Stressed mice alter caloric preference for carbohydrate rich diets: the role of ghrelin during stress

by

By Trevor M. Rodrigues

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, Canada
©2013
Trevor M. Rodrigues

Abstract

Under chronic social stress conditions, mice show a preference for carbohydrates. In this present thesis we hypothesize that this behavior is critical for blunting the stress response and that ghrelin contributes to this change in preference. Using the chronic social defeat stress paradigm, we showed that stressed mice increase their total caloric intake, particularly the intake of carbohydrate rich diets and sucrose solutions over palatable high fat diets. Furthermore, mice that are prevented from increasing their caloric intake show exaggerated hormonal responses to the social stressor. Finally, mice with mutations to the ghrelin receptor gene (GHSR KO) do not show increased caloric intake and show more depressive like symptoms in spite of increasing their consumption of a sucrose solution. This data suggests that stress increases the intake of carbohydrate rich foods to attenuate the endocrine response to social stress and that ghrelin plays an important role in this process.

Acknowledgments

I would like to thank Alfonso Abizaid for being my thesis supervisor and for helping me with everything over the past few years. I would also like to thank Zachary Patterson, whose work, dedication and assistance helped produce the results of this present thesis.

Table of Contents

Abstract.....	ii
Acknowledgments.....	iii
Table of Contents.....	iv
List of Figures.....	vi
Introduction.....	1
<i>Obesity and stress as a challenge to human health</i>	1
<i>Hypothalamic Regulation of Food Intake and energy balance</i>	2
<i>Extra-Hypothalamic Regulation of Feeding</i>	4
<i>Metabolism and the role of hormones during food consumption</i>	5
<i>Ghrelin</i>	7
<i>Ghrelin increases food intake and reduces energy expenditure</i>	8
<i>The Stress Response</i>	10
<i>Change in metabolic rate due to stress</i>	15
<i>Ghrelin and the stress response</i>	16
<i>Reducing stress through Food intake</i>	18
<i>Rational for present thesis</i>	20
General Methods.....	21
<i>Animals</i>	21
<i>Chronic social Defeat</i>	22
<i>Daily Measurements</i>	23
<i>Blood Collection</i>	23
<i>ELISA</i>	24
<i>CORT</i>	24
<i>Statistical Analysis</i>	25
<i>Experiment 1</i>	25
<i>Experiment 2</i>	26
<i>Experiment 3</i>	27
<i>Experiment 4</i>	28
<i>Experiment 5</i>	29
Results.....	30
<i>Experiment 1</i>	30
<i>Experiment 2</i>	36
<i>Experiment 3</i>	43
<i>Experiment 4</i>	49
<i>Experiment 5</i>	60
Discussion.....	69
Appendix A – Supplementary Figures.....	75
References.....	83

List of Figures

Table 1. Nutritional value of provided diets.....	29
Experiment 1	
Figure 1A. Daily 24hr chow intake of DBA/2J mice	38
Figure 1B. Average 24hr chow intake of DBA/2J mice.....	38
Figure 2A. Daily 4hr high fat intake of DBA/2J mice.....	39
Figure 2B. Average 4hr high fat intake of DBA/2J mice.....	39
Figure 3A. Daily total calories (high fat and normal chow) of DBA/2J mice.....	40
Figure 3B. Average total calories intake of DBA/2J mice.....	40
Figure 4. Glucose levels of DBA/2J mice sacrificed during morning and Evening.....	41
Experiment 2	
Figure 5A. Daily 24hr chow intake of C57BL/6 mice.....	44
Figure 5B. Average 24hr chow intake of C57BL/6 mice.....	44
Figure 6A. Daily 4hr high fat intake of C57BL/6 mice.....	45
Figure 6B. Average 4hr high fat intake of C57BL/6 mice.....	45
Figure 7A. Daily total calories (high fat and normal chow) of C57BL/6 mice.....	46
Figure 7B. Average total calories intake of C57BL/6 mice.....	46
Figure 8. Corticosterone (CORT) from stress and non-stress C57BL/6 mice.....	47
Figure 9. Active ghrelin of trunk blood gathered from stress and non-stress mice.....	48
Experiment 3	
Figure 10A. Daily 24hr chow intake of C57BL/6 mice.....	51
Figure 10B. Average 24hr chow intake of C57BL/6 mice.....	51
Figure 11A. Daily sucrose intake of C57BL/6 mice.....	52
Figure 11B. Average 4hr sucrose intake of C57BL/6 mice.....	52
Figure 12A. Daily total calories (high fat and normal chow) of C57BL/6 mice.....	53
Figure 12B. Average total calories intake of C57BL/6 mice.....	53
Figure 13. Active ghrelin for non-stress and stress mice.....	54
Experiment 4	
Figure 14A. Daily 24hr chow intake of GHSR KO and WT mice.....	59
Figure 14B. Average 24hr chow intake of GHSR KO and WT mice.....	59
Figure 15A. Daily 4hr high fat intake of GHSR KO and WT mice.....	60
Figure 15B. Average 4hr high fat intake of GHSR KO and WT mice.....	60
Figure 16A. Daily 4hr sucrose intake of GHSR KO and WT mice.....	61
Figure 16B. Average 4hr sucrose intake of GHSR KO and WT mice.....	61
Figure 17A. Daily total calories of GHSR KO and WT mice.....	62
Figure 17B. Average total calories intake of GHSR KO and WT mice.....	62
Figure 18. Average time spent immobile during forced swim test.....	63
Figure 19. CORT for non-stress and stress GHSR KO and WT mice.....	64
Figure 20. Active ghrelin for non-stress and stress GHSR KO and WT mice.....	65
Experiment 5	
Figure 21A. Daily 24hr chow intake of C57BL/6 mice	68
Figure 21B. Average 24hr chow intake of C57BL/6 mice.....	68
Figure 22A. Daily 4hr high fat intake of C57BL/6 mice.....	69
Figure 22B. Average 4hr high fat intake of C57BL/6 mice	69
Figure 23A. Daily total calories) of C57BL/6 mice	70

Figure 23B. Average total calories intake of C57BL/6 mice	70
Figure 24. Forced swim testing for non-stress, stressed and pair fed mice.....	71
Figure 25. Glucose levels for non-stress, stressed and pair fed mice.....	72
Figure 26. CORT for non-stress, stressed and pair-fed mice.....	73
Figure 27. Active ghrelin for non-stress and stress mice.....	74
Appendix	
Figure 1A. Limbic circuitry showing projections of each of the key areas.....	81
Figure 2A. Chronic social defeat stress paradigm.....	82
Table 3A. Feeding related hormones, and their effects on hunger.....	83
Figure 4A. Experimental design of first study.....	84
Figure 5A. Experimental design for second study.....	85
Figure 6A. Experimental design for third study.....	86
Figure 7A. Experimental design of fourth study.....	87
Figure 8A. Experimental design of fifth study.....	88

Introduction. Obesity and stress as a challenge to human health.

Obesity is one of the leading preventable causes of death. With its increase in prevalence in to both adults and children, the threat to public health is growing rapidly. Obesity is an excess of body fat commonly defined through the use of the body mass index (BMI) scale. Typically, a BMI greater than 30 is considered obese. While obesity itself is a cause for alarm, additional health related ailments such as type 2 diabetes, cancer and cardiovascular disease, all appear at a higher incidence in those that are obese or overweight (a BMI less than 30 but higher than 25) (Bose, Olivan, & Laferrer, 2010). Factors contributing to the elevated incidence of obesity worldwide include an increase in the rate of sedentary lifestyles paired with higher consumption of energy dense food. Economically, the costs associated with obesity have the potential to have detrimental effects on society in coming generations especially towards health care costs (Cawley & Meyerhoefer, 2012). In Canada, \$4.3 billion was spent in 2001 towards obesity related ailments, an amount that represents 2.2% of health care costs (P. T. Katzmarzyk & Janssen, 2004). Additionally long-term surveys indicate that the national prevalence of obesity in adults increased from 5.6% to 15% between 1985 and 1998 (P. Katzmarzyk, Hebebrand, & Bouchard, 2002). Between 2007 and 2008, 17% of adults in Canada, approximately 4 million people, were reported as obese (Y. Chen & Qian, 2012). It was also shown through the Canadian Community Health Survey, that 33.77% of Canadians, roughly 8 million individuals, were overweight (Pouliou & Elliott, 2009).

Physiologically, an increase in body weight and adiposity results in metabolic changes that in themselves contribute to the pathological process of obesity (Bose et al., 2010). For instance, as body weight increases, so does oxygen consumption due to the expansion of metabolically active adipose tissue. This results in an increase in cardiac output, and total blood volume as additional

blood is needed to sustain tissue requirements (Kopelman, 2007). With this change in cardiovascular output, coronary artery disease, stroke and respiratory issues all become more prevalent. Similarly, increased levels of triglycerides and low-density lipoprotein (LDL) cholesterol additionally promote coronary heart disease and stroke (Shinton, Sagar, & Beevers, 1995). Obese individuals can also suffer from sleep apnea as fat mass stored around the neck reduces airway diameter, making it difficult to breathe (Kurtz, Krieger, Kowalski, Hoff, & Mangin, 1980). Extra weight from fat storage can also cause cartilage and joints to wear resulting in osteoarthritis. Obesity also increases the risk of developing gallbladder disease and fatty liver disease (Brummer, 1949; Clain & Lefkowitz, 1987; Sokoloff, Mickelsen, Silverstein, Jay, & Yamamoto, 1960). This present thesis aims to better understand how chronic stress can contribute to the development of obesity and how ghrelin is involved in this phenomenon. Specifically, we hope to elucidate how ghrelin possibly plays a role in modulating energy demands in order to counter stress symptoms.

Hypothalamic Regulation of Food Intake and energy balance

The regulation of food intake and energy balance involves a number of processes that include homeostatic mechanisms as well as motivational and cognitive factors associated with procurement and consumption of food. The complexity of these mechanisms is demonstrated by the amount of brain regions involved in regulating these processes. Of these, the hypothalamus has been identified as a critical component for the homeostatic regulation of food intake, whereas other structures including mesolimbic structures and prefrontal cortical regions appear to be related to the incentive regulation of palatable food consumption.

The hypothalamus has been considered to be one of the most important areas for

regulation of food intake and metabolism. Key nuclei such as the arcuate nucleus (ARC), lateral hypothalamic area (LHA), ventromedial nucleus (VMH), dorsomedial nucleus (DMH) and the paraventricular nucleus (PVN) are all involved in the regulation of feeding. The ARC in particular is recognized as a critical center for control of energy balance. Neurons within the ARC respond to peripheral factors such as ghrelin, insulin and leptin since ARC axon terminals have direct contact with the bloodstream (Peruzzo et al., 2000). Furthermore, the ARC contains two sets of neuronal populations. The first set of neurons produces neuropeptide Y (NPY), a peptide that potently increases food intake (Tatemoto, Carlquist, & Mutt, 1982). Interestingly, NPY neurons also produce a second orexigenic peptide known as agouti related peptide (Agrp) (Asakawa et al., 2002). The second set of neurons in the ARC, melanocortin neurons, produce the α -melanocyte stimulating hormone (α -MSH) which is an anorectic peptide formed from proopiomelanocortin (POMC) protein. In addition, α -MSH binds to MC3/4 receptors in the lateral hypothalamus resulting in the reduction of food intake, and promote satiety (Cone et al., 2001; Nemeroff, Lipton, & Kizer, 1978). Interestingly, NPY/Agrp neurons and α -MSH neurons project to the PVN, as well as other hypothalamic nuclei such as, DMH, lateral hypothalamus (LH) and VMH to regulate energy expenditure and food intake (Elmquist, 2001).

In addition to the hypothalamus, the brain stem is important for the regulation of food intake in response to rapid changes in nutrients. When a meal is consumed, the concentrations of glucose, free fatty acids and metabolic hormones are monitored by a number nuclei in the brain stem. These brain stem regions also receive information from the gut via the ascending vagus nerve in order to regulate short-term appetitive responses.

Extra-Hypothalamic Regulation of Feeding

The mesocorticolimbic dopamine system plays an important role in modulating reward-seeking behaviors including those that promote the consumption of palatable foods (see supplementary figure S1, in appendix 1). Given the choice, animals tend to favor palatable foods, and sometimes continue to consume them even after energy requirements are met (Roth, Tam, Ida, Yang, & Deutch, 1988). The mesocorticolimbic dopamine system consists of dopamine producing cells located in the ventral tegmental area (VTA) of the midbrain project axons that release dopamine into the medial prefrontal cortex (mPFC), and nucleus accumbens (NAc), hippocampus (HIPPO), amygdala (AMY), in particular the limbic sub region of this structure often referred to as the nucleus accumbens (Heimer et al., 1997). The two main projections from the nucleus accumbens are to the ventral pallidum and VTA. Both of these projections are GABAergic (Di Chiara, Porceddu, Morelli, Mulas, & Gessa, 1979). Additionally, the VTA and ventral palladium send GABAergic efferent to the medial dorsal thalamus (Maniam & Morris, 2012). Finally the limbic circuit is closed by glutamatergic projections from the medial dorsal thalamus to the mPFC (G. E. Alexander, Crutcher, & DeLong, 1990; Kalivas & Duffy, 1997; Kalivas & Nakamura, 1999) (see figure 1, appendix 1). The role of dopamine in this system is complex as its binding to different receptors can result in a wide array of effects. The physiological actions of DA are quite diverse and are mediated by five distinct G protein-coupled receptor subtypes; D1A-1D and D5 which are coupled to G proteins that activate adenylyl cyclase, while D2 like subfamily (D2, D3, and D4) inhibit adenylyl cyclase and instead activate K⁺ channels. DA receptor subtypes are distributed throughout different parts of the mesocorticolimbic pathway (Niznik, 1987; Vallone, Picetti, & Borrelli, 2000).

Human brain imaging has shown that corticolimbic brain circuits are also implicated in the

reward aspects of food sensation. Most notable are regions in the orbito-frontal cortex (OFC) as well as the insula, amygdala, and striatum. Subjective values of rewards, one's own preference of rewarding stimuli, are encoded by structures such as the striatum, insula, anterior cingulate cortex as well as the midbrain (Sescousse, Redoute, & Dreher, 2010). Activation of these structures is associated with increased motivation to consume palatable meals. These pathways can override one's homeostatic signals, leading to the overconsumption of calories ultimately leading to weight gain and an increase in adiposity.

It is important to note that there are a number of additional regions that can also regulate appetite. One of these is the orbitofrontal cortex, which is involved in sensory-specific satiety (O'Doherty et al., 2000). The amygdala also plays a key role as it is implicated in the evaluation of palatability (Nishijo, Uwano, Tamura, & Ono, 1998). Finally, while key brain regions integrate to regulate feeding, there is also a level of control by the peripheral system in which sensory and gastrointestinal are used to terminate ingestion (Hillebrand, de Wied, & Adan, 2002).

Metabolism and the role of hormones during food consumption

Energy homeostasis is controlled by a complex system in which factors such as food intake, hormonal regulation and sensory input all help to keep the system in balance. Energy provided from food consumption allows for the maintenance of biological function. When nutrient levels are low, organisms seek out food for energy, and enter a metabolic state of catabolism where stored nutrients are oxidized to provide sufficient energy to maintain tissue function. Energy rich foods such as fat and carbohydrates provide the largest amount of energy, which can be consumed to be utilized or stored for later use (see table 1, appendix 2). At the onset of a meal, metabolism is biased towards the oxidation of carbohydrates and excess energy is stored producing an anabolic

state. In living animals, metabolic changes can be measured using indirect calorimetry, a technique that records the amount of oxygen consumed and the amount of carbon dioxide produced by the organism in question over a defined period of time and under constant temperature conditions. Thus, energy homeostasis is achieved not only by changes (increases or decreases of food intake), but also by alterations in metabolism and energy utilization.

Hormones play an important role in conveying metabolic signals from the body to the brain. These signals provide feedback to key brain regions that regulate food intake, body weight, body temperature, blood glucose levels, and fat storage. Changes in hormones are detected by cells in the hindbrain and ventral hypothalamus, both regions of the brain where the blood brain barrier is more porous (van der Lely, Tschop, Heiman, & Ghigo, 2004).

