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THE IMPACT OF WEIGHT-LOSS SURGERY AND THE BUDDING ROLE OF CANNABINOID ACTION IN ADIPOSE TISSUE

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Dedication

To Dr. Brady –

Thank you for the endless support, encouragement, and life lessons you imparted over the years, particularly in the face of unique challenges. Your mentorship cannot be overstated.

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Abstract

The US prevalence of obesity has risen from less than 20% to over 40% in last the 25 years despite all efforts to control it. Obesity represents a major health challenge because it substantially increases the risk of leading causes of death, including diabetes, heart disease, stroke, and several cancers, as well as dementia and poorer mental health, thereby contributing to a decline in both quality of life and life expectancy. Obesity is also associated with increasingly abnormal function of adipose tissue which contributes to the development of insulin resistance. Prevention and treatment through lifestyle and public health intervention have not been successful in the long term. Currently, the most effective and long-lasting treatment for severe obesity is bariatric surgery (a.k.a. weight-loss surgery). Of course, pharmacological agents have also been explored, though these have historically run into safety concerns. One target under active research is the endocannabinoid system, the antagonism of which reduces food intake resulting in weight-loss. However, this effect is driven by central mechanisms, and the role of the peripheral endocannabinoid system in metabolism is much less understood.

The work reported in this dissertation led to novel findings in obesity research. The first chapter provides an overview of obesity, hunger, appetite, and the metabolic role of adipose tissue. It also introduces the endocannabinoid system as a link between food intake and metabolism, and further explores the role of cannabinoid action in adipose tissue. The second chapter describes a clinical study of the impact of vertical sleeve gastrectomy on subjective hunger and appetite. This study revealed that 12-weeks after vertical sleeve

gastrectomy surgery, when subjects still have obesity, subjects' ratings of hunger and appetite do not significantly decline over the course of the day as they did before surgery. Interestingly, this was observed in parallel with post-surgery fasting levels of leptin and ghrelin being altered in favor of increased food intake. Lastly, the third chapter pivots from the clinic to a novel investigation of cannabinoid action in primary murine white adipose tissue. Utilizing mRNA-sequencing and qRT-PCR methods, the study presents evidence that that the synthetic cannabinoid WIN 55,212-2 inhibited expression of insulin and calcium signaling pathway genes and activity in primary adipose tissue. In addition, assessments of lipid metabolism and insulin signaling linked the attenuated antilipolytic action of insulin to the suppression of insulin-dependent Akt phosphorylation by WIN 55,212-2. Taken together, the chapters of this dissertation allude to the immense complexity of obesity, explore the links between neuroendocrine signaling and food intake, and highlight the roles of adipose tissue and the endocannabinoid system as major players in the regulation of energy metabolism.

CHAPTER 1: INTRODUCTION

1.1 Obesity

1.1.1 Epidemiology & Public Health

The epidemic of obesity has continued to grow at an alarming rate despite public health initiatives to curb and reverse its prevalence. The World Health Organization classifies obesity by body mass index (BMI; kg/m^2), with BMI of 25-30 as overweight, and BMI ≥ 30 as obese. An exception is for Asian populations as they develop negative health consequences at a lower BMI, thus the WHO advises lower BMI cut points for overweight/obesity in Asians (BMI of 23–27.5 for overweight; BMI ≥ 27.5 for obese) [1, 2]. In 1990, the CDC reported an obesity prevalence of 12% [3]. 10 years later, by 1999-2000, the age-adjusted US adult prevalence rate of obesity had risen dramatically to 30.5%. Five years later, from 2005-2006, it had increased to 34.3%. Another five years later, the growth rate of obesity seemed to have slowed, with a prevalence of 35.7% from 2009-2010 [4]. In response, in 2010 the CDC Department of Health and Human Services launched the Healthy People 2020 initiative to lower the rate of obesity down to 30.5% by 2020. Instead, the most recent report from the CDC saw the US rate of obesity rise to 42.4% of adults in 2017-2018 [4]. This alarming trend highlights the limited effectiveness of lifestyle and behavioral interventions based on reduced caloric intake and increased energy expenditure. Current health recommendations rely on the fact that obesity is the consequence of small, cumulative imbalances between energy intake and energy expenditure that result in a positive energy balance and weight gain. However, at the individual level, weight-loss interventions aimed at reducing calorie intake

and increasing energy expenditure are frequently unsuccessful in the long term [5-7].

Obesity represents a major health challenge because it substantially increases the risk of metabolic diseases (type-II diabetes mellitus and fatty liver), cardiovascular diseases (hypertension, myocardial infarction, and stroke), and is also strongly associated with the development of dementia, osteoarthritis, obstructive sleep apnea, and several cancers [4, 8-12]. The elevated risks of this chronic progressive disease, if left unmitigated, can lead to reduced quality of life and economic burden. Obesity has been challenging to address because its causal factors and their interrelationships are enormously complex. While current weight-loss strategies target the individual, it is also clear that there are significant environmental and societal factors involved such that energy balance is strongly linked to the relationship between an individual and their environment [13]. Individual factors, such as genetic background or the gut-brain axis, influence susceptibility to obesity, which may develop in an obesogenic environment (e.g., shift work, drug use, fast food culture). Even from a solely biological perspective, the pathogenesis of obesity cannot be attributed to the disruption of any individual physiological system—it is clear that obesity develops from a combination of factors that vary among individuals and over time [14-16].

1.1.2 Pathophysiology & Links to Diabetes

Over the course of evolution, humans have developed complex, persistent adaptations to defend against weight-loss and promote weight-gain in the face of limited resources for the sake of survival. The storage of energy as fat is an adaptive response to surplus caloric intake. In fact, this process protects against the negative consequences of

prolonged high levels of sugars and lipids in the blood. The storage of energy as fat is thus critical for systemic energy homeostasis such that a severe lack of body fat leads to negative outcomes. In adults, a body fat percentage of less than 5% in men and 15% in women results in a breakdown of normal bodily functions, including impaired immune function and thermal regulation. Women have a higher threshold of essential body fat due to reproductive requirements, and exceedingly low body fat percentage results in a disruption of menstruation. Similarly, the issue of obesity arises when increasing body fat mass reaches a point that the body's ability to store fat properly becomes impaired and existing fat functions abnormally.

Long-term consumption of a diet high in sugars and fat is known to promote weight-gain in the form of fat mass, and the consistently high levels of glucose and lipids in the blood require proportionally greater amounts of insulin to control. The development of obesity can thus be characterized by the onset of hyperinsulinemia, hyperglycemia, and hyperlipidemia, which are conditions associated with "metabolic syndrome." Hyperglycemia is defined as chronic high blood sugar, and results from insufficient glucose uptake from the blood stream. This condition can develop due to insufficient insulin production or insufficient insulin action (i.e., insulin resistance). Chronic hyperglycemia can lead to impaired vasodilation and diabetic neuropathy, which, if left untreated, may require amputation of affected areas. Hyperlipidemia results from a combination of impaired liver and adipose tissue lipid catabolism. It presents as abnormally high levels of low-density lipoproteins (LDL) and/or triglycerides. Continued consumption of a high-fat, high-sugar diet under conditions of

hyperglycemia and hyperlipidemia exacerbates the abnormally high levels of glucose and lipids in the blood such that the capacity of adipose tissues to handle excess glucose and lipid is exceeded. Chronic overburdening of adipocytes (fat cells) impairs their normal functions and insulin sensitivity, leading to high levels of lipolysis and circulating lipids. These excess lipids can be deposited ectopically in non-adipose tissues such as the kidneys, liver, heart, and skeletal muscles. The accumulation of fat in or around these tissues that are not properly equipped to handle fat promotes lipotoxicity, inflammation, insulin resistance, and aberrant function of affected tissues. One such manifestation of this is lipodystrophy, a rare condition defined by dysfunction, loss, and/or inability to make adipose tissue leading to excess circulating lipids and ectopic deposition of fat. As in chronic obesity, the inappropriate accumulation of lipid in multiple organs leads to profound insulin resistance [17].

Unaddressed, these conditions lead to a state of systemic insulin resistance that can ultimately develop into Type-II Diabetes Mellitus. Type-II diabetes mellitus (T2DM) is the combination of the aforementioned factors such that high levels of insulin secretion (hyperinsulinemia) can no longer be maintained by the kidneys to sufficiently compensate for increasing levels of insulin resistance. The magnitude of insulin resistance associated with T2DM is such that they body struggles to utilize glucose and significantly relies on body fat for energy, which compounds the existing metabolic dysregulation. Taken together, these factors can promote the development of atherosclerosis, hypertension, and combined with continued lipotoxicity can further lead to liver, kidney, and heart failure.

1.1.3 Interventions

Lifestyle

Lifestyle intervention (diet and exercise) represents the cornerstone of weight management, and according to one estimate, every year, 42% of the world population tries to lose weight [18, 19]. Yet, even if the initial weight-loss is successful, most individuals will regain weight over time, and only a small proportion of dieters will be able to maintain the reduced weight over the next years. For example, a meta-analysis of weight loss studies in the USA, which included 29 studies that applied hypocaloric diets with or without exercise with long-term (≥ 2 years) follow-up, showed that on average more than half of the weight lost is regained after 2 years and more than three-quarters is regained after 5 years [20]. Another study in patients with overweight/obesity without diabetes showed that one year after initial weight loss, levels of the circulating mediators of appetite that encourage weight regain after diet-induced weight loss do not revert to the levels recorded before weight loss, suggesting a persistent drive towards food intake in defense of the previous body fat mass [21]. A third example is the Look Ahead study, which demonstrated that intensive lifestyle intervention that included group sessions and structured meal plans resulted in maintained weight loss. Yet, the study reported that the average weight regain in the intensive lifestyle intervention group was still 50% of initial weight loss after 4 years, which was maintained until 8 years [22]. Thus, one of the biggest challenges in the management of obesity is the prevention of weight-regain after successful weight loss.

Pharmacological

Historically, pharmacological interventions for the treatment of obesity and insulin resistance have largely failed as long-term solutions owing to a host of negative side-effects, contraindications, lack of effectiveness, and poor adherence such that there remains unclear guidance for clinicians regarding choice of individual drugs [23-27]. One example is the drug rimonabant, an endocannabinoid system inhibitor that targets the hunger drive, which was highly successful at reducing food intake, but was ultimately banned due to its negative psychological side-effects including depression and suicide [24]. A second example is the drug lorcaserin, a selective serotonin 2C receptor agonist that modulates appetite, which was withdrawn after the FDA found that it increased the risk of cancer [28]. A final example is the class of drugs known as thiazolidinediones. Used in the treatment of T2DM, thiazolidinediones (TZDs) are PPARγ agonists that work by promoting adipogenesis thereby reducing circulating fatty acids, enhancing adiponectin expression, and improving insulin sensitivity. Though TZDs are effective, they are relegated to second-line treatment in part due to increased risk of cancer (pioglitazone) and cardiovascular events (rosiglitazone) [29, 30].

Currently, the first-line medication for the treatment of T2DM is metformin. While its primary indication is for the reduction of blood glucose, is also prescribed off-label for weight management in overweight and obese patients without diabetes [31]. The molecular mechanisms underlying the effects of metformin are not fully understood. Metformin thought to exert its primary antidiabetic action through the suppression of hepatic glucose

production via AMPK-mediated effects or inhibition of mitochondrial complex I, yet not all of its effects can be explained by these mechanisms. This suggests that metformin likely acts on multiple tissues through various underlying mechanisms, and highlights an area of continued research [32].

Another class of drugs that has risen in prominence are incretin-based medications that target insulin resistance. Incretins are gut-derived hormones that are released after eating and stimulate the secretion of insulin. The two major incretins are GLP-1 (glucagon-like peptide-1) and GIP (gastric inhibitory peptide, or glucose-dependent insulinotropic polypeptide). Incretin-based medications, like the GLP-1 receptor agonists liraglutide and semaglutide, have shown high clinical efficacy in the treatment of T2DM and in weight management [33-37]. Most recently, semaglutide was approved by the FDA for chronic weight management in adults with obesity or overweight with at least one weight-related condition (e.g., hypertension, T2DM, or hypercholesterolemia [38]. However, clinicians may be hesitant to adopt GLP-1 receptor agonists given their relative infancy and the currently limited data on long-term weight loss and cardiovascular outcomes, though a large ongoing trial is poised to address these concerns [39].

