobese Diabetic	*45% fat diet-induced transient hyper-glycemia with severe obesity.*	*Not reported*
New Zealand Obese	*Resembles metabolic syndrome in humans; HFD DIO and hyperglycemia*	*Not reported*
FVB	*High glucose levels with lower levels of insulin on normal chow*	*Lep $^{db/ob}$ mice display insulin resistance, hyperglycemia and severe hyperinsulinemia compared to C56BL/6*
Kuo Kondo	*Obesity: hyperleptinemia; increase glucose and hbA1c; hypersinsulinemia Similar to C57BLKS/J Lep $^{ob/ob}$ mice*	*A^{y} mutation in agouti results in diabetes nephropathy*
TallyHo	*Natural model of obesity with type 2 diabetes*	*Not reported*
Nagoya	*Exhibit obesity; all males and 1/3 of females exhibit glucose intolerance*	*Not reported*

Modified from Keizo Kanasaki and Daisuke Koya, (2011). Biology of Obesity: Lessons from Animal Models of Obesity.

Appendix B.

Tecklad Global 14% Protein Rodent Maintenance Diet.

Macronutrients	*Percentage*	*Amount*
Crude Protein	*%*	*14.3*
Fat (either extract)	*%*	*4.0*
Carbohydrate	*%*	*48.0*
Crude Fiber	*%*	*4.1*
Neutral Detergent Fiber	*%*	*18.0*
Ash	*%*	*4.7*
Energy Density	*kcal/g (KJ/g)*	*3.4 (12.9)*
Calories from Protein	*%*	*20*
Calories from Fat	*%*	*13*
Calories from Carbohydrate	*%*	*67*

Appendix C.

Open Source 60% High-Fat Diet.

Macronutrients	*gm%*	*kcal%*
Protein	*26.2*	*20*
Fat (either extract)	*34.9*	*60*
Carbohydrate	*26.3*	*20*
Total		
Kcal/gm	*5.2*	

Appendix D.

Gene	*Primer Sequence*
NPY	
Sense	5′-TCCGCTCTGCGACACTACAT-3′
Antisense	5′-GGAAGGGTCTTCAAGCCTTGT-3′
AgRP	
Sense	5′-GCTCCACTGAAGGGCATCA-3′
Antisense	5′-TAGCACCTCCGCCAAAGCT-3′
POMC	
Sense	5′-GCTCAAGGTCCTTCCTGGTG-3′
Antisense	5′-GCCCTGGATTGAATCACGCC-3′
OB-Rb	
Sense	5′-GCTGGAAGCCTGTCGTACTCTTCAC-3′
Antisense	5′-TACACTGCGTCATAGGTAAACTTCCCTC-3′
Beta-Actin	
Sense	5′-GTGCCACCAGACAGCACTGTGTTG-3′
Antisense	5′-TGGAGAAGAGCTATGAGCTGCCTG-3′
GAPDH	
Sense	5′-AGCAATGCCTCCTGTACCAC-3′
Antisense	5′-AAGCAGGGATGATGTTCTGG-3′

qRT-PCR primers: NPY, neuropeptide Y; AgRp, agouti-related protein; POMC, pro-opriomelanocortin; LepR, leptin receptor; Control Primers: Beta-Actin; GAPDH.