In the periphery and upon the consumption of a meal, higher levels of glucose in the blood bind to the GLUT2 receptor protein in pancreatic β cells, which stimulate the production and release of insulin. As a result, the liver, skeletal muscles, take up glucose and store it as glycogen while in adipocytes it is stored as triglycerides. This begins a cascade that eventually activates hormone-sensitive lipase in fat tissue, releasing glycerol and fatty acids into the blood (B. M. King, 1988). The anabolic effects of insulin can be antagonized by hormones found in the adrenal glands, thyroid gland and anterior pituitary gland. In the brain, insulin targets the hypothalamus to increase the activity of α -MSH neurons ultimately leading to an increase in metabolic rate and a decrease in food intake (Perez-Tilve et al., 2010).

Another anorectic hormone is cholecystokinin (CCK), which is released by the proximal small intestine and is important in meal termination or rather satiation (Greenough, Cole, Lewis, Lockton, & Blundell, 1998). The primary brain target for CCK is the Area Postrema, a region that lies outside of the blood-brain barrier, and one that is also sensitive to glucose and free fatty acids.

Another important peptide hormone is glucagon, a peptide produced by alpha cells in the pancreas, and one that is important for optimal responses to meal ingestion. Glucagon promotes lipolysis and ketogenesis particularly when glucocorticoids levels are elevated (Porte, 1967).

While there are literally hundreds of hormones that regulate feeding (see table 2 in appendix), two key hormones that play a role in the regulation of obesity are leptin and ghrelin. Leptin is a protein produced and secreted by adipocytes. Its secretion targets the brain to decrease appetite and increase energy expenditure (Zhang et al., 1994). Leptin travels through the bloodstream, past the blood brain barrier (BBB) and binds to leptin receptors in a number of hypothalamic nuclei including the ARC, providing information about the status of the body's fat stores (Golden, Maccagnan, & Pardridge, 1997; Satoh et al., 1997; M. W. Schwartz, Peskind, Raskind, Boyko, & Porte, 1996). Like insulin, leptin targets and stimulates α -MSH neurons, while inhibiting NPY/AGRP neurons resulting in the decrease of food intake as well as an increase in the expenditure of energy.

Ghrelin

Ghrelin is a 28 amino acid hormone peptide produced in the gastric oxyntic mucosa by P/D1 cells (Sakata et al., 2002). The ghrelin message is translated into a peptide that is cleaved and then modified by the addition of n-octanoic acid to the third serine residue. This modification is critical for the biological effects of this hormone. Other than the fundus, a second major source of ghrelin synthesis is the small intestine. Lesser quantities can also be synthesized in the anterior pituitary gland, lymphocytes, placenta, testis, and ovaries (Gaytan et al., 2003; Gualillo et al., 2001; Hattori et al., 2001; Korbonits, Bustin, et al., 2001; Korbonits, Kojima, Kangawa, & Grossman, 2001; Tena-Sempere et al., 2002; Ueberberg, Unger, Saeger, Mann, & Petersenn, 2009).

Additionally ghrelin has been found in the hypothalamus and cerebral cortex (Cowley et al., 2003; Horvath, Diano, & Tschop, 2003; Hosoda, Kojima, Matsuo, & Kangawa, 2000; Hou, Miao, Gao, Pan, & Zhu, 2006). The role of ghrelin in these various regions however remains unclear and somewhat controversial (Castaneda, Tong, Datta, Culler, & Tschop, 2010). Ghrelin O-Acyltransferase (GOAT) is the enzyme responsible for ghrelin acylation, in which an acyl group or acetyl group is added. GOAT is expressed in the pancreas, adrenal cortex, colon, small intestine, gallbladder, brain and stomach of humans (Yang, Brown, Liang, Grishin, & Goldstein, 2008). In comparison, a high expression of GOAT is observed in the stomach and intestines of mice (Kojima et al., 1999).

Ghrelin increases food intake and reduces energy expenditure.

Upon its discovery, it was evident that, in contrast to leptin and other peripheral hormones, ghrelin actually stimulated food intake and adiposity (Tschop et al. 2000). These effects could in part be attributed to the ability of ghrelin to stimulate growth hormone, but they are still evident in mice that have a mutation preventing them from secreting growth hormone (Kojima et al., 1999; Tschop, Smiley, & Heiman, 2000). Released during periods of fasting, ghrelin plays an important role in signaling energy insufficiency. Interestingly the adipogenic effect of ghrelin is only partly due to increased feeding, and mostly due to reduced utilization of fat as a substrate, promoting the accumulation of fat stores (Tschop et al., 2000).

The effects of ghrelin are mediated through its actions on the growth hormone secretagogue receptor (GHSR), the only known receptor identified for ghrelin so far. GHSR is a G-protein linked receptor that is expressed in the hypothalamus, dorsal vagal complex, and the nucleus of the solitary tract as well as numerous other brain regions as well as various peripheral tissues (Zigman, Jones, Lee, Saper, & Elmquist, 2006). Some of these additional tissues include the

stomach, heart, kidney, intestine, and adipose tissue (Kojima & Kangawa, 2005; Korbonsits, Goldstone, Gueorguiev, & Grossman, 2004). Two splice variants exist of the receptor in humans: GHSR type 1a which is capable of binding ghrelin, and GHSR type 1b, which is not activated by ghrelin (Kojima & Kangawa, 2005). These variants have not been reported in rodents.

The GHSR plays an important role in energy homeostasis and body weight control, as activation of this receptor promotes nutrient incorporation into muscles and other tissue (Castaneda et al., 2010). Nevertheless, studies examining the phenotype of mice with targeted deletion of ghrelin or its receptors have shown only subtle differences in body weight, or in the intake of high fat diet (HFD) or normal diets between KO and WT animals (Sun, Wang, Zheng, & Smith, 2004). Additionally GHSR KO mice show only slightly lower body weight than WT mice fed standard chow (Sun, Butte, Garcia, & Smith, 2008). GHSR KO mice, however, do have less body fat than WT mice after being exposed to a high fat diet for prolonged periods of time (Zigman et al., 2005).

Ghrelin increases food intake through the activation of hypothalamic nuclei, most notably the ARC. Here, ghrelin promotes the secretion of the orexigenic peptides neuropeptide Y (NPY) and agouti-related protein (AgRP) while inhibiting α -MSH expression (Nakazato et al., 2001). Ghrelin has also been shown to reduce the inhibitory tone of CRH neurons by activating pre-synaptic NPY receptors Y1 and Y5 (Ferrini, Salio, Lossi, & Merighi, 2009). This disinhibits the important orexigenic pathway, which is mediated by these neurons (H. Y. Chen et al., 2004). The role of ghrelin towards increasing food intake can have multiple effects on nuclei of the hypothalamus and other associated regions. Ghrelin also targets the PVN to alter substrate utilization which favors carbohydrate metabolism (Bagnasco, Dube, Katz, Kalra, & Kalra, 2003).

In addition, ghrelin also plays a role in reward and motivation. For instance, expression of

the GHSR has been detected in regions such as the VTA, NAc, AMY and HIPPO (Abizaid et al., 2006; Carlini et al., 2002; Diano et al., 2006). In humans, ghrelin increases neuronal activity in reward related areas of the brain for individuals presented with images of palatable foods (Malik, McGlone, Bedrossian, & Dagher, 2008). In mice fed *ad-libitum*, ghrelin has been shown to increase the rewarding value of a high fat palatable diet. Ghrelin may increase the motivation to consume more palatable food after chronic social defeat through VTA dopamine neuron stimulation (Chuang et al., 2011; S. J. King, Isaacs, O'Farrell, & Abizaid, 2011; Perello et al., 2010). The effects of ghrelin on reward related mechanisms are associated with increases in the action potential frequencies of VTA dopaminergic neurons in vitro for GHSR WT but not GHSR KO animals. Interestingly ghrelin increases excitatory input onto dopamine neurons, accelerates synapse formation and dopamine turnover in the nucleus accumbens (Abizaid et al., 2006).

The effects of ghrelin on food intake also involves the binding of this hormone to receptors in the hindbrain, specifically the nucleus tractus solitarius (NTS), area postrema (AP), and dorsal vagal complex (DVC)(Faulconbridge, Cummings, Kaplan, & Grill, 2003). These regions receive visceral afferent inputs and regulate efferents that control autonomic functions including heart rate, respiration and gut motility (Rinaman, Card, Schwaber, & Miselis, 1989; G. J. Schwartz & Moran, 2002).

The Stress Response

While lack of exercise and excess eating are key components of weight gain, there are other environmental factors that can also contribute to the development of obesity. Among them, psychological stress has been linked to changes in both behavior and metabolism that can ultimately lead to an obese phenotype (Black, 2006). In this way, stress can be considered a

mental or emotional state caused by an adverse or demanding circumstance. The body counters, often with exaggerated responses, in order to meet the demands posed by stressful conditions, a process often referred to as allostasis. This process aims to achieve homeostasis, even when set points and other boundaries of control are altered due to different environmental conditions (McEwen, 1998). While it might seem as though stress causes negative effects, it is also vital for survival. When facing an acute stressor, a behavioral and physiological change is initiated which helps to ensure an animal's survival (De Vriendt, Moreno, & De Henauw, 2009). There are two different components of the stress response: a fast stress response and a slow stress response. The fast stress response is mediated by the sympathetic nervous system (SNS). Initially upon perception of a stressful situation, the amygdala processes this information and relays it to the hypothalamus. The hypothalamus mediates both the fast and slow stress responses by sending signals through the autonomic nerves to the adrenal glands (Goldstein, 1987). Preganglionic sympathetic fibers found in the adrenal medulla secrete acetylcholine, which acts on nicotinic acetylcholine receptors causing depolarization and sequentially, influx of calcium by voltage-gated channels. Calcium results in the exocytosis and release of noradrenaline and adrenaline into circulation (Goldstein, 1987). The SNS has direct contact with a wide variety of glands and smooth muscles, which with the addition of adrenaline release, causes a fight or flight response. This response allows the animal to react appropriately to dangerous or alarming situations (Goldstein, 1987; Sainsbury & Zhang, 2012).

The slow stress response involves activation of the hypothalamic pituitary adrenal (HPA) axis. The activation of the HPA axis starts with the stimulation of neurons within the PVN in response to a stressor, physical activity or even illness (P. E. Stokes & Sikes, 1991). When an acute stressor occurs, corticotrophin-releasing hormone (CRH) and vasopressin are released from the

PVN (Buckingham & Hodges, 1979; Makino, Hashimoto, & Gold, 2002). CRH is a peptide hormone and neurotransmitter that acts upon corticotrophin releasing hormone receptors 1 and 2 (CRHR1 & CRHR2). CRHR1 is expressed in the neocortex, hippocampus, basolateral amygdala, ventral tegmental area (VTA), pontine gray, lateral dorsal tegmentum, pedunculopontine tegmental nucleus, and the anterior pituitary (Kishimoto, Pearce, Lin, & Rosenfeld, 1995; Lovenberg et al., 1995; Perrin et al., 1995; Stenzel et al., 1995). CRHR2, however, is expressed in the lateral septum, bed nucleus of the stria terminalis, medial and cortical nuclei of the amygdala, dorsal raphe, and nucleus of the solitary tract (Holsboer & Ising, 2010). While CRHR1 is important for endocrine, autonomic and immune responses to stress, the role of CRHR2 in stress is unclear. CRH enters the portal blood vessel system of the hypophyseal stalk and is transported to the anterior pituitary (Makino et al., 2002). CRH and vasopressin together stimulate CRH responsive corticotrope cells, which are responsible for synthesizing and releasing adrenocorticotrophic hormone (ACTH) into systemic circulation. ACTH is derived from a larger precursor protein called POMC (Eipper & Mains, 1980; Smith & Funder, 1988). ACTH travels in the systemic circulation and then binds to adrenal cortical cells located on the zona fasciculata of the adrenal cortex. The binding of ACTH to receptors on adrenal cortical cells results in the synthesis and secretion of glucocorticoids from precursors such as cholesterol (Peter E. Stokes, 1991). Cortisol is the main glucocorticoid in humans and corticosterone (CORT) acts as the main glucocorticoid in rodents. The functions of CORT include maintenance of vascular tone and permeability, endothelial integrity and mediating the vasoconstrictory actions of catecholamines (Habib, Gold, & Chrousos, 2001; Lamberts, Bruining, & deJong, 1997). Both the CRH and locus coeruleus-norepinephrine (LC-NE) system of the brain are important effectors of the stress response. When one is activated, the other is additionally activated as they participate in a positive feedback loop (Tsigos & Chrousos, 2002).

Circulating cortisol feeds back to the hypothalamus and pituitary to inhibit the secretion of CRH and ACTH. This reduces the amount of CRH secreted, thus controlling the HPA axis by negative feedback (George, Khan, Briggs, & Abelson, 2010). Regulation of adrenaline and norepinephrine, which is controlled by the adrenal medulla through stimulation of the sympathetic nervous system and cortisol, is also an important regulatory system (Porcelli, Lewis, & Delgado, 2012; Stjarne, 1973).

While regulation is vital to controlling the function of the HPA axis, anatomical connections between the HPA axis and areas such as amygdala, hypothalamus and hippocampus demonstrate the importance of the axis in its influence on a wide array of behaviors. For example, sensory information from stress is filtered for significance by the amygdala, while the hypothalamus processes fear signaling, relaying this information to the SNS. The hypothalamus receives input from the amygdala via connections from the ventral amygdalofugal pathway and stria terminalis. Another crucial factor in stress is fear conditioning which consists of learning about the emotional significance of stimuli, a process governed by the amygdala (Davis & Whalen, 2001). The hippocampus on the other hand, appears to be important in preparing the organism to cope with future stressors (Markowitsch & Pritzel, 1985).

Cortisol is known to influence the energetic state of an individual (Dallman et al, 1995; Nieuwenhuizen and Rutters, 2008). For example, cortisol stimulates gluconeogenesis in the liver, and the resulting increase in glucose concentrations causes a surge in insulin release from the pancreas (Huebener, 1960). Various studies have shown increased cortisol in obese individuals, however explaining how this results in an increase in food intake is complicated. Recent studies have shown that when healthy individuals are given CRH, their food consumption and calorie intake decreases, unless they are given access to a palatable food (Adam & Epel, 2007; Foster et al., 2009;

Tomiyama, Dallman, & Epel, 2011). Cortisol production seems to affect non-homeostatic feeding, resulting in an increase in intake of palatable foods (Adam & Epel, 2007).

Stressors vary in nature and can be chemical, biological, psychological and/or physical but they often elicit physiological and/or psychological responses. In many ways, any stimuli that can cause deviations from homeostasis can be considered a stressor. Rodent models of stress include a wide variety of stimuli such as exposure to cold, inescapable foot shock, tail pinching, food restriction, restraint and exposure to socially dominant conspecifics. These stressors can elicit a number of different psychological responses as well as varied effects on food intake (Bartolomucci et al., 2004; Henry, 1997).

An example of one such stress is tail pinching, which elicits feeding as well as situation specific behavior, suggesting that the increase in food intake is due in part to a general stress arousal response (Adam & Epel, 2007). Tail pinching increases feeding during the event, but feeding later decreases when stress has subsided (Dickerson, Gruenewald, & Kemeny, 2004). Studies comparing chronic physical stress to emotional stress showed that rats exposed to chronic physical stress in the form of foot-shock showed reduced consumption and preference for a saccharin sweetened drink as compared to water. Emotional stress, on the other hand, increased saccharin preference and consumption compared with water (Boggiano et al., 2005; Pijlman, Wolterink, & Van Ree, 2003).

While a number of stressors cause a decrease in caloric intake and body weight, social stressors seem to be particularly effective in increasing caloric intake and body weight in rodents. An example of this is the visible burrow system (VBS). VBS is a social stressor in which two male rats are housed in cages that resemble burrows. A social hierarchy is established when a male becomes dominant and the second becomes subordinate. This stressor results in various

behavioral effects ranging from social withdrawal to increased caloric consumption (Bartolomucci et al., 2004; Dallman, Warne, Foster, & Pecoraro, 2007; Koolhaas, De Boer, De Rutter, Meerlo, & Sgoifo, 1997). Also, this model is an ecologically valid stressor for rodents and can be relevant in understanding how social stressors affect human populations. The paradigm is considered dynamic as the animals do not habituate and as a result it is a potent stressor that can have adverse effects on homeostasis (Blanchard et al., 1995).