Surgical

Bariatric surgery (also known as weight-loss surgery) is widely prescribed for those with class-III obesity (BMI >40), and is extremely effective in reducing body weight, improving overt measures of metabolic health, and reducing all-cause mortality among obese adults [40-42]. Indeed, a recent meta-analysis of 174772 participants found that bariatric

surgery was associated with 59% and 30% reduction in all-cause mortality among obese adults with or without T2DM, respectively [42]. This meta-analysis also found that median life-expectancy was 9.3 years longer for obese adults with diabetes who received bariatric surgery as compared to routine (non-surgical) care, whereas the life expectancy gain was 5.1 years longer for obese adults without diabetes [42]. There is little question that bariatric surgical procedures can produce profound and long-lasting effects on body weight. Chief among these procedures are Roux-en-Y gastric bypass (RYGB) and vertical sleeve gastrectomy (VSG), each of which can produce profound and sustained weight loss that cannot be reliably achieved by other means. Both RYGB and VSG involve a significant reduction in the size of the stomach to restrict the volume of food which can be eaten. RYGB further implements re-construction of the GI tract to attach the stomach "pouch" directly to jejunum, bypassing the duodenum, which significantly reduces the degree of nutritional absorption. Other bariatric surgery procedures include gastric banding, which restricts and slow the flow of food into the stomach at the cost of potential scarring, and duodenal switch, which adds intestinal rearrangement to VSG. These procedures have mostly fallen out of favor for VSG, which has demonstrated effectiveness in weight loss as a stand-alone procedure and is now the most commonly performed bariatric surgery worldwide [43, 44]

The effects of bariatric surgery have historically been attributed to either caloric restriction through via the reduction of stomach capacity or through malabsorption via intestinal rearrangement. However, it has been frequently observed that these mechanisms do not fully account for fact that patients report being less hungry after surgery despite rapid

and significant weight loss, which is typically a potent stimulator of appetite and food intake [16]. This suggests that bariatric surgery may affect appetite through additional mechanisms independent of caloric intake and absorption. It is hypothesized that such mechanisms may be through alterations to gut-brain communication, gut hormone signaling, and/or leptin sensitivity [45-50]. Yet, as with lifestyle interventions, weight regain can occur even after bariatric surgery [51-53]. Thus, identifying the factors that make long term weight loss maintenance so challenging is an important step for researchers and clinicians.

1.2 Adipose Tissue & Metabolism

Though adipocytes make up the majority of adipose tissue volume and cell types, the composition of vasculature, immune cells, progenitor cells, fibroblasts, and connective tissues can vary and are important to adipose tissue function. These differences are further reflected in distinctions between the various adipose tissue depots, between visceral and subcutaneous adipose tissues, and between types of adipocytes (white, brown, and beige).

1.2.1 Distribution & Composition

Adipose tissue can be classified by morphology into white, brown, or beige subsets. White adipose tissue is further classified by location, and is largely defined as subcutaneous and visceral. In humans, while subcutaneous adipose tissue (SAT) is distributed throughout the body, the main depots are in the hips, buttocks, and thighs (gluteofemoral), whereas in rodents, subcutaneous adipose depots are the interscapular and inguinal fat pads [54]. Visceral adipose tissue (VAT) in both humans and rodents is largely intra-abdominal (e.g., mesenteric, omental, perirenal, pericardial, perigonadal). Increased accumulation of VAT, as

is seen in the development of obesity, is associated with an increased risk for insulin resistance, T2DM, hypertension and all-cause mortality [54-56]. In contrast, SAT is associated with improved or preserved insulin sensitivity and mitigated risk of developing T2DM [57, 58]. The benefits associated with SAT may be attributed to its ability to serve as a primary energy sink for nutrient deposit, thereby protecting against ectopic fat deposition and associated lipotoxicity [54]. Thus, body fat distribution, irrespective of BMI and/or body fat percentage, can predict risk of obesity-related diseases and complications [59]. Risk can further be stratified by fat distribution, as individuals with a higher waist-to-hip ratio (i.e., more abdominal/visceral fat than lower body/subcutaneous fat) are more predisposed to obesity-related metabolic dysfunction [60]. For example, individuals with gynoid obesity, characterized by subcutaneous fat in the gluteofemoral region, have less risk of developing metabolic dysfunction, whereas individuals with android obesity, which is characterized by abdominal visceral fat, have a greater risk of metabolic dysfunction [60-62].

Adipose tissue is comprised of many different cell types that secrete numerous cytokines, chemokines, and hormones. About a third of the cells within lean, healthy adipose tissue are adipocytes, with the rest of the cells comprised of fibroblasts, endothelial cells, macrophages and other immune cells, stromal cells, and preadipocytes. White adipocytes are the primary cell type for fat storage. They are capable of expanding to accommodate increased lipids through hypertrophy of existing adipocytes and through proliferation and differentiation of pre-adipocytes into mature white adipocytes. Brown adipose tissue (BAT), composed primarily of brown adipocytes, is localized to distinct anatomical regions that have

been well-characterized in rodents, with the majority located in the interscapular region [63]. Thought to be only present to some degree in human infants, it has been shown that BAT represents between 1-2% of total fat stores in adult humans [64, 65]. Brown adipocytes contain a particularly high density of mitochondria and function to generate heat by uncoupling energy production via oxidative phosphorylation into heat production (non-shivering thermogenesis). Beige/brite adipose tissue on the other hand is difficult to quantify but represents cells interspersed within WAT that are capable of transforming into brown-like adipocytes following cold exposure or adrenergic stimulation [66, 67]. The conversion of excess white adipocytes to a beige or brown-like phenotype represents an active area of research with the goal of raising energy expenditure to combat weight gain [68].

White adipose tissue also contains a significant population of macrophages. These immune cells represent less than 10% of total cells in lean adipose tissue, with one report estimating that they represent up to 40-50% of adipose tissue cells in rodents and humans with obesity [69]. Furthermore, it has been shown that in obesity these adipose tissue macrophages are activated via a mechanism distinct from that of classically activated M1 macrophages, which are primarily associated with pathogen-induced inflammation [70, 71]. Considered "metabolically activated" by the hyperglycemic, hyperinsulinemic, and hyperlipidemic microenvironment in obese adipose tissue, these macrophages, in addition to obese adipocytes, are known to produce inflammatory cytokines despite their distinct activation [70-72]. Although these cytokines are mostly restricted to autocrine and paracrine effects, some of them make significant contributions to systemic inflammation and insulin

sensitivity [70-72].

1.2.2 Endocrine Function

Adipose tissue had long been considered simply a site of fat storage. However, our understanding of its complexity has grown over time. It is now known that adipose tissue has a complex distribution, composition, and hormone secretion profile as a major endocrine organ. As an endocrine organ, adipose tissue synthesizes and secretes hormones that are active in a range of processes, such as control of food intake, control of insulin sensitivity, and inflammatory processes [73]. Two highly-studied adipose tissue hormones are leptin and adiponectin. The primary role of leptin is as a homeostatic driver of energy balance, which is demonstrated by its satiety-inducing effects that act to counter the role of ghrelin. Indeed, leptin or leptin receptor deficient humans and rodents exhibit profound hyperphagia, hyperglycemia, and insulin resistance [74, 75]. Leptin is synthesized in response to insulin-action and glucose metabolism, and has been shown to be secreted in proportion to insulin-stimulated glucose uptake in adipocytes [76, 77]. Total leptin levels are also proportional to body fat mass, with greater fat mass correlated with greater circulating leptin, and decreased levels resulting from a loss of fat mass [73, 78, 79].

The role of adiponectin is similarly characterized by metabolic regulation, but is also involved in mediating inflammation. Studies have shown that circulating adiponectin levels are inversely correlated with BMI, and increase during caloric restriction. Adiponectin positively regulates glucose and lipid metabolism by improving insulin sensitivity, and deficiency in adiponectin or its receptor is highly associated with obesity and insulin

resistance [80, 81]. The beneficial effects of adiponectin are thought to be through enhanced insulin signal transduction and by promoting the phosphorylation/activation of AMPK and PPARα [82, 83].

The metabolic stress imposed on adipocytes by the conditions of metabolic syndrome and obesity contributes to a state of low-grade systemic inflammation. Numerous studies have shown that this is due to enhanced expression and secretion of pro-inflammatory cytokines, particularly TNFα, from adipose tissue, and is associated with significant macrophage accumulation and adipocyte hypertrophy [69, 73, 84-93].

TNFα is a potent proinflammatory cytokine that can stimulate the release of other inflammatory cytokines, and was the first WAT-derived inflammatory cytokine reported to be implicated in the initiation and progression of insulin resistance [84]. In humans, TNFα levels are higher in plasma and adipose tissue of obese individuals, and circulating levels reduce with weight loss. A key mechanism by which TNFα induces insulin resistance involves phosphorylation of IRS1 that interferes with insulin-induced phosphorylation of IRS1 [94]. TNFα has also been shown interfere with adipocyte metabolism through suppression of lipolysis and the expression adipogenic genes thus promoting adipocyte hypertrophy and expansion of adipose tissue mass [95].

Another inflammatory cytokine associated with obesity and inflammation is resistin. Like TNFα, resistin is thought to be secreted by macrophages residing in inflamed adipose tissue, and is correlated with insulin resistance in humans and animal models [73, 96, 97]. Key evidence for its involvement in modulating insulin resistance comes from studies

demonstrating that resistin levels are elevated in DIO mice, and blocking its action improves insulin resistance [96, 98]. Moreover, resistin is believed to propagate inflammatory responses by upregulating expression of inflammatory cytokines such as TNFα and IL-6 in monocytes and macrophages in a nuclear factor kappa-B (NFκB)-dependent manner, and is positively associated with circulating inflammatory markers such as C-reactive protein (CRP) and TNFα [97, 99]. Resistin appears to interfere with normal insulin signaling by decreasing insulin receptor and IRS protein expression and phosphorylation in 3T3-L1 cells [100]. It has also been showed to decrease AMPK activation, which is implicated in the regulation of insulin action [101, 102].

Thus, inflammatory cytokine secretion from adipose tissue can promote a vicious cycle of increasing adipose tissue and systemic inflammation, and the worsening of both local and systemic insulin resistance.

1.2.3 Metabolic Function

In parallel with the liver and skeletal muscle, adipose tissue contributes to the maintenance of glucose and lipid homeostasis metabolism. In the fed state, such as after a meal, glucose sensing by the pancreas leads to the secretion of insulin from β-cells into circulation. Insulin subsequently stimulates glucose uptake into skeletal muscle and liver, where it can be stored as glycogen, and the excess into adipose tissue, where it can be converted to and stored as fat. Over time and without further food intake, a drop in blood glucose is sensed by the pancreas, which leads to the secretion of glucagon from pancreatic α-cells. Glucagon stimulates the breakdown of glycogen (glycogenolysis) stored in the liver

and skeletal muscle to maintain blood glucose. Extended fasting depletes muscle and liver glycogen, and sympathetic nervous system activity in addition to glucagon enhances hepatic gluconeogenesis to maintain blood glucose and adipocyte lipolysis to liberate fatty acids for beta-oxidation as an additional source of ATP generation. Refeeding will once again stimulate the secretion of insulin, which suppresses sympathetic signaling, gluconeogenesis, and lipolysis, and the fed-fasted cycle of glucose homeostasis begins again [103].

The majority of excess weight associated with obesity is composed of adipose tissue. Adipocytes store energy in the form of triacylglycerols (TAGs) that are formed from fatty acids and glucose taken up from circulation. These functions are carried out by adipocytes through the processes of adipogenesis (differentiation of preadipocytes into new adipocytes), lipogenesis (synthesis and storage of fatty acids leading to adipocyte hypertrophy), lipid/fatty acid and glucose uptake, lipolysis (breakdown of TAGs and release of lipids to circulation), and to a lesser degree thermogenesis (largely associated with the brown adipocyte phenotype). Insulin action on adipocytes modulates lipid metabolism by stimulating glucose uptake for de novo lipogenesis during positive energy balance, and by inhibiting lipolysis and the subsequent mobilization of fatty acids into circulation during energy deficit. Both lipogenesis and lipolysis can be active simultaneously, though the rates at which they occur are regulated by cellular cAMP concentrations, with increasing levels correlated with greater rates of lipolysis [104-106].