Another paradigm used is the chronic social defeat stress model, is when a mouse is placed in the cage of a bigger, sexually experienced (and therefore more territorial) mouse. Within seconds the resident mouse establishes his dominance and the smaller mouse becomes submissive (see figure S2, Appendix 2). Animals are then separated by a mesh divider but housed together for prolonged periods of time and allowed to interact physically once daily. This chronic interaction leads to increased food intake and body weight in the submissive mouse, and can lead to obesity in older mice (Patterson et al. 2013).

Change in metabolic rate due to stress

Basal metabolic rate refers to the amount of energy expended by an animal at rest. Metabolic rate can increase during strenuous activities such as running or decrease to basal levels when an animal is asleep. The metabolic response to a stressor includes an increase in plasma glucose levels, gluconeogenesis in the liver, and a reduction in insulin stimulated glucose uptake. This energy is fundamental for coping with the energetic demands of stress (Apple et al., 1995). Chronic stress changes the metabolism of an organism by increasing metabolic rate. Additionally, protein catabolism increases, while fat stores remain intact. This outcome is a result of continuous stimulation of the sympathetic nervous system and HPA axis. The HPA axis can also

affect metabolism by altering the production of glucocorticoids and insulin, which both influence blood glucose levels.

Cortisol has been shown to increase the release of neuropeptide Y (NPY) in the ARC, which increases food intake. Glucocorticoids can also decrease the expression of other neuropeptides that are important to feeding such as POMC and agouti-related peptide (AgRP)(Belgardt, Okamura, & Bruning, 2009). The effect of stress on metabolic rate is influenced by the type of stressor, as food intake is multifactorial and influenced by the stress severity (Weststrate, Van der Kooy, Deurenberg, & Hautvast, 1990).

Ghrelin and the stress response

Evidence points to an involvement of ghrelin signaling in stress-induced obesity (Abizaid et al., 2006; Chuang & Zigman, 2010; Lutter, Sakata, et al., 2008; M. Patterson, Bloom, & Gardiner, 2011; Z. R. Patterson, Ducharme, Anisman, & Abizaid, 2010). Like glucocorticoids, ghrelin is secreted after acute and chronic stressors (Patterson et al., 2010) and interacts with glucocorticoids to increase food intake (Otto et al., 2004). Moreover, some research suggests that glucocorticoids themselves are able to influence circulating ghrelin (Otto, Tschop, Heldwein, Pfeiffer, & Diederich, 2004). While glucocorticoids are important for ghrelin induced food intake, ghrelin's adipogenic effects seem to be glucocorticoid-independent (Tung, Hewson, & Dickson, 2004).

Stress-induced increases in ghrelin secretion are due to the activation of the sympathetic nervous system (Chuang and Zigman, 2010). In turn, ghrelin may potentiate the neuroendocrine response to stress given that central and intra-paraventricular ghrelin administration causes activation of CRH neurons leading to enhanced HPA axis responses (Cabral, Suescun, Zigman, &

Perello, 2012). Peripheral ghrelin injection increases CRH mRNA expression within the hypothalamus of mice. Interestingly ghrelin has shown potent ACTH-releasing activity. The role of ghrelin might mediate the effects of stress on food intake by influencing the CRF, serotonin and melanocortin systems (Maniam & Morris, 2012). Stress induced ghrelin secretion also alters sympathetic responses as well as glucose-regulatory processes. These actions are mediated by GHSR located at the nucleus of the solitary tract (Scott et al., 2012).

Given that ghrelin is released in response to stress it is not surprising that ghrelin can alter behavioral responses associated with emotional states. For example, mice lacking GHSR show more depressive-like symptoms following chronic social defeat stress (Lutter et al, 2008). This evidence suggests that ghrelin signaling pathways might provide homeostatic adaptations intended to alleviate the effects of stress (Chuang & Zigman, 2010). This effect, however, carries with it the cost of an adverse metabolic profile (Lutter, Krishnan, et al., 2008)(Patterson et al, 2013). Interestingly, several studies show that repeated stressors including repeated social defeat stress lead to an increase in plasma ghrelin concentrations that remain elevated for days after the termination of the stressor (Kristensson et al., 2006; Lutter, Sakata, et al., 2008; Tassone et al., 2003). The GHSR is expressed in the hypothalamus, specifically the ARC, where food intake, energy expenditure and metabolism are delicately controlled (Kirchner et al., 2009; Tschop et al., 2000) and these effects are mediated by ghrelin acting on NPY/ AGRP neurons in the ARC. This is supported by data demonstrating that ghrelin affects food intake by stimulating the release of NPY and AgRP from the ARC (Cowley et al., 2003; Holst & Schwartz, 2004). Ghrelin also possess protective attributes such as attenuation anxiety and depressive like behavior, which protect the stomach against ulcers (Ochi et al., 2008). Also ghrelin might play a role in protective mechanisms in which ghrelin favors utilization of carbohydrates, enabling a rapid source of energy in the

presence of a chronic stressor (Patterson et al, 2013).

Reducing stress through Food intake

Given the potential protective role of ghrelin, it would be useful to determine the mechanisms underlying these beneficial effects. Several studies have shown that animals with access to sucrose solutions during chronic stress exposure show an attenuated HPA response (Dallman, 2007). One could argue that an increase in the consumption of sucrose or carbohydrate rich foods occurs under conditions of stress, as a means to rapidly meet the energetic challenges of stressors. Furthermore, given that these substances have a palatable taste, and reduce stress levels, both combine to enhance the reinforcing effects of the foods and promote their future intake as a means of coping with new stressors. The tendency for stress to promote preference for palatable foods and non-homeostatic eating may be due in part to its effects on the mesocorticolimbic dopamine system.

The release of CRH into the VTA as a response to stress hints at such a connection (Rodaros, Caruana, Amir, & Stewart, 2007). Stress-induced CRH release, in addition to stimulating corticosterone release, has also been shown to target the VTA and facilitate dopamine neuron firing in a dose-dependent manner (Wanat, Hopf, Stuber, Phillips, & Bonci, 2008). Similarly, palatable food activates the brain's reward system, and this effect is dependent on preference and quantity of food ingested (Cota, Tschop, Horvath, & Levine, 2006). Interestingly, the stress response can be reduced when a palatable food is ingested (Kreek & Koob, 1998). In this sense, chronic stress along with free access to palatable foods may lead to repeated stimulation of the reward pathways and ultimately lead to constant overeating and obesity (Rada, Avena, & Hoebel, 2005).

Accumulating evidence shows that palatable food promotes dependence similar to the addiction found in drug abuse. The consumption of palatable food sustains endogenous opioid release which in turn increases palatable food intake (Colantuoni et al., 2002). On the other hand, access to palatable foods rich in fats or sugars has been shown to attenuate certain effects of stress. Stressed mice show lower corticosterone levels when given access to a palatable meal, however anxiety-like behaviors remain (Dallman, 2007; Ortolani, Oyama, Ferrari, Melo, & Spadari-Bratfisch, 2011). Consumption of palatable foods is a possible mechanism for reducing stress-induced HPA axis activation, which ultimately alters metabolism (Dallman, Akana, et al., 2003). Thus the adaptive benefits of palatable food include providing energy necessary for overcoming the stressor, and allowing the organism to modulate its own stress response (Maniam & Morris, 2012). An unfortunate side effect of this is the tendency for stressed animals to develop obesity when placed in an environment with limitless access to palatable foods.

Ghrelin might play a role in reducing stress symptoms through its mediation of caloric intake, or rather promotion of carbohydrate as an energy substrate, sparing body fat usage (Tschop et al., 2000). This change in energy homeostasis, favors adiposity but also alleviates stress symptoms and allows the animal to refuel lost energy through increased food intake. Finally, given that ghrelin receptors are found in the VTA, and that stress results in increased ghrelin secretion leads to the hypothesis that stress may increase the intake of palatable foods (S. J. King et al., 2011; Perello et al., 2010).

Rational for present thesis

Previous work in our lab hypothesized that stressed WT mice would eat more of a diet rich in high fat due to the effects of ghrelin on palatable foods. We however observed an increase in normal chow (high in carbohydrates) and thus believe this to be the effects of stress on carbohydrate utilization (Z. R. Patterson, Khazall, Mackay, Anisman, & Abizaid, 2013a). Given these results, we reasoned that stressed mice favor the consumption of a rapid fuel source such as carbohydrates in order to manage the stressful conditions. Because this effect depends in part on functional ghrelin signaling, the purpose of ghrelin in the stress response may be to direct feeding behavior toward carbohydrate rich food sources. Given that ghrelin is released in response to stress, one would hypothesize that animals in response to stress would consume palatable food. However previous data has shown that chronic social stress increases normal chow intake, even when a palatable source such as high fat is present.

Given that stress increases the use of carbohydrates as a source of fuel, and that this effect is ghrelin dependent, we hypothesis that access to carbohydrate use is important in alleviating stress symptoms. In order to test whether this effect is strain dependent, we will be testing to see whether a different strain such as DBA/2J mice will show similar food preference behavior under the same stressor. Previous work had shown that C57BL/6 mice tend to prefer carbohydrates over fat, and so we will be using DBA/2J, a strain of mice that is known for their preference for fat (J. Alexander, Chang, Dourmashkin, & Leibowitz, 2006; Lewis et al., 2005; Lewis et al., 2007). One alternative hypothesis could have been that high fat exposure and the stressor were too close, and that the mice were associating the two, reducing high fat intake. In order to test this, we will place the stressor earlier in their day cycle, and high fat 4 hours prior to initiation of their dark cycle. Seeing that utilization of carbohydrates as a rapid fuel source is vital to reducing symptoms of

stress, we will use sucrose as a means to test whether a palatable carbohydrate diet would additionally be favored by stressed mice. Circulating ghrelin is increased in stressed mice however the function of this increase is unknown. Given that ghrelin is able to both increase food intake and protect fat stores, we reasoned that ghrelin signaling may be a key factor in bringing about the change in dietary preference we've observed among stressed mice. To test this hypothesis, we will repeat the conditions of Experiment 3 using animals genetically engineered to lack the GHSR gene, and compare their food intake patterns to wild-type animals. We expect that the stressed GHSR KO mice would decrease their consumption of HFD, sucrose and normal chow in comparison to GHSR WT animals. Studies have shown that access to palatable food high in fat or sugar, attenuates certain effects of stress. Pair feeding, in which food intake is limited to the non-stress group, will be used in our final study in order to determine whether the additional calories are necessary in order to manage stress. Seeing as pair fed mice will have limited access to calories which are needed to alleviate stress symptoms, we hypothesis that pair fed mice will show depression and social anxiety when we measure these behaviors through behavioral testing.

General Methods

Animals

Animals used in the following experiments were all males of the strain DBA/2J (Jackson Labs, Bar Harbour, Maine, USA), C57BL/6 (Charles River, St. Constant, Quebec, Canada), or GHSR KO and WT male mice bred using breeding pairs obtained from Regeneron Pharmaceutical Inc, Tarrytown, NY, USA. The metabolic phenotype of this strain has been previously characterized (Pfluger et al., 2008). Additionally CD1 retired breeders males were purchased from Charles River and used as the resident mice that served as social stressors. All mice were individually housed for 1 week prior to conducting a study with access to water bottles and normal chow (Tekland TD06414). All

procedures followed Carleton University Animal Care Committee guidelines of the Canadian Council on Animal Care (CCAC) under the protocol p10-39.

Upon arrival, mice were placed in acrylic cages (L 11in x W 6.75 in x H 5in) and given time to habituate as well as access to ad libitum normal chow, which was placed in a steel food hopper. Water was initially given in plastic bottles however once the experiment began special lids were used in which only test tube could be utilized to dispense water. Factors such as humidity (63%), temperature (22°C) ventilation were all kept within normal range. Bedding was changed once a week, however during the experimental phase, the stress group cages needed to be changed twice a week. Bedding consisted of bet-o-cob bedding. Non-stress mice were given access to nestlets.

Chronic Social Defeat Paradigm

Initially a baseline of 21 days was conducted in which high fat, normal chow and body weight were all measured. At the end of the baseline of each experiment, animals were weight matched, and assigned into groups (one stress, and one non-stress). Stressed animals were housed in a different room than non-stressed mice so that they are not exposed to the social stress that the retired breeders cause. The stressor consists of housing experimental animals in the same standard cage with an acrylic top and divider (see figure S2 for details in appendix 1) inhabited by a retired breeder CD-1 male mouse. This divider had an acrylic frame and metal wire mesh, which allows the exchange of social cues however it prohibited physical interaction. Each cage additionally had an acrylic lid with holes for test tubes. Bedding was changed twice a week for mice housed with a CD1. Normal chow will be given in steel food hoppers. Each day of the experimental period, the divider was removed (timing depending on experiment) and the animals were allowed to interact for 10 minutes or until the experimental mouse exhibited a submissive

pose. All mice were carefully monitored, and interactions terminated before possible injury could occur.

Daily Measurements

Normal chow intake, 60 % high fat, and body weight was measured throughout the experiment. High fat was presented in ceramic dishes. In cases where sucrose was measured, both water and sucrose were given in test tubes, with stoppers. These were weighed individually, in order to record the intake of each animal individually. Normal chow and water was also measured every 24hrs while sucrose and high fat was measured for 4 hours. Fresh sucrose was prepared every day, 1 hr. prior to feeding. The nutritional information of normal chow, high fat, sucrose (see table 1) was used to calculate the total calories.

Diet	Provider	kcal·g⁻¹	Fat (%)	Carbohydrate (%)	Protein (%)
High Fat	Harlan Diets	5.1kcal·g ⁻¹	34.3	27.3	23.5
Normal Chow	Harlan Diets	3.3kcal·g ⁻¹	4	48	14.3
Sucrose	n/a	4 kcal·g ⁻¹ , 2.5%	0	100	0

Table 1. Nutritional value of provided diets

Blood collection

At the end of each study mice were rapidly decapitated. Trunk blood was collected in borosilicate tubes coated with EDTA (BD, Vacutainer, REF 367884). The blood glucose was

measured and recorded using a glucometer (Bayer Contour) and equivalent glucose strips. Upon collection, the EDTA tubes were placed on ice and then centrifuged for 15 minutes at 3,000g. The separated blood plasma was collected and placed into two 0.2mL eppendorf tubes, one for acylated ghrelin analyses (see ELISA) and the other for CORT analyses. Plasma samples collected for ghrelin concentrations were protected against degradation by adding 2.7µL of 1.0N HCl and 5µL of 10mM PHMB. This was done to preserve the acyl side chain on the ghrelin molecule in order to detect levels of acylated (biologically active) ghrelin. These tubes were then be stored at -80°C until they were assayed.

ELISA

An ELISA (Millipore, Rat/Mouse Active Ghrelin kit, EZRGRA-90K) was conducted by taking the acidified samples and pipetting them into the appropriate wells of an ELISA plate. Samples were incubated in antibody for 2 hours, decanted, and washed 3 times with a wash buffer. 100 µL of enzyme solution was then added to each well and then incubated for 30 minutes. The samples were again rinsed with wash buffer, six consecutive times and then 100 µL of substrate solution was added. The solution was then permitted to develop for 15 minutes and then stopped with acidic stop solution. The plate is then read at 450nm and then compared to standards using a 4th order parametric calibration curve.

CORT

Plasma corticosterone levels were assayed using a radioimmunoassay kit according to the manufacturer's protocol (ICN Biomedicals, CA, USA).

Statistical Analyses

Statistical analyses was conducted on all of the following data collected: body weight, chow, high fat, sucrose (when applicable), total calories, ghrelin and CORT. Experiments one through three were analyzed using 1-way ANOVA's with treatment (stressed vs. non-stressed) as the between group factor. Experiment four was analyzed using 2 x 2 ANOVAs with genotype (WT vs. GHSR-KO) and treatment (stressed vs. non-stressed) as the between group factors. Lastly data from experiment 5 were analyzed using 1 way ANOVAs with treatments (non-stress vs. stress vs. paired fed stress) as the between group factor. Tukey's HSD post hoc tests were performed if significant main effects are found.

Experiment 1: Chronic stress affects caloric intake preference, increasing overall body weight.

For the first study we aimed to elucidate how chronic social stress affects the dietary habits of mice. In particular, we wished to elucidate if the decrease in normal chow is strain dependent. Previous work by our lab has shown that chronic social defeat stress increases caloric intake specifically the intake normal chow (high in carbohydrates) while decreasing the intake of high fat (Z. R. Patterson et al., 2013a). Because C57BL/6 mice prefer carbohydrates, we wanted to replicate this data using a strain of mouse that prefers the intake of fat, thus here we repeated previous work this time using male mice of the DBA/2J strain. We hypothesized that if stress required carbohydrates, then, DBA mice would also increase their carbohydrate rich regular chow and not their high fat intake. Alternatively, if stress increased the intake of preferred palatable foods, then these mice should show increased intake of the high fat diet.

Twenty-five male DBA/2J mice were given ad lib access to normal chow and water during habituation (see figure 3). High fat was presented early morning, two hours after initiation of their light cycle. Access to high fat was permitted for 4 hours upon which it was removed. Our standard

baseline comprised of 21 days in order to capture a progression of the change in diet preference and body weight acclimation. Upon completion of baseline, twelve mice were weight matched and placed into a stress group where they were housed with a CD-1 (see social stress paradigm, figure 2). Mice were stressed daily just after removal of high fat. Upon completion of the stress period, a recovery period was performed in order to see if removal of the stress would still produce stressed behavior and dieting. The recovery period consisting of twenty-one days, involved removal of stress animals from their stressors, and placement back into their individually housed cages. Ad lib access to chow and water was continued as well as 4hr exposure to high fat. Upon completion of the recovery period the mice were sacrificed in the morning and blood glucose was collected.

Experiment 2: Presentation of high fat, does not affect diet preference.

Method

Previous work in our lab has shown that chronic social defeat stress leads to a decrease in high fat intake in spite of this diet being palatable to the mice. Nevertheless, in these studies the high fat diet was given just after the animals had interacted with the dominant aggressor, and this negative interaction may have been associated with the diet, ultimately decreasing the overall intake of the diet. In order to determine if this was the case, we propose a study in which the high fat diet is presented several hours after the interaction with the aggressive mouse is terminated each day. If the decrease in the high fat diet seen in previous studies was due to how closely it was presented after the stressor we should see an increase in the intake of the high fat diet if this diet becomes available hours after the animals were exposed to the stressor.

To test this hypothesis, forty male C57BL/6 mice were given ad lib access to normal chow and water during habituation. High fat presentation lasted for 4 hours prior to the initiation of the dark cycle (4 hours after the stressors). Upon completion of baseline (21 days), twenty C57BL/6 mice were placed in stress cages. Stress occurred early in the morning. In this experiment, no recovery phase was conducted. Half of each group was sacrificed at different time periods, one in the morning and the other at night to compare active ghrelin (n=10 per group). Blood and tissue collection was collected as described above however once again half of each group was sacrificed at different time periods, one in the morning and the other at night.

Experiment 3: Effects of Chronic social defeat stress on preference for carbohydrates or fat.

Method

Stress symptoms are reduced when animals have access to a palatable comfort food (Dallman, Akana, et al., 2003). Previous work has shown that stressed mice increase their intake of normal chow, comprising mostly of carbohydrates, when compared to non-stressed mice. While their normal chow intake increased, high fat diet intake remained the same resulting in an increased overall caloric intake. In order to verify that this increase in chow intake was due to its carbohydrate content, we performed the same experiment but offered animal's access to a sucrose solution in addition to the usual high-fat diet. We hypothesized that stressed mice would increase sucrose and normal chow intake, but not high fat diet intake resulting in an overall increase in calories derived from carbohydrates.

We utilized the same baseline and stress conditions used in the last two experiments with twenty male C57BL/6 mice. Additionally, mice were given access to sucrose solution (2%) at the same time as high fat presentation. Upon completion of the experimental phase, two different

recovery phases were conducted. The first 7 days of recovery consisted of placing the stressed mice back into their individual cages and giving the usual 4hr access to sucrose and high fat. The last 14 days of the recovery consisted of giving the mice ad lib access to high fat and sucrose. All mice were sacrificed in the morning, and our standard blood and tissue collection was conducted.

Study 4: Ghrelin receptor KO decrease overall caloric consumption as well as preference in carbohydrate rich diets.

Method

Given that ghrelin is able to increase food intake and protect fat stores, we investigated its role in the preference of carbohydrates during stress. Eighteen GHSR KO and GHSR WT were allowed to habituate with ad lib access to normal chow and water. During our initial baseline (14 days), mice were given access to high fat and a sucrose solution for 4hr, as well as ad lib normal chow and water. Our stress phase (21 days) consisted of our usually stress period involving the experimental mice being housed with a CD1 retired breeder. After the experimental phase, a behavioral test (forced swim) was conducted. This test involved placing a test mouse in a cage half-filled with room temperature water. This event was videotaped for 5 minutes. The amount of time-spent swimming was compared to the time spent floating. Time spent floating is correlated to negative mood. All mice were once again sacrificed in the morning over a period of two days. Blood and tissue collection followed standard procedure (see above).

Study 5: *Pair fed mice, display reduced ability to alleviate symptoms associated with stress*

Aim: To investigate how pair fed stress GHSR KO and WT cope with stress when caloric consumption is reduced.

Method

Previous work has shown that when stressed, mice increase their overall calories. These calories have shown to be vital in alleviating stress-associated symptoms. In order to test whether lack of additional calories are detrimental towards this process, we conducted another study using thirty male C57BL/6 mice. Our usual baseline (21 days) was implemented, after which the mice were weight matched and split into three groups: non-stress, stress, pair fed. Pair fed mice were given access to calories that match the dietary consumption of non-stress mice. The stress period (21 days) placed the pair fed mice as well as the stress group mice into our usual social stress paradigm. After the experimental phase, a forced swim behavioral test was conducted. All mice were sacrificed the next morning, blood and tissue collection followed our standard procedure (see above).

Results

Experiment 1 - Stress causes an overall change in diet preference and caloric intake

In experiment 1, twenty-five male DBA/2J mice were used to inquire whether there is strain difference in caloric preference with regards to the stress paradigm used in previous studies by our lab. No significant difference was seen between the two experimental groups in terms of body weight for the experimental phase ($F(1,23) = 0.22, p > 0.05$) and recovery phase ($F(1,23) = 0.13, p > 0.05$).

At the start of the study, animals were matched for body weight and food intake, the two groups of animals ate the same amount of chow during baseline when analyzed using a one-way ANOVA ($F(1,23) = 2.20, p > 0.05$). During the experimental phase, however, we found that consumption of chow was significantly higher in stressed mice than in non-stressed mice ($F(1,23) = 60.42, p < 0.05$; See Figure 1A). Stressed mice continued to show increased chow intake compared to non-stressed mice even during the recovery phase ($F(1,23) = 28.31, p < 0.05$). In order to analyze the daily chow intake (figure 1B), a repeated measures ANOVA was used, showing an interaction between stressed and non-stressed mice ($F(1,23) = 1045.86, p < 0.05$). Additional post hoc tests using one-way ANOVAs show that stressed mice consumed more normal chow than non-stress mice from stress day 3 to recovery day 17 ($p < 0.05$).

In contrast, average 4hr consumption of high fat was lower in stressed mice than in non-stressed mice during the stress phase of the experiment ($F(1,23) = 27.09, p < 0.05$; see figure 2A). Stressed mice continued to eat less high fat diet than non-stressed mice during the recovery period ($F(1,23) = 10.39, p < 0.05$). In order to analyze the high fat consumption at a daily level (figure 2B), a repeated measures ANOVA was used, showing an interaction between stressed and non-stressed mice ($F(1,22) = 22.04, p < 0.05$). Additional post hoc tests using one-way ANOVAs

show that stressed mice consumed more normal chow than non-stress mice from stress day 2 to recovery day 10 as well as recovery day 13 and 18 ($p < 0.05$).

Interestingly, the total number of calories consumed over the stress period was greater in stressed mice than in non-stressed controls (see figure 3A), ($F(1,23) = 18.24$, $p < 0.05$).

Furthermore, stressed mice continued to display higher caloric intake during the recovery period. ($F(1,23) = 13.90$, $p < 0.05$). In order to analyze the daily calories consumed by high fat and normal chow (figure 3B), a repeated measures ANOVA was used, showing an interaction between stressed and non-stressed mice ($F(1,22) = 18.35$ $p < 0.05$). Additional post hoc tests using one-way ANOVAs show that stressed mice consumed more normal chow than non-stress mice at S2, S8-17, S21-R2, R4-R7, R9-R12, R14-R18 ($p < 0.05$).

Using a mixed factor ANOVA, analysis of blood concentrations of glucose showed that blood glucose was found to be significantly higher in non-stress mice (see figure 4) ($F(1,23) = 12.74$, $p < 0.05$).

Figure 1A. Average 24hr chow intake of DBA/2J mice at the end of each experimental condition: baseline, stress and recovery. Figure 1B. Daily 24hr chow intake of DBA/2J mice during experimental conditions over baseline, stress and recovery. Shaded region represents stress period. All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls.

Figure 2A. Average 4hr high fat intake of DBA/2J mice at the end of each experimental condition: baseline, stress and recovery. Figure 2B. Daily 4hr high fat intake of DBA/2J mice during experimental conditions over baseline, stress and recovery. Shaded region represents stress period. All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls.

Figure 3A. Average total calories (high fat and normal chow) intake of DBA/2J mice at the end of each experimental condition: baseline, stress and recovery. Figure 3B. Daily total calories (high fat and normal chow) of DBA/2J mice during experimental conditions over baseline, stress and recovery. Shaded region represents stress period. All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls.

Figure 4. Glucose levels of DBA/2J mice sacrificed for each experimental group. All values are expressed as mean with SEM.

Experiment 2 - Change in high fat presentation does not affect caloric intake due to stress

In experiment 2, forty C57BL/J6 male mice were used in order to determine whether the timing of high fat presentation altered the diet preference when faced with our stress paradigm. Similar to experiment 1, chronic social defeat did not affect body weight ($F(1,38) = 2.32, p. > 0.05$).

In order to analyze the difference between non-stressed and stress mice consumption of normal chow, a one-way ANOVA was used. As shown in Figure 5A, stressed mice consumed more normal chow than non-stressed controls ($F(1,38) = 6.56, p < 0.05$). We further analyzed normal chow by running a repeated measures ANOVA at the daily level (figure 5B), which showed a significant interaction effect ($F(1,38) = 3.90, p < 0.05$). Additional post hoc tests using one-way ANOVAs show that stressed mice consumed more normal chow than non-stress mice during the following periods of the stress phase: S1, S3-S6, and S11-12 ($p < 0.05$).

In contrast to experiment 1, the average high fat consumption (see figure 6A) was not affected by chronic social defeat, as both stressed and non-stress mice consumed relatively the same amount of high fat ($F(1,38) = 0.22, p. > 0.05$). In order to analyze high fat at the daily level (figure 6B), a repeated measures ANOVA was used, which showed no interaction between stressed and non-stressed mice ($F(1,38) = 0.22, p > 0.05$).

When calculating the total calories consumed from both high fat and normal chow, stress mice consumed more calories than control non-stressed mice ($F(1,38) = 4.76, p < 0.05$; see figure 7A). In order to analyze the caloric intake at the daily level (figure 7B), a repeated measures ANOVA was used, which showed an interaction between stressed and non-stressed mice ($F(1,38) = 4.76, p < 0.05$). Additional post hoc tests using one-way ANOVAs show that stressed mice consumed more calories on the following days: S3-S6, and S10-S12 ($p < 0.05$).

A two way between groups ANOVA with time of sacrifice (Day vs. night) and stress (stress vs. non-stressed) as factors confirmed that social defeat did not result in differences in glucose levels between non-stress and stress mice ($F(1,36) = 0.23, p > 0.05$). There was however a significant main effect for time of sacrifice indicating that overall glucose levels were higher in the morning ($F(1,34) = 5.88, p < 0.05$, NS = 7.46 ± 0.25 , S = 6.62 ± 0.24). No interaction was found between treatment and timing of glucose measurement ($F(1,36) = 0.08, p > 0.05$). Additionally we see this effect again in CORT (see figure 8), in which there is no difference between non-stress and stress mice ($F(1,32) = 1.21, p = 0.27$) but between A.M and P.M $F(1,32) = 7.18, p = 0.01$. No interaction was found between treatment and timing of CORT samples ($F(1,32) = 0.38, p > 0.05$). Interestingly ghrelin (see figure 9) was found to be higher in stressed mice ($F(1,22) = 6.21, p < 0.05$), but was not affected by which time of day it was measured ($F(1,22) = 0.42, p > 0.52$). Once again, no interaction between treatment and timing of acylated ghrelin was found ($F(1,22) = 0.06, p > 0.05$).

Figure 5A. Average 24hr chow intake of C57BL/6 mice at the end of each experimental condition: baseline and stress. Figure 5B. Daily 24hr chow intake of C57BL/6 mice during experimental conditions over baseline and stress. Shaded region represents stress period. All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls.

Figure 6A. Average 4hr high fat intake of C57BL/6 mice at the end of each experimental condition: baseline and stress. All values are expressed as mean with SEM. Figure 6B. Daily 4hr high fat intake of C57BL/6 mice during experimental conditions over baseline and stress. Shaded region represents stress period. All values are expressed as mean with SEM.

Figure 7A. Average total calories (high fat and normal chow) intake of C57BL/6 mice at the end of each experimental condition: baseline and stress. All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls. Figure 7B. Daily total calories (high fat and normal chow) of C57BL/6 mice during experimental conditions over baseline and stress. Shaded region represents stress period. All values are expressed as mean with SEM.

Figure 8. Corticosterone (CORT) of trunk blood gathered from stress and non-stress groups at morning (8 am) and evenings (4 pm). All values are expressed as mean with SEM.

Figure 9. Active ghrelin of trunk blood gathered from stress and non-stress groups at morning (8 am) and evenings (4 pm). All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls.

Experiment 3 - Stress increases carbohydrate preference and overall caloric intake

In experiment 3, twenty-two C57BL/6 male mice were used in order to determine whether stress resulted in a carbohydrate preference when given access to sucrose. Again, experimental groups were matched for body weight and caloric intake at the end of baseline, so there were no differences between groups in these parameters. No difference in body weight was observed between the experimental groups during stress ($F(1,20) = 0.14$, $p > 0.05$) and recovery ($F(1,20) = 0.27$, $p > 0.60$). Animals that were stressed, however, consumed more chow during the stress ($F(1, 20) = 5.32$, $p < 0.05$), and recovery phases ($F(1, 20) = 5.68$, $p < 0.05$; see figure 10A) as determined by one-way ANOVAs. In order to analyze normal chow at the daily level (see figure 10B), a repeated measures ANOVA was used, showing an interaction between stressed and non-stressed mice ($F(1,20) = 5.65$, $p < 0.05$). As a results, additional post hoc tests using one-way ANOVAs show that stressed mice consumed more normal chow on the following days: S1, S2, S5-S6, S12-S14, S16-S17, R7, R13-R14, R16 and R19 ($p < 0.05$).

Sucrose intake (see figure 11A) for stressed mice, exceeded that of non-stress mice during the stress phase of experimental ($F(1,20) = 16.35$, $p < 0.05$), and no difference was detected during recovery ($F(1,20) = 2.66$, $p > 0.05$). In order to analyze the daily sucrose intake (see figure 11B), a repeated measures ANOVA was used, showing an interaction between stressed and non-stressed mice ($F(1,38) = 8.06$, $p < 0.05$). Additional post hoc tests using one-way ANOVAs show that stressed mice consumed more sucrose on the following days: S8-S10, S12, S14, S18-S21, and R5 ($p < 0.05$).

After recovery day 7, mice were given ad lib access to HF, which resulted in a decrease in normal chow for both experimental groups. Stressed mice did not consume more high fat in comparison to non-stress mice, as both experimental groups consumed relatively the same

amount ($F(1,20) = 0.36, p > 0.05$). After recovery day 7, high fat intake decreased for both groups as they were given ad lib access to high fat, and so the usual 4hr exposure was only a sample of their overall consumption. No difference was found between non-stress and stress mice during recovery ($F(1,20) = 2.38, p > 0.05$). In order to analyze the daily high fat intake, a repeated measures ANOVA was used, showing an no interaction between stressed and non-stressed mice ($F(1,38) = 0.055, p > 0.05$).