Lipolysis results in the liberation and secretion of long-chain fatty acids and glycerol to be utilized for fatty acid beta-oxidation. During conditions of stress or energy deficit (e.g.,

fasting or rapid energy expenditure), adipocytes receive lipolytic hormonal signals through the sympathetic nervous system to mobilize energy stores. These signals, mainly from catecholamines and glucocorticoids, act through adrenergic and glucocorticoid receptor signaling to activate adenylyl cyclase to produce cAMP and raise intracellular levels [104-106]. cAMP activates the enzyme PKA (cAMP-dependent protein kinase A), which is a master regulator of catabolic metabolism. PKA can then proceed to phosphorylate perilipin proteins surrounding the lipid droplet, to facilitate the catalytic activity of ATGL (adipose triglyceride lipase; PNPLA2) to begin TAG hydrolysis [104-106]. HSL (hormone-sensitive lipase; LIPE), which catalyzes the hydrolysis of diacylglycerols to monoacylglycerols (after the activity of ATGL), is also phosphorylated by PKA [104-106]. The fatty acids liberated by lipolysis are the fuel for ATP generation via fatty acid beta-oxidation. Suppression of lipolysis is primarily achieved through the reduction of cAMP levels and PKA activity, which is typically accomplished through insulin action. Insulin signaling acts through the insulin receptor (INSR), and activation of phosphatidylinositol-3 kinase (PI3k) leads to the phosphorylation and activation of Akt (a.k.a. PKB, protein kinase-B). Akt is a serine/threonine kinase, and is responsible for the phosphorylation of several proteins involved in multiple cellular processes such as metabolism, glucose uptake, cell cycle, and proliferation, to name a few. In the suppression of lipolysis, active Akt phosphorylates and activates PDE3B (phosphodiesterase 3b), a major phosphodiesterase isoform expressed in adipose tissue, which catalyzes the hydrolysis of cAMP into AMP to decrease PKA activity and the subsequent suppression of lipolysis [104-106].

1.3 Hunger and Appetite

1.3.1 Neuroendocrine Regulation

Within the brain, the arcuate nucleus (ARC) of the lateral hypothalamus (LH) is located close to the third ventricle and is thus in an ideal location to obtain information from circulating signals that cross the blood brain barrier to propagate information from the periphery [107, 108]. The ARC mediates an array of cognitive and physiological processes including regulation of homeostatic mechanisms such as sleep, wakefulness, fatigue, thermoregulation, blood pressure, thirst, gastrointestinal motility, appetite, and hunger [107, 108]. Specifically, the ARC receives information regarding energy need/state to promote or inhibit energy intake. Orexigenic (appetite-stimulating) neurons in the ARC promote hunger via transmission of neuropeptide Y (NPY), melanin-concentrating hormone (MCH), orexins, and agouti-related peptide (AgRP) [107, 108]. Anorexigenic (appetite-repressing) neurons promote satiety via transmission of proopiomelanocortin (POMC), alpha-melanocyte-stimulating hormone (α-MSH), cocaine and amphetamine-regulated transcript (CART), and corticotropin-releasing-hormone (CRH) [107, 108]. AgRP/NPY-transmitting neurons are also capable of inhibiting POMC-transmitting (anorexigenic) neurons through GABAergic neurotransmission, resulting in increased appetite and food intake [109]. Inhibition of either pathway allows for enhanced activity of the other, thus mediating appetite.

Leptin is a circulating hormone originating from adipose tissue that signals satiety and energy sufficiency. It influences food intake and regulates energy balance by suppressing appetite through its binding to and inhibition of AgRP/NPY neurons and stimulation of

POMC neurons [110-112]. Leptin production is proportional to adiposity, yet, during periods of energy deficit, the rate at which leptin levels fall exceeds the rate at which fat stores are depleted. While this reduction in leptin signaling serves to limit weight loss in the lean state, in obesity a decrease in sensitivity to leptin impairs satiety signaling despite energy surplus, high leptin levels, and no reduction of food intake. This leptin resistance is further demonstrated by the lack of effect of exogenous leptin administration on food intake [113]. Similarly, leptin deficiency in humans causes hyperphagia which can lead to obesity or lipodystrophy if left untreated, but can be reversed by leptin administration [114].

Conversely, the circulatory hormone ghrelin, a hunger signal emanating from the gastrointestinal tract, stimulates appetite by binding to and activating AgRP/NPY neurons and inhibiting POMC neurons [115-117]. Mediated in part by mechanoreceptors in the gut, ghrelin secretion occurs when the stomach is empty, such as during fasting, and halts when the stomach is stretched, such as after meals [118, 119]. In obesity, however, plasma levels of ghrelin are decreased compared to the lean state, and do not exhibit the same post-prandial declines [120-122]. The actions of ghrelin also depend on acylation state; acylated ghrelin has high affinity for the ghrelin receptor (GHS-R; growth hormone secretagogue receptor), while nonacylated, or desacylated, ghrelin does not activate GHS-R but is thought to affect metabolism and food intake in opposition to acylated ghrelin through separate mechanisms [123]. Furthermore, greater ratios of acylated ghrelin to desacylated or total ghrelin are associated with increased food intake, insulin resistance, and obesity [46, 123-125].

Another peptide hormone, the incretin glucagon-like peptide-1 (GLP1), is released

from the gastrointestinal tract as well as the nucleus of the solitary tract in the brainstem following nutrient consumption. In the periphery, GLP-1 enhances the secretion of insulin by the pancreas, whereas in the brain GLP-1 has been shown to reduce appetite through its binding to neurons of the mesolimbic reward system [126].

1.3.2 Circadian Rhythm

In recent years, biological rhythms and the circadian clock have been established as significant regulators of metabolism and feeding behavior. Indeed, it has been demonstrated that the optimal timing of food intake should be earlier rather than later in the day [127-129]. Similarly, studies utilizing 24-hour blood sampling have shown that serum ghrelin levels exhibit a 24-hour rhythm that is predominantly driven by food intake as concentrations drop following a meal and subsequently increase in parallel with hunger until the next bout of food intake [130], and serum leptin levels peak before mid-sleep and then decline until mid-morning before increasing throughout daytime in response to accumulated caloric intake during the day [131]. These findings demonstrate that biological rhythms influence food intake both behaviorally and physiologically, thus linking metabolic state to the time of day.

As a major part of circadian function, sleep also plays an important role in energy homeostasis by modulating hunger and appetite. In rodents, food shortage or starvation results in decreased sleep, and, conversely, total sleep deprivation leads to marked hyperphagia [132-134]. Spiegel et al. were one of the first to report a rise in subjective feelings of hunger and appetite following two nights of sleep restriction (4 hours/night) compared to two nights of sleep extension (10 hours/night) in young, healthy, normal-weight men. It was

found that the sleep restricted group had increased hunger and appetite ratings (24% and 23%, respectively), especially for calorie-dense foods with high carbohydrate content (33%–45% preference) [135]. The study also utilized continuous blood sampling to monitor levels of leptin and ghrelin throughout a 24-hour period. Compared to the sleep extension group, the sleep restricted group had 18% decreased 24-hour mean serum leptin and 28% increased mean serum ghrelin—both changed in a manner favoring increased food intake [135]. It was suggested that the decrease in leptin could be due to impaired signaling of energy balance or an adaptative response to increased energy requirements due to extended wakefulness, but the study did not measure energy intake nor energy expenditure, aspects of energy balance critical in the role of endocrine signaling by leptin and ghrelin. A later report in which caloric intake and activity levels were controlled showed that, compared to six days of extended sleep (9 hours), six days of restricted sleep (~4 hours) resulted in 19% decreased 24-hour mean serum leptin [136]. These findings helped establish that sleep modulates the neuroendocrine control of hunger and appetite. Indicating energy state from the periphery, leptin and ghrelin work within the ARC to promote or inhibit subsequent food intake. However, hunger is thought to be driven more by homeostatic endocrine signaling mechanisms, while appetite is understood to be motivated more by hedonic reward pathways.

1.3.3 Appetite and Reward Systems

Eating in the absence of hunger has been explained by various motivational states including boredom, stress, and fatigue, among others. These states are influenced less by homeostatic drivers (i.e., endocrine signaling), and more so by the mesolimbic reward

system. Neighboring the hypothalamus, in the ventral striatum, is the nucleus accumbens (NAc), a target-site of the mesolimbic dopaminergic pathway projecting from the ventral tegmental area, and to which mesolimbic transmission of dopamine regulates motivation and desire for rewarding stimuli [137]. It follows then that activation of neurons projecting from the LH to the NAc may also mediate sensitivity to reward, particularly to that of food. Further, the cortex communicates with both the LH and NAc to influence food intake and choice via processes such as decision making, emotional response, and memory [108, 138, 139]. Brain imaging studies have indeed shown that in lean and obese individuals reward systems are differentially activated in response to food cues. Stoeckel et al. utilized fMRI to investigate activation of reward system and associated brain regions in response to images of high- and low-calorie foods in 12 obese and 12 lean women [140]. Analysis revealed that images of high-calorie foods elicited greater responses in all but one brain region of interest in obese women compared to lean women, and that high-calorie foods stimulated more overall brain activation than low-calorie foods in obese subjects compared to lean women [140]. fMRI has also been used show that sleep restriction not only increases neuronal activity in response to food stimuli, but also increases activity in areas associated with reward. Specifically, in lean healthy subjects, highly-palatable food stimuli (sweet and/or savory foods) elicited greater activation within the insular cortex than did healthy food stimuli (e.g., fruits and vegetables) after a period of sleep restriction [141, 142]. Thus, different metabolic states (lean vs obese), as well as different amounts of sleep, are associated with distinct activation patterns within the reward system when presented with food cues. The reward

system in mammals can be further probed by examining the endocannabinoid system.

1.4 The Endocannabinoid System

While the appetite inducing effects of cannabis have been known for centuries, the identification of the active molecule Δ^9-tetrahydrocannabinol (THC) did not occur until much later [143]. It was through pharmacological studies of the active compounds in cannabis and the mechanisms by which they act that the cannabinoid receptors were discovered, to be followed by the discovery of endogenous cannabinoid receptor ligands and their metabolic enzymes thereafter [143]. These endogenous cannabinoid molecules (2-arachidonoylglycerol, or 2-AG; N-arachidonoylethanolamine, or AEA), aptly named endocannabinoids (eCBs), the cannabinoid receptors (CB1 and CB2), and the enzymes involved in endocannabinoid synthesis and hydrolysis constitute what has been known as the endocannabinoid system (ECS). Since the identification of the ECS, research has led to an understanding that it plays significant roles in mediating various aspects of physiology and behavior [144].

For decades, brain reward processes have been considered to be under the control of endogenous dopaminergic and opioid systems. It is now understood that central CB1 activation enhances the activity of the brain reward system [145-147]. Cannabinoids stimulate appetite by activating the central ECS, though neuronal targets are not limited to the arcuate nucleus in the lateral hypothalamus; neuronal systems involved in mediating reward also express the CB1 receptor [148-150]. Furthermore, circulating endocannabinoid concentrations increase with fasting and energy deficit, such as following exercise, suggesting an increased motivation for food-derived reward [151]. These findings further

implicate the endocannabinoid system in the regulation of appetite and feeding behavior. Given the highly rewarding nature of palatable foods, evidence suggests that activation of reward pathways by palatable food stimuli in the satiated state promotes food intake motivated by food-derived reward, or hedonic eating, which has also been shown to be associated with enhanced plasma ghrelin and 2-AG [152, 153]. The highly rewarding nature of palatable foods (i.e., sweet and fatty) are significant motivating factors in food-seeking during periods of energy deficit, but has now become a factor contributing to the development of obesity and diabetes. Indeed, several studies have shown that increased levels of circulating eCBs as well as tissue eCB concentrations are positively correlated with BMI in both humans and rodents [154].