Consumption of normal chow, high fat and sucrose, contributed to an overall increase in caloric intake in stressed mice during the stress phase ($F(1,20) = 16.35, p < 0.05$; see figure 12A) and the recovery phase ($F(1,20) = 7.97, p < 0.05$). This was due to the increase consumption of mostly chow which comprised of 3.3 kcal per gram (see table 1 in methods), however sucrose also increased but as the solution was 2.5% sucrose, very little calories attributed towards overall caloric intake. Caloric intake decreased after recovery day 7, as both experimental groups are given ad lib access to all diets, and only 4-hour daily exposure was measured. In order to analyze the overall caloric intake at the daily level (see figure 12B), a repeated measures ANOVA was used, showing an interaction between stressed and non-stressed mice ($F(1,38) = 6.65, p < 0.05$). Additional post hoc tests using one-way ANOVAs show that stressed mice consumed more overall calories on the following days: S2, S5-S6, S12-S15, S17, R6-R7, R10, R16 and R 18 ($p < 0.05$).

No differences were found between stressed mice and non-stressed mice, in terms of blood glucose levels ($F(1,20) = 0.08, p > 0.05$) or plasma corticosterone concentrations ($F(1,20) = 1.521, p > 0.05$). As usual, acylated ghrelin (see figure 13) was found to be higher in stressed mice in comparison to non-stress mice ($F(1, 15) = 5.64, p > 0.05$).

Figure 10A. Average 24hr chow intake of C57BL/6 mice at the end of each experimental condition: baseline, stress and recovery. Figure 10B. Daily 24hr chow intake of C57BL/6 mice during experimental conditions over baseline, stress, and recovery. Shaded region represents stress period. All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls.

Figure 11A. Average 4hr sucrose intake of C57BL/6 mice at the end of each experimental condition: baseline, stress and recovery. Figure 11B. Daily sucrose intake of C57BL/6 mice during experimental conditions over baseline, stress and recovery. B1-R7 used a 4hr exposure to sucrose while R8-R21 used a 24hr exposure to sucrose. Shaded region represents stress period. All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls.

Figure 12A. Average total calories (high fat and normal chow) intake of C57BL/6 mice at the end of each experimental condition: baseline, stress and recovery. Figure 13B. Daily total calories (high fat and normal chow) of C57BL/6 mice during experimental conditions over baseline, stress and recovery periods. Shaded region represents stress period. All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls.

Figure 13. Active ghrelin for non-stress and stress mice from collected trunk blood. * $P < .05$ relative to non-stressed controls

Experiment 4 – Ghrelin's role in diet preference

Experiment 4 was conducted to elucidate the role of ghrelin on the increase in carbohydrate (sucrose and normal chow) intake seen during chronic social defeat stress.

As with previous experiments, all experimental groups were matched for body weight and caloric intake at the end of the baseline. After the stress phase no differences in body weight were observed between KO and WT strains ($F(1,32) = 0.33, p.>0.05$), as well as non-stress vs. stress ($F(1,32) = 0.19, p.>0.05$). Additionally no interaction effect was found ($F(1,32) = 0.12, p.>0.05$).

A multifactorial ANOVA with two between group factors (treatment and strain), determined that, stressed mice increased their normal chow intake during the stress phase in comparison to control mice ($F(1, 36) = 10.60, p.<0.05$; see figure 14A), however there is no main effect in normal chow intake with regards to strain difference (WT or KO) ($F(1,32) = 3.11, p.>0.05$). No interaction was found between treatment and strain ($F(1, 32) = 0.96, p.>0.05$). Additional one-way ANOVAs revealed that WT stress mice's average chow intake was higher than that of WT non-stress mice ($F(1,18) = 12.47, p.<0.05$). In contrast, stressed KO mice consumed similar amounts of chow than non-stressed mice. Further analysis shows that WT stress mice consume more normal chow than KO stress mice $F(1,16) = 4.61, p.<0.05$. In order to analyze normal chow intake at the daily level (see figure 14B), a repeated measures ANOVA was used, showing a main effect between stressed and non-stressed mice ($F(1,32) = 13.21, p.<0.05$). However while there is a trend for strain effect between KO and WT mice, there was no overall significance ($F(1,32) = 3.32, p.>0.078$). Additionally interaction was not found between strain and treatment ($F(1,32) = 1.68, p > 0.203$). Seeing that there was a trend for strain, repeated measures ANOVAs were used to conduct a further analysis of chow intake data for each week of treatment. While treatment (stress vs nonstress) was found to be significant each week, strain showed a trend only in week one ($F(1,32)$

= 3.56, $p > 0.06$). Seeing as this trend was only found in week one, additional post hoc tests using two-way multi factorial ANOVAs were used, showing that stressed mice consumed more normal chow on the following days: S5-S7

WT mice showed lower intake of the high fat diet during the stress period, but this effect did not achieve statistical significance ($F(1, 16) = 3.33$, $p > 0.092$; see figure 15A). All other experimental groups consumed relatively the same high fat when measured over an average four-hour exposure. In order to measure high fat intake at the daily level (figure 15B), a repeated measures ANOVA was used, showing no main effect between stress vs. non-stress ($F(1,32) = 2.62$, $p > 0.05$), and strain KO vs. WT ($F(1,32) = 0.85$, $p > 0.05$). Additionally, no interaction was found ($F(1,32) = 0.53$, $p > 0.05$).

Average sucrose consumption (see figure 16A), much like normal chow intake, shows a significant difference between stress and non-stress experimental (treatment) groups ($F(1, 36) = 11.00$, $p < 0.01$), but no difference between KO and WT (strain) in their respective groups ($F(1,32) = 0.04$, $p > 0.05$). No interaction effect was found between the strain and treatment ($F(1,32) = 0.02$, $p > 0.05$). Additional post hoc tests using one-way ANOVAs revealed that, WT stress mice consume more average sucrose than WT non-stress mice ($F(1,18) = 7.81$, $p < 0.05$). While KO stressed mice seemed to consume more sucrose than non-stressed KO mice, this effect was not significantly different ($F(1,16) = 4.07$, $p > 0.06$). Using a repeated measures ANOVA to analyze daily sucrose intake (see figure 16B), a main effect between stressed and non-stressed mice was found ($F(1,32) = 11.00$, $p < 0.05$), however no difference between KO and WT strains was found ($F(1,32) = 0.04$, $p > 0.05$). Additionally no interaction between strain and treatment was found ($F(1,32) = 0.02$, $p > 0.05$).

Total caloric intake was calculated as the sum of calories consumed from the high fat diet, regular chow diet and sucrose solution. Using mixed factor ANOVAs, no difference was observed between strains ($F(1,32) = 2.03$, $p > 0.05$; see figure 17A), however there was a significant difference between stress mice and non-stress mice, as stressed mice consumed more calories ($F(1,36) = 18.20$, $p < 0.001$). Further post hoc tests using one-way ANOVAs show this effect was evident in both WT mice ($F(1,16) = 11.137$, $p < 0.01$, and to a lesser extent in KO mice $F(1,16) = 7.08$, $p < 0.05$). In order to analyze total caloric intake at the daily level, a repeated measures ANOVA was used (see figure 17B), showing a main effect between stressed and non-stressed mice ($F(1,32) = 12.74$, $p < 0.05$). No main effect was found between WT and KO strain mice ($F(1,32) = 2.46$, $p > 0.05$). Additionally no interaction was found between strain and treatment ($F(1,32) = 0.98$, $p > 0.05$).

Behavioral tests were conducted in order to measure depression as a result of the stress paradigm. Mice depressive behavior was tested using forced swim test where a mouse is placed in a container with water, videotaped for 5 minutes and then scored based on time spent immobile. Using a mixed factor ANOVA, stressed mice in both strains showed an increased in immobile time during forced swim test ($F(1,32) = 24.55$, $p < 0.05$; see figure 18). Additionally a strain effect was indicated between KO and WT mice ($F(1,32) = 6.22$, $p < 0.05$) in which post hoc one-way ANOVAs revealed that non-stress KO display higher immobile time than non-stress WT, however this difference is not significantly different for stressed mice $F(1, 18) = 4.40$, $p > 0.05$. Additionally, both KO ($F(1,18) = 7.70$, $p < 0.05$), and WT stressed mice ($F(1,18) = 16.56$, $p < 0.01$) showed a higher time spent immobile in comparison to non-stress counterparts. No interaction was found however between treatment and strain ($F(1,32) = 0.15$, $p > 0.05$).

Additional blood work showed no difference in glucose levels between strains ($F(1,32) = 1.39$, $p > 0.05$), treatment ($F(1,32) = 2.46$, $p > 0.05$) or interaction ($F(1,32) = 2.639$, $p > 0.05$). CORT produced (see figure 19) similar results as there was no difference in strain ($F(1,32) = 0.87$, $p > 0.05$), treatment ($F(1,32) = 0.24$, $p > 0.05$), or interaction ($F(1,32) = 1.02$, $p > 0.05$). Interestingly, with ghrelin, we observed a treatment effect as stressed mice have higher active ghrelin than non-stress mice ($F(1,23) = 8.35$, $p < 0.05$; see figure 20), however no strain difference ($F(1,23) = 3.46$, $p > 0.05$), or interaction ($F(1,23) = 3.80$, $p > 0.05$) was found. Additional post hoc test using one-way ANOVAs, show that WT stressed mice have higher active ghrelin than KO stressed mice ($F(1,11) = 5.09$, $p < 0.05$).

Figure 14A. Average 24hr chow intake of GHSR KO and WT mice at the end of each experimental condition: baseline and stress periods. Figure 14B. Daily 24hr chow intake of GHSR KO and WT mice during experimental conditions over baseline and stress periods. Shaded region represents stress period. Dashed lines indicate weekly period. All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls

Figure 15A. Average 4hr high fat intake of GHSR KO and WT mice at the end of each experimental condition: baseline and stress periods. Figure 15B. Daily 4hr high fat intake of GHSR KO and WT mice during experimental conditions over baseline and stress periods. Shaded region represents stress period. . All values are expressed as mean with SEM.

Figure 16A. Average 4hr sucrose intake of GHRSR KO and WT mice at the end of each experimental condition: baseline and stress periods. Figure 16B. Daily 4hr sucrose intake of GHRSR KO and WT mice during experimental conditions over baseline and stress periods. Shaded region represents stress period. All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls.

Figure 17A. Average total calories (high fat, normal chow and sucrose) intake of GHRSR KO and WT mice at the end of each experimental condition: baseline and stress periods.. Figure 17B. Daily total calories (high fat, normal chow and sucrose) of GHRSR KO and WT mice during experimental conditions over baseline and stress periods. Shaded region represents stress period. All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls

Figure 18. Average time spent immobile during forced swim test. All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls

Figure 19. CORT for non-stress and stress GHSR KO and WT mice.

Figure 20. Active ghrelin for WT and KO mice under either stressed, or non-stressed conditions. *
P < .05 relative to non-stressed controls

Experiment 5 – Caloric consumption alleviates stress symptoms

In experiment 5, thirty-two C57BL/6 male mice were assigned to one of three groups (non-stress, stress and pair fed), and went through a baseline and stress phase of our usual stress paradigm.

As with previous studies, using one-way ANOVAs no difference between body weight of stressed and non-stressed control mice was found during the stress phase of the experiment ($F(1,29) = 2.63$, $p.>0.05$).

Also similar to the previous experiments, using one-way ANOVAs, we observed that stressed mice consumed more chow during their stress phase than non-stressed mice ($F(1, 29) = 5.54$, $p.<0.05$; see figure 21A). Stressed mice in the pair fed group only received the same amount of chow as the mice in the control non-stress group, which was confirmed by follow up Bonferonni post hoc tests ($p.>0.05$). In order to analyze the daily chow intake (see figure 21B), a repeated measures ANOVA was used, showing no interaction between the three groups ($F(1,29) = 1.28$, $p.>0.05$). Additional post hoc tests using one-way ANOVAs with Bonferonni tests, show that stressed mice consumed more chow than pair fed and non-stress mice on the following days: S5 and S16 ($p.<0.05$).

In contrast, using one-way ANOVAs, stressed mice did not consume more high fat, than both pair fed mice, and non-stress mice ($F(1,29) = 1.77$, $p.>0.05$; see figure 22A). Pair fed mice only received the same amount of high fat as the mice in the control non-stress group, which once again was confirmed with follow up bonferonni post hoc tests ($p.>0.05$). In order to analyze the high fat intake at a daily level (see figure 22B), a repeated measures ANOVA was used, showing no interaction between the three groups ($F(1,29) = 2.59$, $p.>0.05$). Additional post hoc tests using

one-way ANOVAs with Bonferroni tests, show that pair fed mice consumed less high fat than both non-stress and stress mice on the following days: S13-S14 and S18($p < 0.05$).

Consequently, stressed mice consumed more overall average calories ($F(1, 29) = 16.10$, $p < 0.05$; see figure 23A), than both pair fed mice and non-stress mice during the stress phase of the experiment. In order to analyze the caloric intake at a daily level(see figure 23B), a repeated measures ANOVA was used, showing an interaction between the three groups ($F(1,29) = 19.62$, $p < 0.05$). Additional post hoc tests using one-way ANOVAs with Bonferroni test, showed that stressed mice consumed more calories than pair fed and non-stress mice on the following days: S2, S3, S5-S7, S12-S21 ($p < 0.05$).

Stressed mice, whether they were pair fed or allowed to increase their caloric intake, spent more time immobile in the forced swim tests, in comparison to non-stress mice ($F(2,26) = 19.97$, $p < 0.05$), but no difference was observed between the stressed and pair fed group themselves ($F(1,19) = 1.18$, $p > 0.05$ (see figure 24).

Blood glucose was found to be significantly lower in pair-fed mice in comparison to both non-stress and stressed mice as determined by a one-way ANOVAs with follow up bonferroni post hoc tests ($F(1,27) = 5.45$, $p < 0.05$; see figure 25). Interestingly, CORT was shown to be highest in pair-fed mice when compared to both stress and non-stress mice as determined by one-way ANOVAs followed by Bonferroni post hoc tests ($F(2,25) = 18.74$, $p < 0.05$; see figure 26). A similar effect was found with plasma active ghrelin concentrations, where pair fed mice showed the highest active ghrelin levels after the stress phase compared to stressed mice fed ad lib and non-stress mice ($F(2,23) = 17.66$, $p < 0.05$; Bonferroni post hoc tests; see figure 27).

Figure 21A. Average 24hr chow intake of C57BL/6 mice at the end of each experimental condition: baseline and stress periods. All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls. Figure 22B. Daily 24hr chow intake of C57BL/6 mice during experimental conditions over baseline and stress periods. Shaded region represents stress period. All values are expressed as mean with SEM.

Figure 22A. Average 4hr high fat intake of C57BL/6 mice at the end of each experimental condition: baseline and stress periods. All values are expressed as mean with SEM. Figure 23B. Daily 4hr high fat intake of C57BL/6 mice during experimental conditions over baseline and stress periods. Shaded region represents stress period. All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls

Figure 23A. Average total calories (high fat and normal chow) intake of C57BL/6 mice at the end of each experimental condition: baseline and stress periods. All values are expressed as mean with SEM. Figure 24B. Daily total calories (high fat and normal chow) of C57BL/6 mice during experimental conditions over baseline and stress periods. Shaded region represents stress period. All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls

Figure 24. Forced swim testing for non-stress, stress and pair fed mice. * $P < .05$ relative to non-stressed controls

Figure 25. Glucose levels of C57BL/6 mice sacrificed for each experimental group. All values are expressed as mean with SEM. * $P < .05$ relative to non-stressed controls.

Figure 26. CORT (ug/dl) for non-stress, stress and pair-fed mice.
* $P < .05$ relative to non-stressed controls.

Figure 27. Active ghrelin for non-stress, stress and pair-fed mice from collected trunk blood.
* $P < .05$ relative to non-stressed controls.

Discussion

This current thesis, aimed to further investigate ghrelin, a hormone vital to the regulation of hunger, and its effects on stress and diet preference. In response to stress, physiological change enables an organism to meet the energetic demands of a wide array of challenges. Previous work by Patterson et al, showed that when mice are stressed, their consumption of normal chow, a diet rich in carbohydrates, increases. Ghrelin was shown to increase food intake and promote the utilization of these carbohydrates over the use of a palatable fat (Z. R. Patterson, Khazall, Mackay, Anisman, & Abizaid, 2013b). These changes were important in order to meet the energetic demands of chronic and acute stress. Our findings showed that presenting the palatable diet soon after the stressor caused a decrease in the intake of the high fat diet, possibly through the formation of an association between this diet and the stressor. Once the high fat diet was presented several hours after the stressor, animals no longer decreased their intake of this diet but rather consumed the same amount as non-stress individuals. In contrast, when stress mice were given a sucrose solution they increased the intake of this solution along with that of the normal chow. This effect was also shown in GHSR KO and WT mice, however GHSR WT mice consumed more normal chow than GHSR KO, whereas GHSR KO consumed more high fat. Finally, when the additional calories from high fat and normal chow are restricted to that of non-stress mice, both CORT and ghrelin levels rise.

As with previous studies in our lab, results for our first study showed that stressed mice consumed more normal chow than non-stressed mice, however they additionally decreased their high fat diet intake. This effect was tested with a different strain of mice (DBA/2J) which have a preference for high fat, in order to determine if the decrease in high fat previously seen in our lab was due to the strain of mice (J. Alexander et al., 2006). Our findings showed that this stress effect

was not strain dependent but rather due to the placement of the stressor, which was associated with the high fat, resulting in a decrease of that particular diet. With our second experiment, we found that when the stressor was moved to an earlier time point, the stressed mice consumed just as much high fat as non-stressed mice. It became clear at this point that the stressed mice were associating the acute stressor with high fat, resulting in a decrease in high fat.

Our second experiment, as in previous studies, showed once again that stress caused an increase in circulating acylated ghrelin levels which may be in part responsible for the increases in caloric intake seen in stressed mice from our experiments (Z. R. Patterson et al., 2013a). Ghrelin levels were found to be higher in stressed animals regardless of when these animals were sacrificed. Additional blood work showed that both glucose and CORT were significantly higher when mice were sacrificed in the early evening compared to animals sacrificed in the early morning, but not when comparing stressed mice to non-stressed mice. Corticosterone is a steroid hormone known to be able to activate the GHS-R, and might be a link to the gut-brain relationship (Broglia, Grottoli, Arvat, & Ghigo, 2008). Additionally, access to additional calories when under stress conditions, might attenuate the stress response, producing lower CORT levels than expected for stressed mice (Dallman, Pecoraro, et al., 2003). However our experiments show that regular chow, with its high percentage of carbohydrates might be all that is required for attenuating stress

The increase in caloric intake may be in part due to the increases in ghrelin secretion seen in stressed mice. Indeed, ghrelin levels increase both after acute and chronic stress in humans and rodents, persisting well after the stressor is no longer present (Kanehisa et al., 2006; Kristensson et al., 2006; Lutter, Sakata, et al., 2008). One could argue that this is because ghrelin protects against the effects of chronic stress given that previous studies have shown that GHSR KO mice are more prone to show depressive like behavior when subjected to chronic social defeat. Our fourth

experiment was aimed at understanding the role of ghrelin in this process. Results indicated that stressed WT mice consumed more calories primarily through an increase in the intake of normal chow, but decreased their intake of high fat compared to stressed GHSR KO mice and control non-stressed GHSR KO and WT mice. This was in parallel with behavioral data showing that stressed GHSR KO mice tended to show more stress induced helplessness in the forced swim test than stressed WT mice, non-stressed GHSR KO and WT mice.

It is possible that, in addition to neuroprotection, the rise in ghrelin helps to attenuate anxiety and depressive like behaviors in addition to promoting the increase of appetite in order to relieve the high energetic costs of stress. In fact previous studies have shown that ghrelin has an anxiolytic effect (Alvarez-Crespo et al., 2012; Kanehisa et al., 2006; Lutter, Sakata, et al., 2008), although in some case ghrelin appears to stimulate anxiety as well as the activity of the amygdala (Currie, Mirza, Fuld, Park, & Vasselli, 2005; Malik et al., 2008). More work is necessary to solve these discrepancies, but it is possible that ghrelin produces both the negative emotions associated with hunger, while at the same time reducing the anxiety that comes from going into the environment to search for food.

Lastly, our fifth experiment attempted to determine what would be the effect of preventing animals from increasing their caloric intake when under stress conditions. Results from these studies failed to find differences in the amount of time pair-fed mice (mice that were only allowed to eat what non stressed mice ate under ad lib conditions) spent struggling in the forced swim test compared to stress mice that were allowed to increase their caloric intake. These mice, however, had a more pronounced endocrine response to the chronic stressor as reflected by higher CORT and ghrelin levels. Whether this relates to animals that are in a heavier allostatic load remains to be determined. Nevertheless, mice that are not allowed to supplement their caloric intake to meet

the demands of the social stressor result in more elevated levels of CORT and ghrelin. This state may in the long run be detrimental to their health.

There are a number of reasons why chronic social stress leads to an overconsumption of carbohydrate rich foods. The first is that stress causes a depletion of carbohydrate stores, as these are nutrients that can be utilized rapidly to meet the increased energetic demands posed by the stressor (Depke et al., 2008). Second, the increase in the intake of the carbohydrate diet may also reflect a mechanism by which stressed mice (and perhaps humans) attempt to up-regulate serotonin levels. Indeed, carbohydrate consumption increases the plasma tryptophan ratio, as well as serotonin release via insulin secretion (Wurtman & Wurtman, 1995). Finally, the intake of carbohydrates may produce a rapid activation of the reward circuits, an event known to dampen the activity of the HPA axis (Dallman, Pecoraro, et al., 2003). The relief obtained from the reduction of HPA activity may lead to the formation of a coping mechanism that in the short term is helpful in controlling the effects of the stressor, but in the long term may lead to metabolic abnormalities.

Stress induced ghrelin secretion may play an important role in all of these mechanisms. For instance, ghrelin facilitates the oxidation of carbohydrates as fuel while sparing fat (Tschop et al, 2000), and stressed GHSR KO mice eat less of a diet with high carbohydrate content than WT stressed mice (Z. R. Patterson et al., 2013a). Ghrelin also affects the serotonergic system to facilitate carbohydrate consumption while promoting obesity. In fact, stress produces an increase in serotonergic utilization which is not seen in GHSR KO mice (Z. R. Patterson et al., 2010). This along with the fact GHSR KO mice have in general more serotonin in the ARC nucleus presumably exerting increased anorectic tone, may promote an overall increased caloric intake in stressed WT mice but not in GHSR KO mice. Finally, ghrelin directly acts on the reward system and may

promote carbohydrate intake through the stimulation of VTA dopamine neurons, an effect that may not be seen in GHSR KO mice (Abizaid et al., 2006; Chuang & Zigman, 2010; Z. R. Patterson et al., 2010)

There are some limitations to this study that must be considered. For instance, presentation of high fat can have a major effect on four-hour consumption. As seen in our first experiment, placing an acute stressor right after presentation of high fat resulted in a decrease in high fat, as mice associated stress with the diet. Additionally, mice might prefer consuming high fat more readily during the night, when they are in their most active state as they are considered nocturnal, and all of the experiments conducted in this thesis had only four-hour exposure to high fat, presented during their day cycle. Additionally ghrelin levels are generally considered highest prior to initiation of the mice's sleep cycle. A small factor such as this can affect all diet preference. Additionally the use of sucrose as a diet can also be difficult to measure as difference percentages of sucrose can be used, and testing is required to determine which percentage works best. Measuring the consumption of sucrose can also be a difficult process as it is a liquid, which might not provide the same comparison in texture to normal chow, which is hard, or high fat, which is soft. The texture of the diet itself might coerce a mouse to be more interested in one over another. Additionally, our stress model is limited in that it demonstrates only one type of stressor, while in reality there is a multitude of different stressors, each of which vary in its effect, timing and duration. Also while this data shows that mice prefer carbohydrate rich diets when in our stress paradigm, and that ghrelin levels rise in the stressed group, it does not show any gain in weight leading to obesity. This might be an effect that occurs with longitudinal studies, as there could be a change in metabolic profile, leading to obesity. While this data show that stressed mice consumed more carbohydrate rich diets, it does not explain fully how and for which processes the mice

require these additional calories. We hypothesize that they are necessary to attenuate the stress response and cope, however there might be a more complex metabolic mechanism occurring.

The ramifications of ghrelin towards the stress response are at its early stages. Future studies should further extend to understand ghrelin's effects on anxiety-like behavior, and how this affects food intake. Not only is ghrelin crucial to the development of anti-obesity agents, it may also help towards resolving anxiety-like behaviors. Long-term studies should be implemented to see how stress affects body weight over time, and whether the preference in diet is altered towards a certain preference. However, it is important to note that altering active ghrelin might lead to further implications towards stress. While ghrelin increases caloric intake, its protective effects might outweigh the possible psychological effects of stress, which could be more harmful in the long run. Although ghrelin's short-term effects could prove to be beneficial, its long-term implications might cause a change in metabolic profile, which is harmful.

The stress response generates a multitude of physiological changes that lead to an adaptation towards a threat real or perceived. These changes are adaptive for short-term periods, as they promote the survival of the organism. In the long term, however, chronic stress poses challenges that cannot be met by the compensatory mechanisms elicited by the stress response, and this can lead to numerous health effects such as obesity or anxiety. Stress is correlated with the increase in calories, particularly carbohydrate rich diets. In conclusion, our data suggests that ghrelin increases during stress and plays a role in promoting the consumption of carbohydrates. This alteration in metabolic demands is helpful in alleviating stress like symptoms.

Appendix A- Supplementary Figures

Figure 1A. Limbic circuitry showing projections of each of the key areas.

Figure 2A. Chronic social defeat stress paradigm.

		Effect on Hunger	
Hormone	Location	Hunger	Satiety
Ghrelin	Stomach	Decreased	Increased
Leptin	Adipose tissue	Increased	Decreased
Cholecystokinin	Foregut and midgut	Decreased	Increased
Glucagon-like peptide-1	Hindgut	Decreased	Increased
Oxyntomodulin	Hindgut	Decreased	Increased
Peptide YY	Hindgut	Decreased	Increased
Somatostatin	Gut and pancreas	Decreased	Increased

Table 3A. Feeding related hormones, and their effects on hunger.

Figure 4A. *Experimental design of first study. Experiment consists of three phases; baseline, stress and recovery. Colors indicate two different experimental groups.*

Figure 5A. *Experimental design for second study. Experiment consists of two phases; baseline and stress. Colors indicate two different experimental groups.*

Figure 6A. Experimental design for third study. Experiment consists of three phases; baseline, stress and recovery. The recovery phases consisted of two elements in two mice were house 7 days with bully, and then single housed for 14 days. Colors indicate two different experimental groups.

Experiment 4

Figure 7A. Experimental design of fourth study. Experiment consists of two phases; baseline and stress. Each stress and non-stress group have their own KO and WT. Colors indicate four different experimental groups.

Figure 8A. Experimental design of fifth study. Experiment consists of three phases; baseline, stress and behavioral tests. There are three group used in this study. Pair fed group matches caloric intake of baseline group. Colors indicate three different experimental groups.

References

- Abizaid, A., Liu, Z. W., Andrews, Z. B., Shanabrough, M., Borok, E., Elsworth, J. D., . . . Horvath, T. L. (2006). Ghrelin modulates the activity and synaptic input organization of midbrain dopamine neurons while promoting appetite. *J Clin Invest*, 116(12), 3229-3239.
- Adam, T. C., & Epel, E. S. (2007). Stress, eating and the reward system. *Physiol Behav*, 91(4), 449-458. doi: 10.1016/j.physbeh.2007.04.011
- Alexander, G. E., Crutcher, M. D., & DeLong, M. R. (1990). Basal ganglia-thalamocortical circuits: parallel substrates for motor, oculomotor, "prefrontal" and "limbic" functions. *Prog Brain Res*, 85, 119-146.
- Alexander, J., Chang, G. Q., Dourmashkin, J. T., & Leibowitz, S. F. (2006). Distinct phenotypes of obesity-prone AKR/J, DBA/2J and C57BL/6J mice compared to control strains. *Int J Obes (Lond)*, 30(1), 50-59. doi: 10.1038/sj.ijo.0803110
- Alvarez-Crespo, M., Skibicka, K. P., Farkas, I., Molnar, C. S., Egecioglu, E., Hrabovszky, E., . . . Dickson, S. L. (2012). The amygdala as a neurobiological target for ghrelin in rats: neuroanatomical, electrophysiological and behavioral evidence. *PLoS One*, 7(10), e46321. doi: 10.1371/journal.pone.0046321PONE-D-12-19030 [pii]
- Apple, K.K., Dikeman, M.E., Minton, J.E., McMurphy, R.M., Fedde, M.R., Leith, D.E., & Unruh, J.A. (1995). Effects of restraint and isolation stress and epidural blockade on endocrine and blood metabolite status, muscle glycogen metabolism, and incidence of dark-cutting. *Journal of Animal Science*, 73, 2295-2307.
- Asakawa, A., Inui, A., Goto, K., Yuzuriha, H., Takimoto, Y., Inui, T., . . . Kasuga, M. (2002). Effects of agouti-related protein, orexin and melanin-concentrating hormone on oxygen consumption in mice. *Int J Mol Med*, 10(4), 523-525.

- Bagnasco, M., Dube, M. G., Katz, A., Kalra, P. S., & Kalra, S. P. (2003). Leptin expression in hypothalamic PVN reverses dietary obesity and hyperinsulinemia but stimulates ghrelin. *Obes Res*, 11(12), 1463-1470. doi: 10.1038/oby.2003.196
- Bartolomucci, A., Pederzani, T., Sacerdote, P., Panerai, A. E., Parmigiani, S., & Palanza, P. (2004). Behavioral and physiological characterization of male mice under chronic psychosocial stress. *Psychoneuroendocrinology*, 29(7), 899-910.
- Belgardt, B. F., Okamura, T., & Bruning, J. C. (2009). Hormone and glucose signalling in POMC and AgRP neurons. *J Physiol*, 587(Pt 22), 5305-5314. doi: 10.1113/jphysiol.2009.179192
- Black, P. H. (2006). The inflammatory consequences of psychologic stress: relationship to insulin resistance, obesity, atherosclerosis and diabetes mellitus, type II. *Med Hypotheses*, 67(4), 879-891.
- Blanchard, D. C., Spencer, R. L., Weiss, S. M., Blanchard, R. J., McEwen, B., & Sakai, R. R. (1995). Visible burrow system as a model of chronic social stress: behavioral and neuroendocrine correlates. *Psychoneuroendocrinology*, 20(2), 117-134. doi: 0306453094E0045B [pii]
- Boggiano, M. M., Chandler, P. C., Viana, J. B., Oswald, K. D., Maldonado, C. R., & Wauford, P. K. (2005). Combined dieting and stress evoke exaggerated responses to opioids in binge-eating rats. *Behav Neurosci*, 119(5), 1207-1214.
- Bose, M., Olivan, B., & Laferrer, B. (2010). Stress and obesity - role of the hypothalamic-pituitary-adrenal axis in metabolic disease. *Curr Opin Endocrinol Diabetes*, 16(5), 340-346. doi: 10.1097/med
- Broglia, F., Grottoli, S., Arvat, E., & Ghigo, E. (2008). Endocrine actions of cortistatin: in vivo studies. *Mol Cell Endocrinol*, 286(1-2), 123-127. doi: 10.1016/j.mce.2007.12.012S0303-7207(07)00477-7 [pii]

- Brummer, P. (1949). On the correlation between gallbladder disease, obesity, and pregnancy. *Ann Med Intern Fenn*, 38(2), 106-109.
- Buckingham, J. C., & Hodges, J. R. (1979). Hypothalamic receptors influencing the secretion of corticotrophin releasing hormone in the rat. *J Physiol*, 290(2), 421-431.
- Cabral, A., Suescun, O., Zigman, J. M., & Perello, M. (2012). Ghrelin indirectly activates hypophysiotropic CRF neurons in rodents. *PLoS One*, 7(2), e31462. doi: 10.1371/journal.pone.0031462PONE-D-11-18508 [pii]
- Carlini, V. P., Monzon, M. E., Varas, M. M., Cragnolini, A. B., Schioth, H. B., Scimonelli, T. N., & de Barioglio, S. R. (2002). Ghrelin increases anxiety-like behavior and memory retention in rats. *Biochem Biophys Res Commun*, 299(5), 739-743. doi: S0006291X02027407 [pii]
- Castaneda, T. R., Tong, J., Datta, R., Culler, M., & Tschop, M. H. (2010). Ghrelin in the regulation of body weight and metabolism. *Front Neuroendocrinol*, 31(1), 44-60. doi: 10.1016/j.yfrne.2009.10.008
- Cawley, J., & Meyerhoefer, C. (2012). The medical care costs of obesity: an instrumental variables approach. *J Health Econ*, 31(1), 219-230. doi: 10.1016/j.jhealeco.2011.10.003
- Chen, H. Y., Trumbauer, M. E., Chen, A. S., Weingarth, D. T., Adams, J. R., Frazier, E. G., . . . Qian, S. (2004). Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. *Endocrinology*, 145(6), 2607-2612. doi: 10.1210/en.2003-1596en.2003-1596 [pii]
- Chen, Y., & Qian, L. (2012). Association between lifetime stress and obesity in Canadians. *Prev Med*, 55(5), 464-467. doi: 10.1016/j.ypmed.2012.08.013