CB1 receptor antagonists and inverse agonists have been shown to successfully decrease food intake [155]. One drug in particular, a CB1 inverse agonist known as rimonabant (SR141716), showed great promise as an anorectic anti-obesity drug. While the drug succeeded in reducing food intake by suppressing appetite, its inhibitive effects on motivation and desire for reward were reported to cause severely depressive symptoms and even suicide [24, 25]. Ultimately, rimonabant's status as a treatment for obesity was suspended due to these serious psychiatric side-effects. Since then, research into CB1 activation and antagonism outside of the CNS has expanded to mitigate psychiatric side-effects. Indeed, it is known that CB1-deficient mice are resistant to diet-induced obesity (DIO), but it has recently been shown that adipocyte-specific inducible deletion of the CB1 receptor protects against DIO in mice on a high-fat diet (HFD), and reverses the phenotype

in already obese mice [156]. Additional studies in peripheral tissues, particularly adipose tissue, and their analogous cell-lines have shown that peripheral CB1 targeting could be an effective strategy for sustained and clinically significant treatment for obesity and its metabolic complications [154, 157-159].

Besides THC and endocannabinoids, CB1 receptors also bind synthetic cannabimimetic compounds, for example WIN 55,212-2, which produce effects similar to those of cannabinoids but have an entirely different molecular structure. The CB1 receptor is a membrane-bound G protein-coupled receptor (GPCR), with ligand binding associated with activation of the Gαi subunit. The classical signaling mechanism for Gαi is inhibition of cyclic-adenosine monophosphate (cAMP) dependent pathways through inhibition of adenylyl cyclase. CB1 is considered one of the most abundant GPCRs in the central nervous system (CNS), but is also found in neurons of the peripheral nervous system (PNS) and metabolically active tissues such as the liver, kidneys, GI tract, pancreas, and adipose tissues adipose tissue [160]. CB1 receptors appear to mediate most, if not all of the psychoactive effects of cannabinoids, and have a characteristic distribution in the nervous system: It is particularly enriched in cortex, hippocampus, hypothalamus, amygdala, basal ganglia outflow tracts, and cerebellum—a distribution that corresponds to the most prominent behavioral effects of cannabis [161]. Perhaps due to these effects, much of our understanding of the ECS has come from investigating its role in the nervous system. In the nervous system, eCBs act as retrograde inhibitors that suppress the release of neurotransmitters from presynaptic neurons through presynaptic CB1 activation [162]. The synthesis and release of

eCBs from the postsynaptic neuron has been shown to be calcium dependent [163]. Whether these mechanisms hold up in the peripheral ECS have only recently begun to be elucidated.

Centrally, the ECS regulates food intake via the hypothalamus; in the periphery, the ECS mediates energy metabolism through action in metabolically active tissues [157, 164]. Strong associations between a highly active ECS and obesity have been shown, with adipocyte CB1 expression and circulating endocannabinoid levels higher in subjects with obesity relative to their non-obese counterparts [154]. Differential expression or modulation of the activity of ECS enzymes can also impact ECS tone to affect health and disease. For example, mice lacking FAAH (fatty acid amide hydrolase), which is the primary hydrolytic enzyme of AEA, have higher high-fat food consumption, gained more weight and adipose tissue, and showed signs of insulin resistance compared to controls [165].

As previously discussed, sleep plays a major role in regulating food intake and even food choice [166]. Lack of sleep leads to increased hunger and appetite due in part to changes in leptin and ghrelin levels favoring increased food intake. More recent studies have demonstrated that sleep restriction also affects endocannabinoid levels. In a randomized crossover study comparing four nights of normal sleep (8.5 hours) to four nights of restricted sleep (4.5 hours) in healthy young adults, Hanlon et al. examined the 24-hour profiles of circulating 2-AG. Levels of leptin, ghrelin, and food intake, as well as ratings for hunger and appetite, were assessed [167]. In both sleep conditions, there was a daily variation of 2-AG concentration with a nadir during the middle of the sleep period and a peak in the early afternoon. However, sleep restriction resulted in an amplified 2-AG profile with a delayed,

yet greater, maximum value. Additionally, in line with previous reports, leptin was found to be decreased following sleep restriction compared to normal sleep, and ghrelin showed an overall elevated concentration over the 24-hour period during sleep restriction relative to normal sleep, even after meals. Moreover, under conditions of sleep restriction, subjects reported increases in hunger and appetite concurrent with the afternoon elevation of 2-AG levels and pre-prandial ghrelin levels, and were observed to consume more calories from palatable snacks after lunch compared to normal sleep conditions [167].

Circadian profiles of serum leptin, ghrelin, and 2-AG have also been investigated within the context of obesity. 24-hour blood sampling has shown that in lean adults serum concentrations of 2-AG vary throughout the day. Controlling for food intake, light-dark cycles, and sleep opportunity, Hanlon et al. showed that 2-AG levels increased during the morning to a peak in the midafternoon, and declined to a nadir during midsleep, coincident with the middle of the overnight fast [168]. Similarly, leptin was at its lowest upon waking, and rose over the course of the day with a peak during early sleep. Ghrelin also varied throughout the day, exhibiting pre-prandial rise, postprandial decline, and peak levels during the overnight fast [168]. In a later study, Hanlon et al. demonstrated that the circadian profile 2-AG is altered in obesity. Compared to nonobese adults, the amplitude of 2-AG was dampened (i.e., the profile was flatter) and the timings of the nadir and peak were delayed by 4 to 5 hours, indicating significant circadian misalignment [169]. Interestingly, the circadian profile of leptin was not significantly different in obese adults compared to nonobese adults, though the serum concentrations were significantly higher, as expected.

These findings suggest that obesity is associated with altered endocannabinoid system activity, which may play a role in the pathophysiology of obesity.

1.5 Insulin Signaling and Resistance

Insulin is the primary hormone responsible for signaling the cells of the body to increase glucose uptake and shift energy utilization towards storage. A key role of insulin in fat and muscle is to promote glucose uptake shortly after a meal to reduce blood glucose levels and provide essential fuel for these organs. Following a meal, in response to elevated blood glucose and levels of other metabolites, pancreatic β-cells secrete insulin to coordinate systemic glucose homeostasis. This homeostasis is driven by tissue insulin sensitivity, which, at a systemic level, describes the efficiency of a given concentration of insulin to normalize blood glucose levels. This physiological mechanism involves several processes across multiple organs, including suppression of liver and adipocyte energy mobilization (glycogenolysis and lipolysis, respectively), and enhanced glucose and fatty acid uptake into liver, muscle, and adipocytes. These cellular processes are regulated by insulin-dependent signal transduction [170].

Insulin signaling begins with binding and activation of the insulin receptor by insulin, followed by a cascade of phosphorylation and dephosphorylation events, second-messenger generation, and protein-protein interactions that result in diverse metabolic events in almost every tissue [170]. There are a number of downstream pathways that are regulated by insulin receptor activity, but most pathways involved in regulating cellular energy metabolism act through Akt [170]. Overall, insulin action serves to suppress catabolic metabolism and

promote anabolic metabolism (energy uptake, synthesis, and storage). Insulin stimulates glucose uptake by inducing the translocation of GLUT-4 storage vesicles to the plasma membrane, an event directly mediated by Akt activity. In addition, as a result of insulin action, active Akt promotes glycogen and fatty acid synthesis by phosphorylating and inactivating GSK-3 (glycogen synthase kinase-3), which facilitates the dephosphorylation and activation of glycogen synthase by PP1 (protein phosphatase-1), and phosphorylating and activating both ATP citrate lyase and SREBP-1 (sterol-response-element binding protein-1) [170]. In parallel, active Akt also suppresses gluconeogenesis and fatty acid oxidation via the phosphorylation of FOXO1 (Forkhead box protein O1), a transcription factor that regulates the expression of lipogenic and gluconeogenic genes [170, 171]. At a cellular level, insulin sensitivity can be described as the efficiency by which insulin signal transduction propagates. Insulin resistance can then be described as a degradation in transduction efficiency. This degradation can be the result of changes in the activity of positive and/or negative effectors, including the insulin receptor substrate proteins IRS1 and IRS2, various protein kinases, and protein phosphatases.

IRS1 is a convergence point for the insulin signaling pathway. It is an important docking site for both the insulin receptor and PI3k, which respectively sit directly upstream and downstream of IRS1, and is thus a major regulator of insulin sensitivity. IRS1 activity is subject to phosphorylation by multiple kinases and phosphatases, and whether IRS1 is activated or inhibited depends on the site(s) of phosphorylation [172, 173]. Tyrosine phosphorylation of IRS1 by the insulin receptor facilitates the binding of PI3k to promote the

activation of Akt and subsequent insulin signal transduction. Activation of serine/threonine kinases and phosphatases, some of which are insulin/IRS-dependent, can exert positive or negative feedback on the pathway through their actions on IRS1 [173, 174]. For example, PP2A (protein phosphatase-2A) downregulates insulin signaling by dephosphorylating and inactivating Akt, but has also been shown to upregulate insulin signaling through serine dephosphorylation of IRS1 [174-176]. Furthermore, the high levels of circulating inflammatory cytokines, free fatty acids, and insulin associated with obesity lead to the activation of several serine/threonine kinases that negatively regulate IRS1 [172].

Thus, insulin resistance can lead to a dysregulation of the metabolic processes mediated by insulin-Akt signaling, and contributes to the development of hyperglycemia, hyperlipidemia, which can further lead to atherosclerosis, fatty-liver, and ultimately T2DM.

1.6 Calcium & Energy Metabolism

1.6.1 The Role of Calcium in Energy Homeostasis and Adipocyte Function

Epidemiological findings, accounting for physical activity and caloric intake, suggest that people with high calcium intake have a lower prevalence of overweight, obesity, and insulin resistance, while lower calcium intake is associated with greater prevalence rates [177, 178]. Moreover, calcium supplementation has been shown to aid weight-loss in humans and mice with obesity while on a calorie-restricted diet, inhibit weight-gain and improve glucose homeostasis in HFD-fed mice, and inhibit weight-regain during refeeding in mice [179-182]. These findings are consistent with studies of supplemental calcium in rats [183, 184]. Another study in mice demonstrated that high calcium intake depresses levels of parathyroid and,

subsequently, the conversion of 25-hydroxy cholecalciferol to 1,25-hydroxy vitamin D_3 ($1,25D_3$) [177]. The same study also showed that isolated primary human adipocytes treated with $1,25D_3$ exhibited significant increases in intracellular calcium that were correlated with a marked inhibition of lipolysis [177]. However, the molecular connections between dietary calcium intake and metabolic improvements remain poorly understood.

Calcium (Ca^{2+}) is one of the most abundant ions in the body regulating multiple cellular processes. Along with cAMP, the calcium ion is a major second messenger that differs from its counterpart in that it is neither created nor destroyed, but rather is moved around inside and outside the cell via transporters, pumps, and channels to alter its concentration. Homeostatic maintenance of intracellular calcium is accomplished by the coordination of several mechanisms and pathways that help to establish a calcium gradient and respond to changes in this gradient [185]. Changes in Ca^{2+} concentrations are detected by a wide array of Ca^{2+}-binding effector proteins that work in parallel with or modulate the activity of other signaling pathways to impact cellular and systemic physiology. For example, changes in levels of intracellular Ca^{2+} drive action potentials, muscle contraction, hormone secretion, certain immune responses, and regulate enzyme activity to coordinate extracellular and intracellular signals [186]. In non-excitable cells, Ca^{2+} signaling typically results from the activation of GPCRs or tyrosine kinase receptors, which leads to phospholipase C (PLC) phosphorylation and stimulation. Calcium-dependent hydrolysis of PIP2 (phosphatidylinositol 4,5-bisphosphate) by PLC produces two second messengers: IP3 (inositol 1,4,5-trisphosphate) and diacylglycerol. IP3 induces Ca^{2+} release from the ER

through activation of its receptor (IP3R), and diacylglycerol and Ca^{2+} then activate PKC (protein kinase-C). Downstream calcium signaling is also dependent on the activity of the protein calmodulin. Calmodulin (CaM) is a calcium-binding messenger protein that plays a fundamental role in cells through the amplification of the Ca^{2+} signal [186]. Ca^{2+} binding to calmodulin induces a conformational change to an active Ca^{2+}/CaM complex. Activated calmodulin propagates Ca^{2+} signals by binding to and modulating the activity of a vast number of proteins. In particular, a major partner of the Ca^{2+}/CaM complex is the Ca^{2+}/calmodulin-dependent serine/threonine protein kinase class of enzymes (CaMKs). CaMKs have been shown to be responsible for the phosphorylation and activation of various transcription factors, enzymes, and proteins involved in numerous processes. For example, CaMKII (Ca^{2+}/CaM-dependent Kinase-II) is critical for modulating cardiomyocyte contraction (heart beat) by phosphorylating several important regulatory proteins, and is essential for synaptic plasticity and memory formation [186-188].