- 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. *J Clin Invest*, 121(7), 2684-2692. doi: 10.1172/JCI5766057660 [pii]
- Chuang, J. C., & Zigman, J. M. (2010). Ghrelin's Roles in Stress, Mood, and Anxiety Regulation. *Int J Pept*, 2010. doi: 10.1155/2010/460549
- Clain, D. J., & Lefkowitz, J. H. (1987). Fatty liver disease in morbid obesity. *Gastroenterol Clin North Am*, 16(2), 239-252.
- Colantuoni, C., Rada, P., McCarthy, J., Patten, C., Avena, N. M., Chadeayne, A., & Hoebel, B. G. (2002). Evidence that intermittent, excessive sugar intake causes endogenous opioid dependence. *Obes Res*, 10(6), 478-488. doi: 10.1038/oby.2002.66
- Cone, R. D., Cowley, M. A., 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 Disord*, 25 Suppl 5, S63-67. doi: 10.1038/sj.ijo.0801913
- Cota, D., Tschop, M. H., Horvath, T. L., & Levine, A. S. (2006). Cannabinoids, opioids and eating behavior: the molecular face of hedonism? *Brain Res Rev*, 51(1), 85-107. doi: 10.1016/j.brainresrev.2005.10.004
- Cowley, M. A., Smith, R. G., Diano, S., Tschop, M., Pronchuk, N., Grove, K. L., . . . Horvath, T. L. (2003). The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron*, 37(4), 649-661.
- Currie, P. J., Mirza, A., Fuld, R., Park, D., & Vasselli, J. R. (2005). Ghrelin is an orexigenic and metabolic signaling peptide in the arcuate and paraventricular nuclei. *Am J Physiol Regul Integr Comp Physiol*, 289(2), R353-R358. doi: 10.1152/ajpregu.00756.2004

- Dallman, M. F. (2007). Modulation of stress responses: how we cope with excess glucocorticoids. *Exp Neurol*, 206(2), 179-182. doi: 10.1016/j.expneurol.2007.06.002
- Dallman, M. F., Akana, S. F., Laugero, K. D., Gomez, F., Manalo, S., Bell, M. E., & Bhatnagar, S. (2003). A spoonful of sugar: feedback signals of energy stores and corticosterone regulate responses to chronic stress. *Physiol Behav*, 79(1), 3-12. doi: S0031938403001008 [pii]
- Dallman, M. F., Pecoraro, N., Akana, S. F., La Fleur, S. E., Gomez, F., Houshyar, H., . . . Manalo, S. (2003). Chronic stress and obesity: a new view of "comfort food". *Proc Natl Acad Sci U S A*, 100(20), 11696-11701. doi: 10.1073/pnas.19346661001934666100 [pii]
- Dallman, M. F., Warne, J. P., Foster, M. T., & Pecoraro, N. C. (2007). Glucocorticoids and insulin both modulate caloric intake through actions on the brain. *J Physiol*, 583(Pt 2), 431-436. doi: jphysiol.2007.136051 [pii]10.1113/jphysiol.2007.136051
- Davis, M., & Whalen, P. J. (2001). The amygdala: vigilance and emotion. *Mol Psychiatry*, 6(1), 13-34.
- De Vriendt, T., Moreno, L. A., & De Henauw, S. (2009). Chronic stress and obesity in adolescents: scientific evidence and methodological issues for epidemiological research. *Nutr Metab Cardiovasc Dis*, 19(7), 511-519. doi: 10.1016/j.numecd.2009.02.009
- Depke, M., Fusch, G., Domanska, G., Geffers, R., Volker, U., Schuett, C., & Kiank, C. (2008). Hypermetabolic syndrome as a consequence of repeated psychological stress in mice. *Endocrinology*, 149(6), 2714-2723. doi: 10.1210/en.2008-0038
- Di Chiara, G., Porceddu, M. L., Morelli, M., Mulas, M. L., & Gessa, G. L. (1979). Evidence for a GABAergic projection from the substantia nigra to the ventromedial thalamus and to the superior colliculus of the rat. *Brain Res*, 176(2), 273-284. doi: 0006-8993(79)90983-1 [pii]

- Diano, S., Farr, S. A., Benoit, S. C., McNay, E. C., da Silva, I., Horvath, B., . . . Horvath, T. L. (2006). Ghrelin controls hippocampal spine synapse density and memory performance. *Nat Neurosci*, 9(3), 381-388. doi: nn1656 [pii]10.1038/nn1656
- Dickerson, S. S., Gruenewald, T. L., & Kemeny, M. E. (2004). When the social self is threatened: shame, physiology, and health. *J Pers*, 72(6), 1191-1216. doi: JOPY295 [pii]10.1111/j.1467-6494.2004.00295.x
- Eipper, B. A., & Mains, R. E. (1980). Structure and biosynthesis of pro-adrenocorticotropin/endorphin and related peptides. *Endocr Rev*, 1(1), 1-27.
- Elmquist, J. K. (2001). Hypothalamic pathways underlying the endocrine, autonomic, and behavioral effects of leptin. *International Journal of Obesity*, 25, S78-S82.
- Faulconbridge, L. F., Cummings, D. E., Kaplan, J. M., & Grill, H. J. (2003). Hyperphagic effects of brainstem ghrelin administration. *Diabetes*, 52(9), 2260-2265.
- Ferrini, F., Salio, C., Lossi, L., & Merighi, A. (2009). Ghrelin in central neurons. *Curr Neuroparmacol*, 7(1), 37-49. doi: 10.2174/157015909787602779
- Foster, M. T., Warne, J. P., Ginsberg, A. B., Horneman, H. F., Pecoraro, N. C., Akana, S. F., & Dallman, M. F. (2009). Palatable foods, stress, and energy stores sculpt corticotropin-releasing factor, adrenocorticotropin, and corticosterone concentrations after restraint. *Endocrinology*, 150(5), 2325-2333. doi: 10.1210/en.2008-1426
- Gaytan, F., Barreiro, M. L., Chopin, L. K., Herington, A. C., Morales, C., Pinilla, L., . . . Tena-Sempere, M. (2003). Immunolocalization of ghrelin and its functional receptor, the type 1a growth hormone secretagogue receptor, in the cyclic human ovary. *J Clin Endocrinol Metab*, 88(2), 879-887.

- George, S. A., Khan, S., Briggs, H., & Abelson, J. L. (2010). CRH-stimulated cortisol release and food intake in healthy, non-obese adults. *Psychoneuroendocrinology*, 35(4), 607-612. doi: 10.1016/j.psyneuen.2009.09.017
- Golden, P. L., Maccagnan, T. J., & Pardridge, W. M. (1997). Human blood-brain barrier leptin receptor. Binding and endocytosis in isolated human brain microvessels. *J Clin Invest*, 99(1), 14-18. doi: 10.1172/JCI119125
- Goldstein, D. S. (1987). Stress-induced activation of the sympathetic nervous system. *Baillieres Clin Endocrinol Metab*, 1(2), 253-278.
- Greenough, A., Cole, G., Lewis, J., Lockton, A., & Blundell, J. (1998). Untangling the effects of hunger, anxiety, and nausea on energy intake during intravenous cholecystokinin octapeptide (CCK-8) infusion. *Physiol Behav*, 65(2), 303-310. doi: S0031-9384(98)00169-3 [pii]
- Gualillo, O., Caminos, J., Blanco, M., Garcia-Caballero, T., Kojima, M., Kangawa, K., . . . Casanueva, F. (2001). Ghrelin, a novel placental-derived hormone. *Endocrinology*, 142(2), 788-794.
- Habib, K. E., Gold, P. W., & Chrousos, G. P. (2001). Neuroendocrinology of stress. *Endocrinol Metab Clin North Am*, 30(3), 695-728; vii-viii.
- Hattori, N., Saito, T., Yagyu, T., Jiang, B. H., Kitagawa, K., & Inagaki, C. (2001). GH, GH receptor, GH secretagogue receptor, and ghrelin expression in human T cells, B cells, and neutrophils. *J Clin Endocrinol Metab*, 86(9), 4284-4291.
- Heimer, L., Alheid, G. F., de Olmos, J. S., Groenewegen, H. J., Haber, S. N., Harlan, R. E., & Zahm, D. S. (1997). The accumbens: beyond the core-shell dichotomy. *J Neuropsychiatry Clin Neurosci*, 9(3), 354-381.

- Henry, J. P. (1997). Psychological and physiological responses to stress: the right hemisphere and the hypothalamo-pituitary-adrenal axis, an inquiry into problems of human bonding. *Acta Physiol Scand Suppl*, 640, 10-25.
- Hillebrand, J. J., de Wied, D., & Adan, R. A. (2002). Neuropeptides, food intake and body weight regulation: a hypothalamic focus. *Peptides*, 23(12), 2283-2306. doi: S0196978102002693 [pii]
- Holsboer, F., & Ising, M. (2010). Stress hormone regulation: biological role and translation into therapy. *Annu Rev Psychol*, 61, 81-109, C101-111. doi: 10.1146/annurev.psych.093008.100321
- Holst, B., & Schwartz, T. W. (2004). Constitutive ghrelin receptor activity as a signaling set-point in appetite regulation. *Trends Pharmacol Sci*, 25(3), 113-117. doi: S0165-6147(04)00027-6 [pii]10.1016/j.tips.2004.01.010
- Horvath, T. L., Diano, S., & Tschop, M. (2003). Ghrelin in hypothalamic regulation of energy balance. *Curr Top Med Chem*, 3(8), 921-927.
- Hosoda, H., Kojima, M., Matsuo, H., & Kangawa, K. (2000). Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. *Biochem Biophys Res Commun*, 279(3), 909-913. doi: 10.1006/bbrc.2000.4039
- 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(2-3), 126-131. doi: 10.1016/j.regpep.2006.02.005
- Huebener, H. J. (1960). [On the mechanism of gluconeogenesis after the oral administration of cortisol]. *Hoppe Seylers Z Physiol Chem*, 322, 135-141.

- Kalivas, P. W., & Duffy, P. (1997). Dopamine regulation of extracellular glutamate in the nucleus accumbens. *Brain Res*, 761(1), 173-177. doi: S0006-8993(97)00464-2 [pii]
- Kalivas, P. W., & Nakamura, M. (1999). Neural systems for behavioral activation and reward. *Curr Opin Neurobiol*, 9(2), 223-227. doi: S0959-4388(99)80031-2 [pii]
- Kanehisa, M., Akiyoshi, J., Kitaichi, T., Matsushita, H., Tanaka, E., Kodama, K., . . . Isogawa, K. (2006). Administration of antisense DNA for ghrelin causes an antidepressant and anxiolytic response in rats. *Prog Neuropsychopharmacol Biol Psychiatry*, 30(8), 1403-1407. doi: S0278-5846(06)00222-3 [pii]10.1016/j.pnpbp.2006.05.005
- Katzmarzyk, P., Hebebrand, J., & Bouchard, C. (2002). Spousal resemblance in the Canadian Population: implications for obesity epidemic. *International Journal of Obesity*, 26, 241-246.
- Katzmarzyk, P. T., & Janssen, I. (2004). The economic costs associated with physical inactivity and obesity in Canada: an update. *Can J Appl Physiol*, 29(1), 90-115.
- King, B. M. (1988). Glucocorticoids and hypothalamic obesity. *Neurosci Biobehav Rev*, 12(1), 29-37. doi: S0149-7634(88)80071-X [pii]
- King, S. J., Isaacs, A. M., O'Farrell, E., & Abizaid, A. (2011). Motivation to obtain preferred foods is enhanced by ghrelin in the ventral tegmental area. *Horm Behav*, 60(5), 572-580. doi: 10.1016/j.yhbeh.2011.08.006S0018-506X(11)00188-7 [pii]
- Kirchner, H., Gutierrez, J. A., Solenberg, P. J., Pfluger, P. T., Czyzyk, T. A., Willency, J. A., . . . Tschop, M. H. (2009). GOAT links dietary lipids with the endocrine control of energy balance. *Nat Med*, 15(7), 741-745. doi: 10.1038/nm.1997nm.1997 [pii]
- Kishimoto, T., Pearce, R. V., 2nd, Lin, C. R., & Rosenfeld, M. G. (1995). A sauvagine/corticotropin-releasing factor receptor expressed in heart and skeletal muscle. *Proc Natl Acad Sci U S A*, 92(4), 1108-1112.

- 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(6762), 656-660.
- Kojima, M., & Kangawa, K. (2005). Ghrelin: Structure and Function. *Physiol Rev*, 85, 495-522. doi: 10.1152/physrev.00012.2004.-Small
- Koolhaas, J. M., De Boer, S. F., De Rutter, A. J., Meerlo, P., & Sgoifo, A. (1997). Social stress in rats and mice. *Acta Physiol Scand Suppl*, 640, 69-72.
- Kopelman, P. (2007). Health risks associated with overweight and obesity. *Obes Rev*, 8 Suppl 1, 13-17. doi: 10.1111/j.1467-789X.2007.00311.x
- Korbonits, M., Bustin, S. A., Kojima, M., Jordan, S., Adams, E. F., Lowe, D. G., . . . Grossman, A. B. (2001). The expression of the growth hormone secretagogue receptor ligand ghrelin in normal and abnormal human pituitary and other neuroendocrine tumors. *J Clin Endocrinol Metab*, 86(2), 881-887.
- Korbonits, M., Goldstone, A. P., Gueorguiev, M., & Grossman, A. B. (2004). Ghrelin--a hormone with multiple functions. *Front Neuroendocrinol*, 25(1), 27-68. doi: 10.1016/j.yfrne.2004.03.002
- Korbonits, M., Kojima, M., Kangawa, K., & Grossman, A. B. (2001). Presence of ghrelin in normal and adenomatous human pituitary. *Endocrine*, 14(1), 101-104. doi: 10.1385/ENDO:14:1:101
- Kreek, M. J., & Koob, G. F. (1998). Drug dependence: stress and dysregulation of brain reward pathways. *Drug Alcohol Depend*, 51(1-2), 23-47.
- Kristensson, E., Sundqvist, M., Astin, M., Kjerling, M., Mattsson, H., Dornonville de la Cour, C., . . . Lindstrom, E. (2006). Acute psychological stress raises plasma ghrelin in the rat. *Regul Pept*, 134(2-3), 114-117. doi: S0167-0115(06)00030-9 [pii]10.1016/j.regpep.2006.02.003

- Kurtz, D., Krieger, J., Kowalski, J., Hoff, E., & Mangin, P. (1980). [Nycterohemeral variations in plasma growth hormone (GH) levels and sleep apnea syndromes: their relationship with obesity (author's transl)]. *Rev Electroencephalogr Neurophysiol Clin*, 10(4), 366-375.
- Lamberts, S. W. J., Bruining, H. A., & deJong, F. H. (1997). Corticosteroid therapy in severe illness. *New England Journal of Medicine*, 337(18), 1285-1292.
- Lewis, S. R., Ahmed, S., Dym, C., Khaimova, E., Kest, B., & Bodnar, R. J. (2005). Inbred mouse strain survey of sucrose intake. *Physiol Behav*, 85(5), 546-556. doi: S0031-9384(05)00216-7 [pii]10.1016/j.physbeh.2005.06.003
- Lewis, S. R., Dym, C., Chai, C., Singh, A., Kest, B., & Bodnar, R. J. (2007). Genetic variance contributes to ingestive processes: a survey of eleven inbred mouse strains for fat (Intralipid) intake. *Physiol Behav*, 90(1), 82-94. doi: S0031-9384(06)00381-7 [pii]10.1016/j.physbeh.2006.08.028
- Lovenberg, T. W., Liaw, C. W., Grigoriadis, D. E., Clevenger, W., Chalmers, D. T., De Souza, E. B., & Oltersdorf, T. (1995). Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain. *Proc Natl Acad Sci U S A*, 92(3), 836-840.
- Lutter, M., Krishnan, V., Russo, S. J., Jung, S., McClung, C. A., & Nestler, E. J. (2008). Orexin signaling mediates the antidepressant-like effect of calorie restriction. *J Neurosci*, 28(12), 3071-3075. doi: 10.1523/JNEUROSCI.5584-07.200828/12/3071 [pii]
- Lutter, M., Sakata, I., Osborne-Lawrence, S., Rovinsky, S. A., Anderson, J. G., Jung, S., . . . Zigman, J. M. (2008). The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. *Nat Neurosci*, 11(7), 752-753. doi: 10.1038/nn.2139nn.2139 [pii]

- Makino, S., Hashimoto, K., & Gold, P.W. (2002). Multiple feedback mechanisms activating corticotropin-releasing hormone system in the brain during stress. *Pharmacology, Biochemistry and Behavior*, 73, 147-158.
- Malik, S., McGlone, F., Bedrossian, D., & Dagher, A. (2008). Ghrelin modulates brain activity in areas that control appetitive behavior. *Cell Metab*, 7(5), 400-409. doi: 10.1016/j.cmet.2008.03.007S1550-4131(08)00078-8 [pii]
- Maniam, J., & Morris, M. J. (2012). The link between stress and feeding behaviour. *Neuropharmacology*, 63(1), 97-110. doi: 10.1016/j.neuropharm.2012.04.017
- Markowitsch, H. J., & Pritzel, M. (1985). The neuropathology of amnesia. *Prog Neurobiol*, 25(3), 189-287.
- McEwen, B. S. (1998). Stress, adaptation, and disease. Allostasis and allostatic load. *Ann N Y Acad Sci*, 840, 33-44.
- Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., & Matsukura, S. (2001). A role for ghrelin in the central regulation of feeding. *Nature*, 409(6817), 194-198. doi: 10.1038/35051587
- Nemeroff, C. B., Lipton, M. A., & Kizer, J. S. (1978). Models of neuroendocrine regulation: use of monosodium glutamate as an investigational tool. *Dev Neurosci*, 1(2), 102-109.
- Nishijo, H., Uwano, T., Tamura, R., & Ono, T. (1998). Gustatory and multimodal neuronal responses in the amygdala during licking and discrimination of sensory stimuli in awake rats. *J Neurophysiol*, 79(1), 21-36.
- Niznik, H. B. (1987). Dopamine receptors: molecular structure and function. *Mol Cell Endocrinol*, 54(1), 1-22.

- O'Doherty, J., Rolls, E. T., Francis, S., Bowtell, R., McGlone, F., Kobal, G., . . . Ahne, G. (2000). Sensory-specific satiety-related olfactory activation of the human orbitofrontal cortex. *Neuroreport*, 11(2), 399-403.
- Ochi, M., Tominaga, K., Tanaka, F., Tanigawa, T., Shiba, M., Watanabe, T., . . . Arakawa, T. (2008). Effect of chronic stress on gastric emptying and plasma ghrelin levels in rats. *Life Sci*, 82(15-16), 862-868.
- Ortolani, D., Oyama, L. M., Ferrari, E. M., Melo, L. L., & Spadari-Bratfisch, R. C. (2011). Effects of comfort food on food intake, anxiety-like behavior and the stress response in rats. *Physiol Behav*, 103(5), 487-492. doi: 10.1016/j.physbeh.2011.03.028
- Otto, B., Tschop, M., Heldwein, W., Pfeiffer, A. F., & Diederich, S. (2004). Endogenous and exogenous glucocorticoids decrease plasma ghrelin in humans. *Eur J Endocrinol*, 151(1), 113-117.
- Patterson, M., Bloom, S. R., & Gardiner, J. V. (2011). Ghrelin and appetite control in humans--potential application in the treatment of obesity. *Peptides*, 32(11), 2290-2294. doi: 10.1016/j.peptides.2011.07.021
- Patterson, Z. R., Ducharme, R., Anisman, H., & Abizaid, A. (2010). Altered metabolic and neurochemical responses to chronic unpredictable stressors in ghrelin receptor-deficient mice. *Eur J Neurosci*, 32(4), 632-639. doi: 10.1111/j.1460-9568.2010.07310.xEJN7310 [pii]
- Patterson, Z. R., Khazall, R., Mackay, H., Anisman, H., & Abizaid, A. (2013a). Central ghrelin signaling mediates the metabolic response of C57BL/6 male mice to chronic social defeat stress. *Endocrinology*, 154(3), 1080-1091. doi: 10.1210/en.2012-1834en.2012-1834 [pii]

- Patterson, Z. R., Khazall, R., Mackay, H., Anisman, H., & Abizaid, A. (2013b). Central Ghrelin Signaling Mediates the Metabolic Response of C57BL/6 Male Mice to Chronic Social Defeat Stress. *Endocrinology*. doi: en.2012-1834 [pii]10.1210/en.2012-1834
- Perello, M., Sakata, I., Birnbaum, S., Chuang, J. C., Osborne-Lawrence, S., Rovinsky, S. A., . . . Zigman, J. M. (2010). Ghrelin increases the rewarding value of high-fat diet in an orexin-dependent manner. *Biol Psychiatry*, 67(9), 880-886. doi: 10.1016/j.biopsych.2009.10.030S0006-3223(09)01318-3 [pii]
- Perez-Tilve, D., Hofmann, S. M., Basford, J., Nogueiras, R., Pfluger, P. T., Patterson, J. T., . . . Tschop, M. H. (2010). Melanocortin signaling in the CNS directly regulates circulating cholesterol. *Nat Neurosci*, 13(7), 877-882. doi: 10.1038/nn.2569nn.2569 [pii]
- Perrin, M., Donaldson, C., Chen, R., Blount, A., Berggren, T., Bilezikjian, L., . . . Vale, W. (1995). Identification of a second corticotropin-releasing factor receptor gene and characterization of a cDNA expressed in heart. *Proc Natl Acad Sci U S A*, 92(7), 2969-2973.
- Peruzzo, B., Pastor, F. E., Blazquez, J. L., Schobitz, K., Pelaez, B., Amat, P., & Rodriguez, E. M. (2000). A second look at the barriers of the medial basal hypothalamus. *Exp Brain Res*, 132(1), 10-26.
- Pfluger, P. T., Kirchner, H., Gunnel, S., Schrott, B., Perez-Tilve, D., Fu, S., . . . Tschop, M. H. (2008). Simultaneous deletion of ghrelin and its receptor increases motor activity and energy expenditure. *Am J Physiol Gastrointest Liver Physiol*, 294(3), G610-618. doi: 00321.2007 [pii]10.1152/ajpgi.00321.2007
- Pijlman, F. T., Wolterink, G., & Van Ree, J. M. (2003). Physical and emotional stress have differential effects on preference for saccharine and open field behaviour in rats. *Behav Brain Res*, 139(1-2), 131-138. doi: S0166432802001249 [pii]

- Porcelli, A. J., Lewis, A. H., & Delgado, M. R. (2012). Acute stress influences neural circuits of reward processing. *Front Neurosci*, 6, 157. doi: 10.3389/fnins.2012.00157
- Porte, D., Jr. (1967). Beta adrenergic stimulation of insulin release in man. *Diabetes*, 16(3), 150-155.
- Pouliou, T., & Elliott, S. J. (2009). An exploratory spatial analysis of overweight and obesity in Canada. *Prev Med*, 48(4), 362-367. doi: 10.1016/j.ypmed.2009.01.017
- Rada, P., Avena, N. M., & Hoebel, B. G. (2005). Daily bingeing on sugar repeatedly releases dopamine in the accumbens shell. *Neuroscience*, 134(3), 737-744. doi: 10.1016/j.neuroscience.2005.04.043
- Rinaman, L., Card, J. P., Schwaber, J. S., & Miselis, R. R. (1989). Ultrastructural demonstration of a gastric monosynaptic vagal circuit in the nucleus of the solitary tract in rat. *J Neurosci*, 9(6), 1985-1996.
- Rodaros, D., Caruana, D. A., Amir, S., & Stewart, J. (2007). Corticotropin-releasing factor projections from limbic forebrain and paraventricular nucleus of the hypothalamus to the region of the ventral tegmental area. *Neuroscience*, 150(1), 8-13. doi: S0306-4522(07)01098-6 [pii]10.1016/j.neuroscience.2007.09.043
- Roth, R. H., Tam, S. Y., Ida, Y., Yang, J. X., & Deutch, A. Y. (1988). Stress and the mesocorticolimbic dopamine systems. *Ann N Y Acad Sci*, 537, 138-147.
- Sainsbury, A., & Zhang, L. (2012). Role of the hypothalamus in the neuroendocrine regulation of body weight and composition during energy deficit. *Obes Rev*, 13(3), 234-257. doi: 10.1111/j.1467-789X.2011.00948.x

- Sakata, I., Nakamura, K., Yamazaki, M., Matsubara, M., Hayashi, Y., Kangawa, K., & Sakai, T. (2002). Ghrelin-producing cells exist as two types of cells, closed- and opened-type cells, in the rat gastrointestinal tract. *Peptides*, 23, 531-536.
- Satoh, N., Ogawa, Y., Katsuura, G., Tsuji, T., Masuzaki, H., Hiraoka, J., . . . Nakao, K. (1997). Pathophysiological significance of the obese gene product, leptin, in ventromedial hypothalamus (VMH)-lesioned rats: evidence for loss of its satiety effect in VMH-lesioned rats. *Endocrinology*, 138(3), 947-954.
- Schwartz, G. J., & Moran, T. H. (2002). Leptin and neuropeptide y have opposing modulatory effects on nucleus of the solitary tract neurophysiological responses to gastric loads: implications for the control of food intake. *Endocrinology*, 143(10), 3779-3784.
- Schwartz, M. W., Peskind, E., Raskind, M., Boyko, E. J., & Porte, D., Jr. (1996). Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans. *Nat Med*, 2(5), 589-593.
- Scott, M. M., Perello, M., Chuang, J. C., Sakata, I., Gautron, L., Lee, C. E., . . . Zigman, J. M. (2012). Hindbrain ghrelin receptor signaling is sufficient to maintain fasting glucose. *PLoS One*, 7(8), e44089. doi: 10.1371/journal.pone.0044089PONE-D-12-16911 [pii]
- Sescousse, G., Redoute, J., & Dreher, J. C. (2010). The architecture of reward value coding in the human orbitofrontal cortex. *J Neurosci*, 30(39), 13095-13104. doi: 10.1523/JNEUROSCI.3501-10.201030/39/13095 [pii]
- Shinton, R., Sagar, G., & Beevers, G. (1995). Body fat and stroke: unmasking the hazards of overweight and obesity. *J Epidemiol Community Health*, 49(3), 259-264.
- Smith, A. I., & Funder, J. W. (1988). Proopiomelanocortin processing in the pituitary, central nervous system, and peripheral tissues. *Endocr Rev*, 9(1), 159-179.

- Sokoloff, L., Mickelsen, O., Silverstein, E., Jay, G. E., Jr., & Yamamoto, R. S. (1960). Experimental obesity and osteoarthritis. *Am J Physiol*, 198, 765-770.
- Stenzel, P., Kesterson, R., Yeung, W., Cone, R. D., Rittenberg, M. B., & Stenzel-Poore, M. P. (1995). Identification of a novel murine receptor for corticotropin-releasing hormone expressed in the heart. *Mol Endocrinol*, 9(5), 637-645.
- Stjarne, L. (1973). Frequency dependence of dual negative feedback control of secretion of sympathetic neurotransmitter in guinea-pig vas deferens. *Br J Pharmacol*, 49(2), 358-360.
- Stokes, P. E., & Sikes, C. R. (1991). Hypothalamic-pituitary-adrenal axis in psychiatric disorders. *Annu Rev Med*, 42, 519-531. doi: 10.1146/annurev.me.42.020191.002511
- Stokes, Peter E. (1991). Hypothalamic-Pituitary-Adrenal Axis in Psychiatric Disorders. *Annual Reviews*, 42, 519-531.
- 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(2), 843-850. doi: 10.1210/en.2007-0271
- Sun, Y., Wang, P., Zheng, H., & Smith, R. G. (2004). Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. *Proc Natl Acad Sci U S A*, 101(13), 4679-4684. doi: 10.1073/pnas.0305930101
- Tassone, F., Broglio, F., Destefanis, S., Rovere, S., Benso, A., Gottero, C., . . . Maccario, M. (2003). Neuroendocrine and metabolic effects of acute ghrelin administration in human obesity. *J Clin Endocrinol Metab*, 88(11), 5478-5483.
- Tatemoto, K., Carlquist, M., & Mutt, V. (1982). Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature*, 296(5858), 659-660.

- Tena-Sempere, M., Barreiro, M. L., Gonzalez, L. C., Gaytan, F., Zhang, F. P., Caminos, J. E., . . . Aguilar, E. (2002). Novel expression and functional role of ghrelin in rat testis. *Endocrinology*, *143*(2), 717-725.
- Tomiyama, A. J., Dallman, M. F., & Epel, E. S. (2011). Comfort food is comforting to those most stressed: evidence of the chronic stress response network in high stress women. *Psychoneuroendocrinology*, *36*(10), 1513-1519. doi: 10.1016/j.psyneuen.2011.04.005
- Tschop, M., Smiley, D. L., & Heiman, M. L. (2000). Ghrelin induces adiposity in rodents. *Nature*, *407*(6806), 908-913. doi: 10.1038/35038090
- Tsigos, C., & Chrousos, G. P. (2002). Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *J Psychosom Res*, *53*(4), 865-871. doi: S0022399902004294 [pii]
- Tung, Y. L., Hewson, A. K., & Dickson, S. L. (2004). Glucocorticoid-dependent stimulation of adiposity and appetite by a ghrelin mimetic in the rat. *Eur J Endocrinol*, *150*(6), 905-911.
- Ueberberg, B., Unger, N., Saeger, W., Mann, K., & Petersenn, S. (2009). Expression of ghrelin and its receptor in human tissues. *Horm Metab Res*, *41*(11), 814-821. doi: 10.1055/s-0029-1233462
- Vallone, D., Picetti, R., & Borrelli, E. (2000). Structure and function of dopamine receptors. *Neurosci Biobehav Rev*, *24*(1), 125-132. doi: S0149-7634(99)00063-9 [pii]
- van der Lely, A. J., Tschop, M., Heiman, M. L., & Ghigo, E. (2004). Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. *Endocr Rev*, *25*(3), 426-457. doi: 10.1210/er.2002-0029
- Wanat, M. J., Hopf, F. W., Stuber, G. D., Phillips, P. E., & Bonci, A. (2008). Corticotropin-releasing factor increases mouse ventral tegmental area dopamine neuron firing through a protein

kinase C-dependent enhancement of lh. *J Physiol*, 586(8), 2157-2170. doi:

10.1113/jphysiol.2007.150078jphysiol.2007.150078 [pii]

Weststrate, J. A., Van der Kooy, K., Deurenberg, P., & Hautvast, J. G. (1990). The effect of psychological stress on diet-induced thermogenesis and resting metabolic rate. *Eur J Clin Nutr*, 44(4), 269-275.

Wurtman, R. J., & Wurtman, J. J. (1995). Brain serotonin, carbohydrate-craving, obesity and depression. *Obes Res*, 3 Suppl 4, 477S-480S.

Yang, J., Brown, M. S., Liang, G., Grishin, N. V., & Goldstein, J. L. (2008). Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell*, 132(3), 387-396. doi: 10.1016/j.cell.2008.01.017S0092-8674(08)00117-7 [pii]

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., & Friedman, J. M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature*, 372(6505), 425-432. doi: 10.1038/372425a0

Zigman, J. M., Jones, J. E., Lee, C. E., Saper, C. B., & Elmquist, J. K. (2006). Expression of ghrelin receptor mRNA in the rat and the mouse brain. *J Comp Neurol*, 494(3), 528-548. doi: 10.1002/cne.20823

Zigman, J. M., Nakano, Y., Coppari, R., Balthasar, N., Marcus, J. N., Lee, C. E., . . . Elmquist, J. K. (2005). Mice lacking ghrelin receptors resist the development of diet-induced obesity. *J Clin Invest*, 115(12), 3564-3572. doi: 10.1172/JCI26002