With regard to energy homeostasis, CaMKK2 (CaMK Kinase-2 or CaMK Kinase-β, not to be confused with CaMKII) has been shown to be involved in regulation of appetite, adipocyte function, glucose homeostasis, and even immune function [189-192]. In the hypothalamus, CaMKK2 has been shown to regulate the expression of NPY and AgRP, ostensibly through the activation of AMPK (AMP-activated protein kinase). [193]. Specifically, CaMKK2-deficiency is associated with reduced hypothalamic AMPK activity and downregulation of both NPY and AgRP in NPY neurons [193]. Furthermore, intracerebroventricular injection of a CaMKK antagonist reduced food intake in wild-type

mice but not in CaMKK2-deficient mice, which themselves were resistant to diet-induced weight gain and hyperglycemia [193]. These findings implicate CaMKK2 in the regulation of hunger and appetite by functioning as an AMPK kinase to promote orexigenic signaling in the hypothalamus, perhaps by modulating ghrelin action [194]. AMPK is known to plays a role in cellular energy homeostasis by activating glucose and fatty acid uptake and oxidation when cellular energy is low, and separate studies have indeed demonstrated its regulation by CaMKK2 [195-198].

CaMKK2 has further been implicated in regulating hepatic glucose metabolism and adipocyte differentiation. Acute, liver-specific knockdown of CaMKK2 of HFD-fed mice led to a reduction in blood glucose and improved glucose tolerance. Furthermore, primary hepatocytes from CaMKK2-deficient mice produced less glucose in parallel with decreased expression of gluconeogenic genes, yet also exhibited increased rates of lipogenesis and fatty acid oxidation [199]. In preadipocytes, inhibition or deletion of CaMKK2 enhanced differentiation into mature adipocytes, which could be reversed by activation of AMPK [190]. These findings suggests that CaMKK2 is involved in adipogenesis via AMPK regulation, and further illustrates a role for CaMKK2 in mediating energy homeostasis.

A mechanistic *in vitro* study in differentiated human adipocytes reported that potassium induced membrane depolarization causes an influx of Ca^{2+} that inhibits lipolysis induced by adrenergic receptor activation, adenosine receptor inhibition, adenylyl cyclase activation, and phosphodiesterase (PDE) inhibition, suggesting that calcium may mediate lipolysis through PDEs [200]. The study further demonstrated that the observed inhibition of

lipolysis by increased intracellular calcium was abolished by a non-selective PDE inhibitor and a PDE3b inhibitor, but not by PDE1 or PDE4 inhibitors. Interestingly, the PI3k inhibitor wortmannin only minimally blocked the effect of calcium, suggesting that calcium may activate PDE3b by a mechanism different from that of insulin [200]. Surprisingly, however, the study also found that the antilipolytic effect was not affected by the inhibition of CaM, CaMKs, or calcineurin (CaN; a.k.a. protein phosphatase 2B; PP2b), which suggests that the effect may not rely on calcium-dependent signaling. These findings allude to a role of calcium in mediating lipolysis through PDEs, but the mechanism by which PDEs are activated by calcium is unclear. PDE1s have been well-established as Ca^{2+}/calmodulin-dependent PDEs. The inability of PDE1 inhibitors to block the antilipolytic effect of intracellular calcium was hypothesized to be due to low basal PDE1 expression and/or activity in the differentiated adipocytes, though this was not specifically investigated [200].

Calcium channels play fundamental roles in regulating the concentration of cytosolic Ca^{2+}. Cytosolic calcium rises either by influx through channels of the plasma membrane, such as such as voltage-gated Ca^{2+} channels or transient receptor potential (TRP) channels, and/or by release from intracellular stores, such as the endoplasmic reticulum or mitochondria [186]. Ca^{2+} is removed from the cytoplasm by various types of Ca^{2+} pumps, including the Ca^{2+}-ATPase and Na^{+}/ Ca^{2+} exchanger on the plasma membrane, the sarco/endoplasmic reticulum Ca^{2+} pump (SERCA) on the ER membrane, and the mitochondria Ca^{2+} uniporter on the mitochondria membrane [201-204]. Calcium signaling is also a vital function in the process of adipogenesis. Studies have shown that cytosolic Ca^{2+} is increased in 3T3-L1 preadipocytes

after treatment with Ca^{2+}-ATPase inhibitor thapsigargin, which in turn inhibits adipocyte differentiation [205, 206]. This is consistent with reports of enhanced calcineurin activity inhibiting 3T3-L1 differentiation [207, 208]. Similarly, extracellular Ca^{2+} has been shown mediate adipogenesis, with low extracellular levels promoting and high levels suppressing differentiation of 3T3-L1 preadipocytes [209]. This is consistent with studies in rodents fed a calcium supplemented diet [179-181, 183, 184].

Voltage-gated calcium (CaV) channels are one of the major calcium-permeable channels. CaV channels are known to play roles in the release of neurotransmitters in neurons and in the differentiation and function of adipocytes [210]. Blocking of L-type (high-voltage-activated) CaV channels was shown to inhibit high-extracellular-Ca^{2+}-stimulated adipogenesis of porcine bone-marrow stem cells (BMSCs) [182]. In addition, inhibition of T-type (low-voltage-activated) CaV channels was shown to reduce the proliferation of preadipocytes and HFD-induced weight-gain in mice [211].

1.6.2 Transient Receptor Potential (TRP) Channels

The transient receptor potential superfamily of cation channels constitutes another major class of calcium-permeable channels that are ubiquitously expressed in mammalian tissues, including adipose tissue. TRP channels have a variety of physiological functions, such as detection of various mechanical and chemical stimuli in sensory signal transduction such as vision, hearing, olfaction, taste, touch, pain, and thermo-sensation [212]. The TRP superfamily is further classified into TRPV (Vanilloid), TRPA (Ankyrin), TRPC (Canonical), TRPP (Polycystic), TRPM (Melastatin), TRPN (NomPC), and TRPML (Mucolipin), according

to their primary amino acid sequences [212-215]. Numerous studies and review articles further suggest that several TRP channels are involved in regulating energy homeostasis and adipocyte function, and most of these functions are mediated by alterations in intracellular Ca^{2+} levels or in the activity the calcium signaling pathway [216-225]. Within the context of energy homeostasis and obesity, the most well-studied TRPs are the TRPVs. Although most members of the TRPV family are modestly permeable to Ca^{2+}, they may play different roles in the body due to their differences in structure and distribution.

Since the first report of its cloning in 1997, TRPV1 was the first TRPV identified and has been studied extensively as a potential drug target for a variety of applications [226-229]. Also known as the capsaicin receptor, TRPV1 is associated with pain and temperature regulation. Capsaicin is the active ingredient in chili peppers that produces a sensation of heat and burning in any tissue with which it comes into contact. Its involvement in pain management has been leveraged as a therapeutic mechanism: suppression of nociception and induction of analgesia are achieved by TRPV1 desensitization (such as by topical capsaicin creams) or antagonism [230]. Besides capsaicin, TRPV1 is also activated by temperatures above 42°C [229, 231]. Several studies also confirm that TRPV1 plays a key role in regulating adipocyte function and energy homeostasis: TRPV1 activation by capsaicin induces Ca^{2+} influx, inhibits differentiation, and promotes lipolysis in 3T3-L1 adipocytes [232-234]; Differentiation of 3T3-L1 cells results in elevated TRPV1 expression [234]; TRPV1-deficiency exacerbates obesity and promotes insulin resistance in mice [235]; Chronic dietary capsaicin prevents weight-gain in wild-type, but not TRPV1-deficient mice, even when fed a HFD [232,

233, 236]; Long-term dietary capsaicin mitigates the effects of HFD, promotes weight-loss, and prevents weight-regain in mice [236]; Capsaicin promotes adipocyte browning [237, 238]; Adipose tissue of HFD-fed mice and of humans with obesity have reduced TRPV1 expression [232, 236]. Together, these data from multiple studies corroborate a significant role of TRPV1 in regulating energy homeostasis, and suggest that TRPV1 activation and resulting calcium influx promotes energy expenditure through browning and increased thermogenesis.

However, some studies have reported opposing findings. Knockout of TRPV1 was shown to protect against diet-induced obesity (DIO) and its negative effects on blood pressure and markers of inflammation [239, 240]. Furthermore, TRPV1 antagonists have been shown to improve insulin resistance in mice with diabetes [241]. These contradictory results highlight the complexity of the role TRPV1 in regulating energy homeostasis. However, since TRPV1 ligands can exert potent effects in other tissues, results from dietary intervention may be confounded and thus may not reflect the true function of TRPV1.

TRPV2 shows a 50% sequence homology with TRPV1, yet it cannot be activated by capsaicin. TRPV2 is probably the least-understood member of the TRPV subfamily. Existing data on the role of TRPV2, while limited, suggest that it plays a role in adipocyte function. Sun et al. showed that TRPV2-deficient mice on HFD gain have significantly larger white and brown adipose tissue depots than wild-type mice. Additionally, activation of TRPV2 has been found to suppress differentiation of murine brown preadipocytes and 3T3-L1 preadipocytes [234, 242]. One of these studies, also showed that TRPV2 expression was higher in differentiated 3T3-L1 adipocytes compared to preadipocytes, and also higher in the

subcutaneous WAT of DIO mice [234]. These findings suggest that TRPV2 plays a role in adipogenesis.

TRPV4 is broadly expressed and was first identified as an osmolality sensor. One study reported that the amount of Ca^{2+} entering through a single TRPV4 channel is about 100-fold greater than that of a single L-type CaV channel in smooth muscle tissue, highlighting TRPV4 as a major Ca^{2+} influx channel [243]. Ye et al. conducted a comprehensive investigation into the role of TRPV4 in adipocytes and adipose tissue [244]. The presence of TRPV4 in 3T3-F442A adipocytes was determined as both hypotonicity and TRPV4 agonism induced a TRPV4-dependent increase in intracellular calcium. Knockdown of TRPV4 resulted in greater thermogenic gene expression, elevated adipocyte respiration, and decreased expression of genes related to proinflammatory pathways in 3T3-F442A adipocytes [244]. Ye's group also investigated the *in vivo* role of TRPV4 in metabolism and found that TRPV4-deficient mice were resistant to the effects of HFD after 9 weeks on the diet relative to wild-type mice. Specifically, TRPV4-deficient mice gained significantly less weight, were more glucose and insulin tolerant, and their WAT had greater thermogenic gene expression, decreased proinflammatory gene expression, and greater insulin sensitivity compared to wild-type [244]. Furthermore, preadipocytes isolated from TRPV-deficient mice WAT showed a differentiation efficiency consistent with that of wild-type preadipocytes, and these differentiated primary adipocytes showed a trend in gene expression changes consistent with the results in 3T3-F422A cells [244]. Taken together, these findings suggest that TRPV4-deficiency mitigates the effects of HFD due in part to increased thermogenic gene expression,

and illustrate a role in regulating metabolism that is largely in opposition to most reports of TRPV1 function. However, a later study showed that TRPV4-deficient mice were less active and significantly more susceptible to DIO than wild-type mice [245].

Relative to the previously discussed TRPV channels, TRPV6 is much more selective for calcium. TRPV6 (also known as CaT1) was first cloned from intestinal tissue, where it is highly expressed. Quantitative PCR and immunohistochemistry analyses further showed that TRPV6 is localized in tissues associated with high rates of calcium absorption and transcellular transport, namely highly differentiated epithelial tissues in kidney, pancreas, placenta, mammary tissue, and throughout the gastrointestinal tract [246-249]. A recent gene-expression study of 3T3-L1 cells showed elevated TRPV6 expression after differentiation, though additional evidence for the involvement of TRPV6 in metabolism has been limited [234].

TRPA1 is a member of the Ankyrin subfamily of TRP channels. Like TRPV1, TRPA1 is known to be involved in nociception and thermo-sensing, and has been shown to be co-expressed with TRPV1 in sensory nerve endings [250]. However, it is different in that it is a more versatile "chemo-sensor" in that that can be activated by various naturally occurring chemicals including thymol, menthol, nicotine, gingerol, cinnamaldehyde, allicin, oleuropein aglycone (a major compound in extra virgin olive oil), and allyl isothiocyanate (AITC, the active compound in wasabi, horseradish, and mustard oil). These characteristics led to the TRPA1 being known as the wasabi receptor. Studies have shown that TRPA1 is involved in adipocyte thermogenesis and energy metabolism [251]. Lo et al. found that differentiation of

3T3-L1 preadipocytes was suppressed by AITC, and that dietary administration of AITC to DIO mice on HFD reduced weight-gain, hepatic lipid accumulation, and white adipocyte size [252]. Camacho et al. demonstrated a similar effect of dietary cinnamaldehyde in DIO mice on HFD, with the additional finding of increased expression of genes related to fatty acid beta oxidation [253]. A study in DIO rats showed that dietary and IV-administered oleuropein aglycone enhanced UCP1 (uncoupling protein-1) in BAT and reduce visceral adipose tissue mass by stimulating norepinephrine secretion [254]. Similar results have been reported for AITC and cinnamaldehyde administration [251]. These findings bear resemblance to studies of TRPV1, which taken together suggest that TRPA1 and TRPV1 may regulate energy homeostasis via the sympathetic nervous system.

An abundance of evidence confirms the involvement of TRP channels in regulating metabolism and adipocyte function. Despite some contradictory findings, the majority of reports point to TRPs altering intracellular calcium to affect energy metabolism.

1.6.3 Interactions Between Calcium, cAMP, and Insulin Signaling

cAMP signaling is tightly related to calcium signaling, and their intricate regulation contributes to the activation of a wide array of downstream signaling events. The activity of adenylyl cyclase is regulated by G-proteins (Gαs activates; Gαi inhibits) and forskolin (a direct activator), but can also be both directly (via Ca^{2+} binding) and indirectly (Ca^{2+}/CaM/CaMKs/CaN/PKC) influenced by Ca^{2+} [255]. There are nine membrane-bound adenylyl cyclase (AC) isoforms in mammals (AC1-9), each with isoform-specific regulation by Ca^{2+} and G$\beta\gamma$ (Table 1.1).

Table 1.1 Regulation of AC Isoforms by Ca^{2+}

Isoform	Activation	Inhibition
AC1	Ca^{2+}/CaM or PKC	CaMKs, CaN, Gβγ, or Ca^{2+} binding
AC2	PKC or Gβγ	Ca^{2+} binding
AC3	Ca^{2+}/CaM or PKC	CaMKs, CaN, Gβγ, or Ca^{2+} binding
AC4	no data	PKC or Ca^{2+} binding
AC5	ER-Ca^{2+} depletion-STIM1 binding	Ca^{2+} binding
AC6	ER-Ca^{2+} depletion-STIM1 binding	PKC
AC7	PKC or Gβγ	Ca^{2+} binding
AC8	Ca^{2+}/CaM or PKC	CaN, Gβγ, or Ca^{2+} binding
AC9	CaMKs or PKC	CaN or Ca^{2+} binding
AC10	Ca^{2+} binding	Ca^{2+} binding

AC1, AC3, and AC8 are shown to be activated via Ca^{2+}/CaM or PKC, and inhibited via CaMKs (AC1 & AC3), CaN, Gβγ, or Ca^{2+} binding. AC2, AC4, and AC7 are shown to be activated by PKC (AC2 & AC7) or Gβγ, and inhibited by PKC (AC4) or Ca^{2+} binding. AC5 and AC6 are shown to be activated via ER-Ca^{2+} depletion-STIM1 binding (stromal interaction molecule-1, an endoplasmic reticulum calcium sensor), and are inhibited by PKC (AC6) or Ca^{2+} binding (AC5). AC9 is shown to be activated by PKC or CaMKs, and inhibited by CaN or Ca^{2+} binding. AC10 is the only soluble (non-membrane bound) isoform and is not regulated by G-proteins but is sensitive to Ca^{2+} binding and bicarbonate/pH [255]. In neurons, calcium signaling has also been shown to impact typical cAMP signaling via downstream activation of CREB (cAMP-response element binding protein) through CaMK-mediated phosphorylation of PKA [256-258].

In parallel, cAMP has been shown to modulate calcium signaling activity by regulating Ca^{2+} store release as well as extracellular Ca^{2+} influx. Both IP3 receptors and ryanodine receptors (RyR) have been shown to be phosphorylated by PKA or CaMKII,

resulting in potentiation of IP3-stimulated or RyR-mediated Ca^{2+} release from intracellular stores [259-261]. Another major mechanism of modulating calcium signals by cAMP involves the regulation of plasma membrane channels. The regulation of voltage-gated channels by the cAMP pathway has long been described; in fact, PKA-dependent phosphorylation has been reported to protect voltage-dependent Ca^{2+} channels from the inhibitory role exerted by PIP2 [262]. cAMP/PKA-dependent phosphorylation has also been reported to sensitize TRPV1 and TRPV2 channels *in vitro*. Point mutation of predicted TRPV1 phosphorylation sites greatly reduced forskolin-induced current potentiation relative to wild-type [263]. In contrast, phosphorylation by PKA of rat TRPV1 appears to reduce channel desensitization [264]. TRPV2 is another substrate of PKA, which interacts with the channel through ACBD3 (Acyl CoA binding domain protein-3), an A-Kinase anchoring protein (AKAP), and enhances TRPV2-mediated Ca^{2+} entry in response to heat [265]. These findings demonstrate that AC/cAMP signaling, in part via PKA-dependent phosphorylation, plays a role in the regulation of cytosolic calcium and calcium signaling.

cAMP signaling also plays a major role in regulating metabolism through the PKA-mediated activation of CREB proteins, which make up a family of transcriptional effectors that bind to cAMP-responsive elements in the promoter region of several metabolic genes. Of the CREB proteins, two notable examples are ATF3 and ATF4 (activating transcription factor-3 and -4, respectively), which have been shown by a growing number of studies to be involved in mediating glucose homeostasis and insulin sensitivity [266]. An early showed that, compared to wild-type, Atf3-deficient mice were resistant to DIO and had

better glucose tolerance, but had similar insulin tolerance along with similar insulin-stimulated Akt phosphorylation in adipose tissue and skeletal muscle [267]. Further investigations have focused on the role of Atf3 in pancreatic islet cells, where Atf3 has been shown to regulate the expression of insulin [268]. ATF4 has been similarly described. An early study in mice showed Atf4 expression to be induced by insulin and repressed by glucocorticoids, implicating Atf4 as an anabolic transcription factor [269]. Later, Atf4-deficient mice were shown to have lower percent body fat, resistance to DIO, and higher glucose and insulin tolerance than wild-type controls and the liver and skeletal muscle of these mice displayed greater basal Akt phosphorylation, and also appeared to be more responsive to insulin-induced Akt phosphorylation [270, 271]. Consistent with these findings, more recent studies have demonstrated that Atf4 and its metabolic gene targets are stimulated by mTORC1 via the Pi3k-Akt signaling pathway [272, 273]. These findings highlight the interplay of the cAMP/CREB and insulin signaling pathways within the context of obesity and T2DM, and demonstrate that despite the leaps and bounds in our understanding of these pathways, there was and is much more to learn.

There is a growing body of evidence demonstrating that cAMP, Ca^{2+}-, and insulin-dependent pathways participate jointly and cooperatively in the modulation of cell functions. As previously discussed, a major effect of insulin signaling is the suppression of cAMP action through the activation of PDEs. Calcium signaling may modulate this effect through the Ca^{2+}/CaM-dependent activation of PDE1s to potentiate it or activation of adenylyl cyclase to attenuate it. Alternatively, Ca^{2+}/CaN can dephosphorylate Akt and suppress insulin signaling

to facilitate the activation of cAMP/PKA/CREB signaling, which in turn can be calcium-mediated. As we continue discover more about the interplay between these molecular mechanisms and the cross-talk and feedback between the cascades of these pathways, a clearer picture of metabolism develops, though it also becomes ever more complex.

1.6.4 Modulation of TRP Channels by Cannabinoids

Interestingly, cannabinoids have been known to inhibit N-type voltage-gated calcium channels in cell lines and neurons to mediate neuropathic pain, but more recent studies have further demonstrated that cannabinoids interact with target TRP channels and modulate cellular metabolism [274]. Many diseases involve TRP channel dysfunction, including neuropathic pain, inflammation, respiratory disorders, and obesity. In the pursuit of new treatments for these disorders, it was discovered that TRPV and TRPA channels can be modulated by several endogenous, phytogenic, and synthetic cannabinoids. Upon activation, calcium preferentially moves through the channel, enters the cell, and stimulates a series of calcium-dependent processes that ultimately lead to desensitization of the channel. Upon desensitization, the channel enters a refractory period in which it can no longer respond to further stimulation, leading to the paradoxical analgesic effect of cannabinoid compounds [275].

The first endogenous agonist of TRPV1 to be identified was the endocannabinoid anandamide (AEA) [276]. Additionally, THC, perhaps the most well-known cannabinoid, has been shown to act most potently on TRPV2, moderately on TRPV4 and TRPA1, but is not reported to modulate TRPV1. In comparison, cannabidiol (CBD) has much lower affinity for

the CB1 and CB2 receptors, but is reported to act on TRPV1 [277]. Moreover, the synthetic cannabinoid WIN 55,212-2 is a potent CB1 agonist that has been shown to act on and desensitize TRPV1 and TRPA1 to exert analgesic effects [278].

Based on the history and background laid out in this introduction, there are several hypotheses that have become the object of active research. Regarding the effects of bariatric surgery, it is known that food intake and nutrient absorption are dramatically reduced. However, such a state would typically stimulate insatiable hunger. Yet, bariatric surgery patients commonly report having little hunger or appetite. This thesis aimed to compare the hunger and appetite of sleeve gastrectomy patients before and after surgery to assess with greater resolution the changes in feelings and motivation for food.

With regard to food intake, the endocannabinoid system is a well-known stimulator of appetite for highly-palatable foods. However, it has also come to be seen as a significant player in cellular and molecular metabolism throughout the body. Furthermore, the mechanisms by which the endocannabinoid system functions in peripheral tissues to regulate metabolism are poorly understood. To that end, this thesis aimed to explore the role of cannabinoid action in adipose tissue.

CHAPTER 2:

IMPACT OF VERTICAL SLEEVE GASTRECTOMY ON SUBJECTIVE HUNGER AND APPETITE RATINGS AND ENDOCRINE HORMONES

2.1 Introduction

Recent Centers for Disease Control & Prevention estimates report that over 40% American adults have obesity [4], which confers elevated risk of co-morbidities associated with metabolic syndrome like diabetes, cardiovascular disease, cancer, impaired sleep, and depression [4, 8-12]. It is recommended that adults with obesity adopt traditional lifestyle modifications including exercise, healthy diets, and moderate caloric restriction to manage weight [279]. While these strategies can indeed have a profound impact on metabolic health, weight-loss is modest at best, and long-term weight-loss remains difficult to maintain through these methods alone [5]. Moreover, past pharmacological interventions have largely failed as long-term solutions owing to a host of negative side-effects, contraindications, lack of effectiveness, and poor adherence [23-27]. Thus, bariatric surgery (also known as weight-loss surgery) is widely prescribed for those with class II obesity with co-morbidities and class III, IV, and V obesity, and is extremely effective in reducing body weight and reducing obesity-related risk factors for co-morbidities [40, 41]. The impact of bariatric surgery on body weight is driven by physical alteration of the gastrointestinal tract to: restrict food intake through partial resection of the stomach by vertical sleeve gastrectomy (VSG) surgery, and decrease caloric absorption through intestinal shortening by the Roux-en-Y Gastric Bypass procedure [280, 281]. These anatomical modifications facilitate a marked reduction in caloric

intake while also resulting in a significant change in the secretion of gut hormones [282]. Remarkably, improvements have been observed in as little as 72 hours after surgery to hormones responsible for regulating energy homeostasis as well as hunger and appetite [283].

A major axis expected to be perturbed following VSG is the regulation of energy homeostasis by the endocrine hormones: leptin and ghrelin. Leptin is secreted predominantly by adipose tissue in proportion to the amount of lipids stored [284], and is also produced by epithelial chief cells in the lower half of the gastric mucosa fundus [285]. Leptin has a wide repertoire of peripheral effects, mediated indirectly through the central nervous system as well as via direct actions on target tissues. In contrast, ghrelin is secreted in pulses across the day from specialized cells in the stomach and the upper small intestine [286, 287]. The regulatory axis of these two hormones is considered to be exerted through the regulatory role of activated acyl-ghrelin to, respectively, inhibit or stimulate satiety through differential activity of the melanocortin-4 receptor [288, 289]. Progressive weight-loss through caloric restriction has been observed to lead to a decline in the levels of serum leptin as a result of decreased adipose tissue mass [79]. Conversely, VSG, despite the caloric restriction, has been shown to lower serum ghrelin levels at the short-term and long-term follow-up, and it has been postulated that a majority of ghrelin cells are removed during surgery [290, 291]. The simultaneous decline of both hormones in the axis leads to an unclear understanding of how hunger and appetite may be affected by VSG surgery. With that said, it is still not completely clear if bariatric surgery impacts hunger and appetite [292].

Hunger is thought to be driven more by homeostatic endocrine signaling mechanisms, while appetite is understood to be motivated more by hedonic reward pathways. Bariatric surgery may alter the hedonic or rewarding properties of food and that may contribute to changes in hunger, appetite, food preference, and actual consumption [293-295]. The decline in food intake following VSG may also result from the combination of food capacity restriction and alterations in circulating endocrine hormones in opposition to food intake in line with reports confirming overall increased satiety and reduced appetite following bariatric surgery [296, 297].

The purpose of the current study was to examine the relationship between active weight-loss, subjective ratings of hunger and appetite across a day, and fasting levels of the hormones leptin and ghrelin in women undergoing laparoscopic VSG surgery. At the time of the study, VSG was the most common bariatric surgical procedure performed in the hospital, and women accounted for 80% of the patients undergoing the procedure. We aimed to determine how subjects' baseline and post-surgical ratings of hunger and appetite differ throughout the day after VSG surgery compared to fasting leptin and ghrelin levels. We hypothesized that, at twelve weeks post-surgery, before sustained weight loss has been achieved, subjective ratings of hunger and appetite would be reduced overall compared to pre-surgical baseline ratings. In addition, we examined the influence of fasting ghrelin activity and caloric intake on subjective hunger and appetite ratings, and hypothesized that these ratings would be impacted less by ghrelin activity and more by caloric intake.

2.2 Materials and Methods

The studies were approved by the Institutional Review Board of The University of Chicago (IRB#14-0984), and all participants were compensated for their participation. All participants provided written informed consent in accordance with the Declaration of Helsinki.

2.2.1 Subject Characteristics

Screening of potential subjects and the process of enrollment into the clinical study was conducted as previously described [298]. VSG was the most common bariatric surgical procedure performed at the University of Chicago and so we only enrolled those underdoing this type of bariatric surgery. Further, women accounted for 80% of patients undergoing the procedure and thus we have only included women in the current study in order to achieve sufficient power for analysis. Fourteen women with class II obesity with co-morbidities or class III, IV, or V obesity were voluntarily enrolled following approval and scheduling for laparoscopic VSG surgery. Subjects were screened for the absence of malignant physiological or endocrine conditions, pregnancy, and irregular lifestyles. Sleep was monitored by polysomnography to rule out sleep-related disorders and to ensure obstructive sleep apnea was, at minimum, adequately controlled with continuous positive airway pressure. Deidentified subject characteristics can be found in Table 2.1.

Table 2.1 Deidentified Subject Characteristics

Subjects	N = 10	Mean Age (years)	37 ± 10.1	
Race	8 AA, 2 NHW	Mean Height (cm)	168.4 ± 4.6	
Biometric	**Timepoint**	**Mean**	**SEM**	**p-value**
Weight (kg)	Baseline	129.9	± 5.1	
	Post-surgery	109.5	± 5.3	1.2E-09
BMI (kg/m^2)	Baseline	45.6	± 1.8	
	Post-surgery	38.3	± 1.9	1.2E-08
Waist:Hip	Baseline	0.924	± 0.021	
	Post-surgery	0.886	± 0.030	0.28
Body Fat (%)	Baseline	53.5	± 0.8%	
	Post-surgery	48.5	± 1.2%	2.4E-05

AA: African-American; NHW: Non-Hispanic White. Height and age were measured at intake screening appointment. Weight was measured with subject vitals at each intake appointment. Subject BMI, waist:hip ratio, and percent body fat mass were recorded the mornings of baseline and post-surgery laboratory sessions. Statistical significance calculated by two-tailed paired T-Test.

2.2.2 Laboratory Sessions

Subjects were admitted to the University of Chicago Medicine Clinical Resource Center at 1-2 weeks prior to the scheduled surgery date (baseline) and 12-13 weeks following surgery (post-surgery). Individuals were studied at 12-13 weeks post-surgery because at this time point there are no longer post-surgery food restrictions (patients can again eat whatever food types they like) and during active weight loss, rather than after a stable new weight has been achieved. Height and age were recorded at baseline screening appointments. Weight was recorded with subject vitals at both intake appointments. Bioimpedance was assessed using the Quantum X BIA analyzer validated in this population as previously described [299]. BMI, percent body fat mass, and waist:hip ratios were recorded during the morning of baseline and post-surgery in-lab sessions. In-lab sessions were structured as previously

reported [298]. During in-lab sessions, four isocaloric mini-meals were provided per day. Total calories offered to each subject during baseline sessions was determined according to the Mifflin-St. Jeor equation [300]. At post-surgery lab sessions, all subjects were offered 1000 kcal per day distributed over four mini-meals of 250 kcal each. While the pre-surgery meals were calculated and presented based on individual caloric need, the fixed caloric content of the post-surgery meals was presented based on post-surgical guidelines. At 12 weeks post-surgery, patients are instructed to eat around 1000 kcal per day, thus our presentation of four mini-meals of 250 kcal each. A registered dietitian from the Clinical Resource Center Metabolic Kitchen supervised the preparation of all meals. Subjects were not allowed to consume any foods or beverages that were not provided by the metabolic kitchen, and were given thirty minutes to eat. If the entire meal was not consumed, leftover food was weighed to determine calories and macronutrients consumed (Table 2.2). For both sessions, mini-meals were served at 0900, 1230, 1530, and 1900 hours.

2.2.3. Fasting Serum and Plasma Hormone Levels

Morning fasting blood samples were collected by nursing staff in gold top, serum separator tubes. Acylated ghrelin and total ghrelin were measured via ELISA (Millipore Sigma EZGRT-89K, EZGRA-88K) with a lower limit of detection of 8 pg/mL and CV of 3.5-6.6%. Leptin was measured via radioimmunoassay (Millipore Sigma HL-81K) with a lower limit of detection of 0.5 ng/mL and CV of 3.6-6.2%.

2.2.4. Assessment of Hunger & Appetite

During laboratory sessions, subjects completed four validated, computerized visual

analog scales (VAS) to assess hunger and appetite at eight time points: 25 minutes before, and 1hour and 35 minutes after each of the four mini-meals. With computerized VAS (0cm, "Not at all", to 10cm, "Extremely" or "A very large amount"), subjects scored their hunger and appetite based on the following questions: 1) "How hungry do you feel right now?", 2) "How thirsty do you feel right now?", 3) "How full does your stomach feel right now?", 4) "How strong is your desire to eat right now?", 5) "How much do you think you could eat right now?", and 6) "How nauseated do you feel right now?".

To assess appetite at each time point, subjects used a computerized VAS (0cm, "Not at all", to 10cm, "Very much") to rate how much they would enjoy eating foods, at the time of the questionnaire, from 7 different categories. The appetite questionnaire was prompted as follows: "Please answer the following questions based on how you feel at this very moment. Based only on your appetite right now, without concern for calories, fat or a 'healthy diet', how much would you enjoy eating foods from each of the following 7 categories?: 1) SWEETS such as cake, candy, cookies, ice cream, pastry; 2) SALTY FOODS such as chips, slated nuts, pickles; 3) STARCHY FOODS such as bread, pasta, cereal, potatoes; 4) FRUITS & FRUIT JUICES; 5) VEGETABLES; 6) MEAT, POULTRY, FISH, OR EGGS; 7) DAIRY PRODUCTS such as milk, cheese, or yogurt." Composite appetite results were computed arithmetically by summation of the respective basic appetite categories. Composite appetite categories were: A) Global Appetite: all 7 categories; B) High Carb-High-Fat: categories 1 & 2; C) High Carb-Low-Fat: categories 3, 4, & 5; D) Sweet & Starchy: categories 1 & 3; and E) High Protein: categories 6 & 7.

2.2.5. Mixed Model Construction

Mixed modeling was utilized to estimate the degree of difference in ratings between baseline and post-surgery. Model fitting was conducted using the R package *lme4* [301]. In order to ensure that the dependence derived from time series collection was accounted for in the statistics, the random vector was a simple intercept set as the Subject Identification Number. Experimental ratings were unaltered at entry into the data set.

The surgery-associated parsimonious model was calculated by using surgery as a binomial variable (Pre [0], Post [1]) and iterative inclusion of factors representing potential corrections for: 1) age (integer, years), 2) pre-surgery weight loss, 3) post-surgery weight loss, or 4) time of day. Given the known effect of meals to affect hunger and appetite, we represented time of day as the best combination of factors including 1) prandial (binomial, pre-meal [0], post-meal [1]); and 2) time point (integer, {1,2, ..., 8}), meal (integer, {1,2,3,4}), latter part of day (binomial, first half [0], latter half [1]). To allow for correction by weight loss, it was held constant for all respective points of a given subject. An enhanced model was chosen by: 1) an improved Akaike Information Criterion of the N+1 model (compared to N model without the addition of the factor), 2) each factor effect has low Type S error rate ($p < 0.05$), and low inflation of standard error of estimation (< 50%).

2.3 Results

2.3.1. Laboratory Session Calorie & Macronutrient Intake

Ten of the fourteen women enrolled in the study completed a minimum of 5 out of 8 surveys during both baseline and post-surgery lab sessions to be analyzed in the study.

Following screening, subjects participated in two lab sessions: 1-2 weeks before (baseline) and 12-13 weeks after VSG surgery. Post-surgery, subjects consumed on average significantly fewer calories per day than at baseline (p = 2.7E-06), and also fewer than the 1000 kcal offered. Average baseline quantities of protein, carbohydrates, and fat consumed represent, respectively, 18.7%, 50.1%, and 32.2% of average total calories at baseline. Post-surgery average quantities of protein, carbohydrate, and fat consumed represent, respectively, 32.3%, 41.1%, and 26.6% of average total calories at post-surgery. Total macronutrient intake was significantly decreased post-surgery (p = 9.5E-07, 1.7E-06, and 2.3E-05 for protein, carbohydrate, and fat, respectively; Table 2.2).

Table 2.2 Laboratory Session Diet & Food Consumption Information

Macronutrient	Timepoint	Mean	SEM	p-value
Total Food Eaten (g)	Baseline	1920	± 109	
	Post-surgery*	583	± 76	9.7E-08
Calories (kcal)	Baseline	2223	± 168	
	Post-surgery*	591	± 90	2.7E-06
Protein (g)	Baseline	103.8	± 4.9	
	Post-surgery*	48.0	± 4.6	9.5E-07
Carbohydrate (g)	Baseline	278.9	± 22.4	
	Post-surgery*	60.7	± 9.5	1.7E-06
Fat (g)	Baseline	79.5	± 7.3	
	Post-surgery*	17.5	± 4.6	2.3E-05

Four isocaloric meals were prepared for each subject for each day of laboratory sessions. Proportion of macronutrients matched between otherwise identical laboratory sessions at baseline or post-surgery. Total calories offered at baseline were calculated according to the Mifflin-St. Jeor equation. Subjects were given thirty minutes to eat. Leftover food was weighed to calculate calories consumed. Statistical significance calculated by two-tailed paired T-Test. *One subject received one incorrect meal the night before the full day of surveys; this data is included in the analysis.

One subject was provided an incorrect meal for their first post-surgery in-lab dinner (the night before the full day of surveys that are included in the study). Calories eaten for this meal was approximately 450kcal (~200 more kcal than intended). Data for this subject and this meal were not excluded from the analysis since calories eaten was still greatly reduced relative to corresponding pre-surgery data. We did not see an effect from removal of this data; in all cases post-surgery calories eaten was greatly reduced relative to baseline, so the observed trend was affected only somewhat. Moreover, morning fasting ratings from this subject were consistent with overall observed trends thus confirming our decision to include this individual in subsequent analysis.

2.3.2. Subjective Ratings of Hunger & Appetite

Surgery Effect

The independent effect of surgery on subjective ratings of hunger and appetite was estimated using corrected linear mixed-modeling. Surgery was revealed to significantly decrease ratings of appetite for high-carbohydrate, low-fat (HCLF) foods ($p = 0.018$) and of global appetite ($p = 0.038$; Figure 2.1A). Specifically, appetite ratings were significantly decreased for protein ($p = 0.047$), fruits ($p = 2.7e\text{-}4$), and vegetables ($p = 0.023$; Figure 2.1B). With regard to subjective ratings of hunger, surgery was revealed to significantly decrease ratings of thirst ($p = 5.4e\text{-}3$) and quantity can eat ($p = 1.6e\text{-}3$), and significantly increased ratings of nausea ($p = 0.046$; Figure 2.1C).

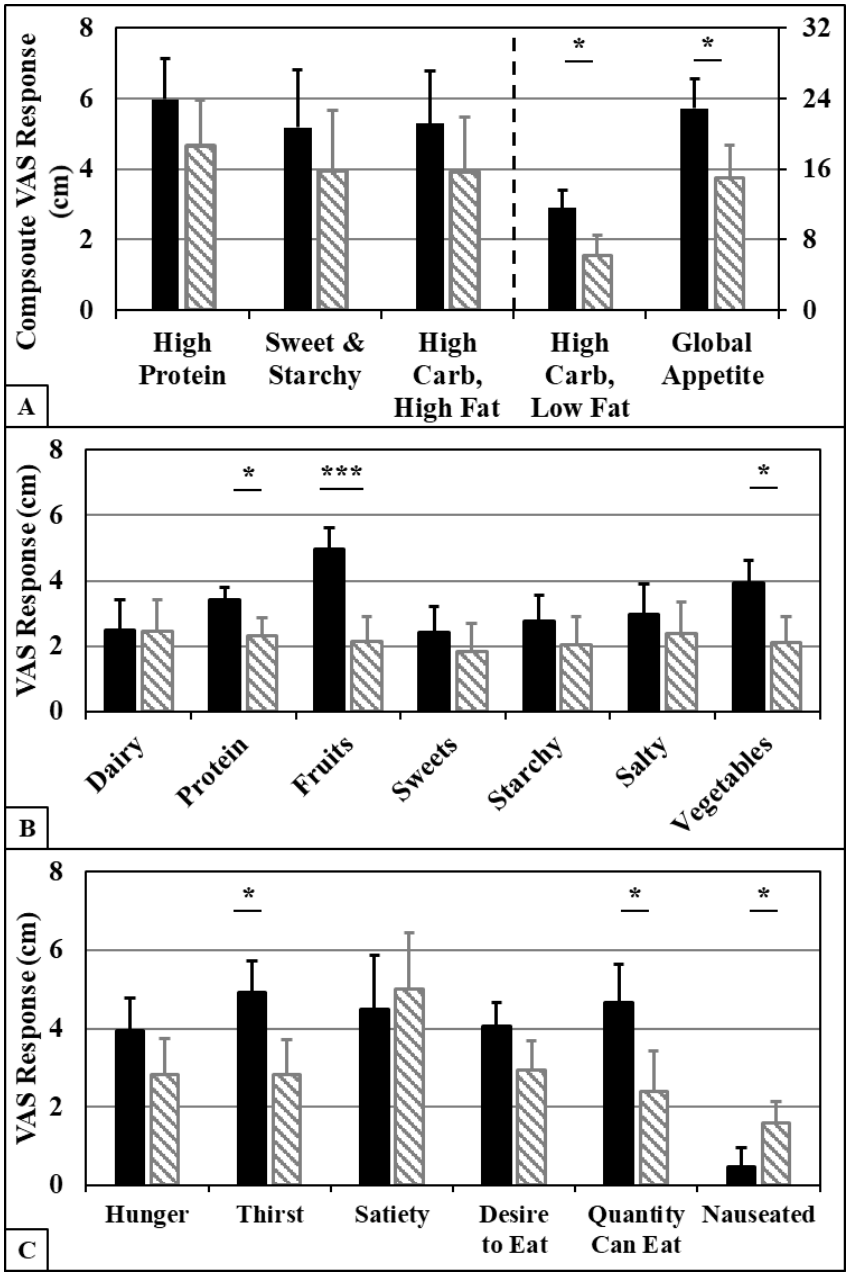

Figure 2.1. Hunger and appetite ratings at baseline and at post-surgery.
(A) Composite Appetite Ratings. **(B)** Subjective appetite ratings for various food groups. **(C)** Subjective hunger ratings. Survey responses were collected before and after each of four isocaloric mini-meals at baseline (black) and twelve weeks following surgery (grey stripes) for each subject (n=10). Average estimated survey responses for each survey question were estimated through mixed linear modeling with subject ID as the random intercept and the effect of surgery assigned as a fixed binomial parameter. Models corrected for effects of age, weight loss before or after surgery, and time of day relative to meals if present. Composite appetite ratings were summated, and then modelled separately. Significance of differences were determined by two-tailed t-tests with propagated parameter uncertainties. P-values were, respectively: * < 0.05, ** < 0.01 and *** < 0.001.

Weight-loss

The interaction of post-surgery weight-loss (as a percent of baseline) with subjective ratings of hunger and appetite was evaluated using linear mixed-modeling. Relative to unchanged ratings, greater weight-loss was predicted by significant decreases in average ratings of global appetite ($p = 1.1e-4$), appetite for high-carbohydrate, high-fat (HCHF) foods ($p = 1.9e-4$), HCLF foods ($p = 1.9e-4$), fruits & sweets ($p = 2.2e-4$), and high-carbohydrate foods ($p = 6.8e-6$; Figure 2.2A). Specifically, greater weight-loss was predicted by changes in average ratings of appetite for fruits ($p = 2.2e-3$ 0.01) and for sweets ($p = 0.043$; Figure 2.2B). With regard to subjective ratings of hunger, greater weight-loss was predicted by changes in both quantity can eat ($p = 6.7e-4$) and nausea ($p = 0.05$; Figure 2.2C).

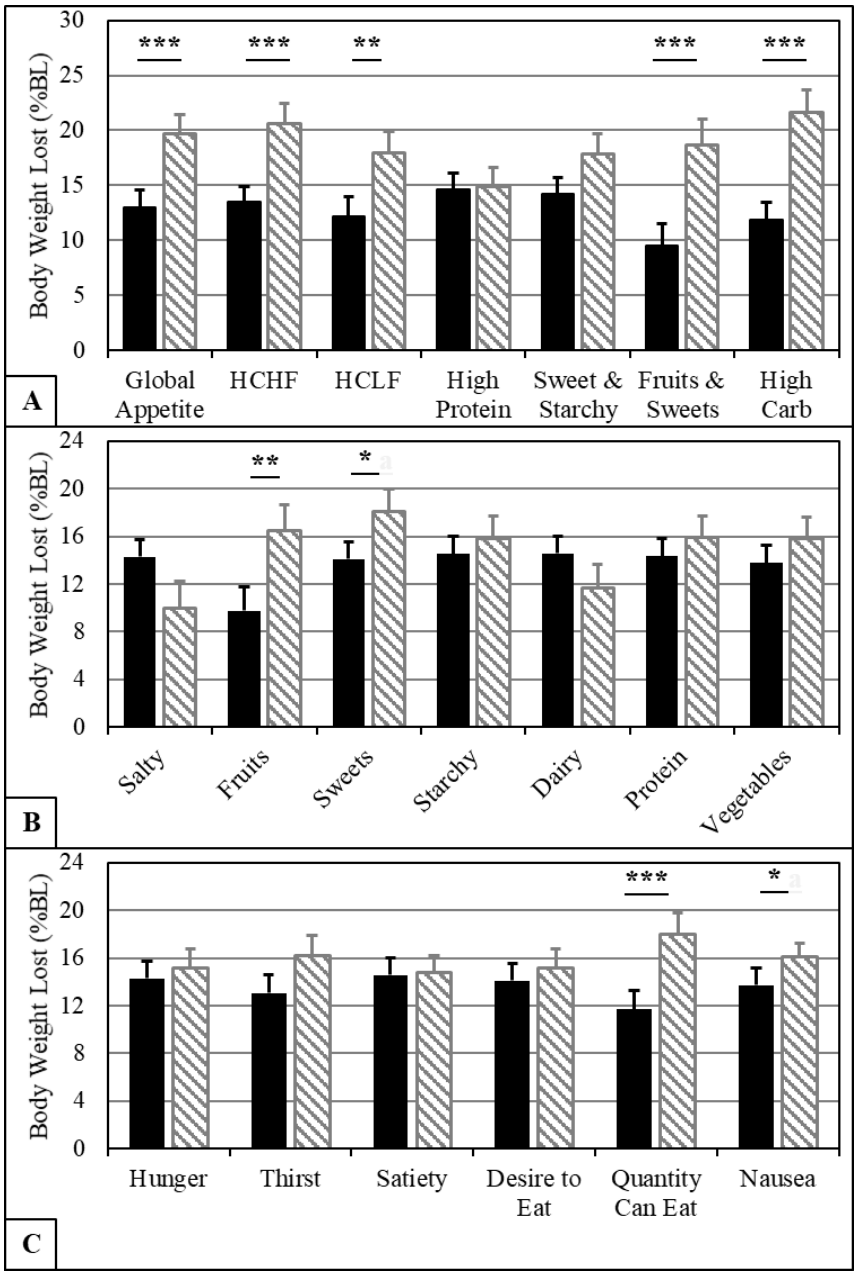

Figure 2.2 Influence of change in hunger and appetite ratings on post-surgery weight loss
Correlations between change in subjective ratings of (**A**) Composite Appetite, (**B**) Various food groups, and (**C**) Hunger with weight-loss. Data from eight surveys during first day of identical laboratory sessions at baseline and after surgery. Estimated effects were computed using mixed linear modeling. Models were parameterized with random factor of Subject ID, and the univariate fixed model representing the change in response between baseline (black) to post-surgery (grey stripes) to the respective question. Significance was determined by two-tailed t-tests with propagated error rates comparing predicted weight-loss at post-surgery associated with unchanged baseline ratings to the estimated weight loss at post-surgery associated with the actual post-surgery ratings. P-values were, respectively: * < 0.05, ** < 0.01, and *** < 0.001.

Calories Eaten

The correlative relationship between subjective ratings of hunger and appetite and calories eaten was estimated using linear mixed-modeling. A significant decrease in average ratings of appetite for HCLF foods was predicted by the decrease in calories eaten ($p = 0.028$; Figure 2.3A). Specifically, this decrease was in ratings of appetite for fruits ($p = 5.0e\text{-}3$; Figure 2.3B). The decrease in calories eaten was also estimated to predict a significant decrease in ratings of quantity can eat ($p = 7.6e\text{-}4$) and a significant increase in ratings of nausea ($p = 0.034$; Figure 2.3C).

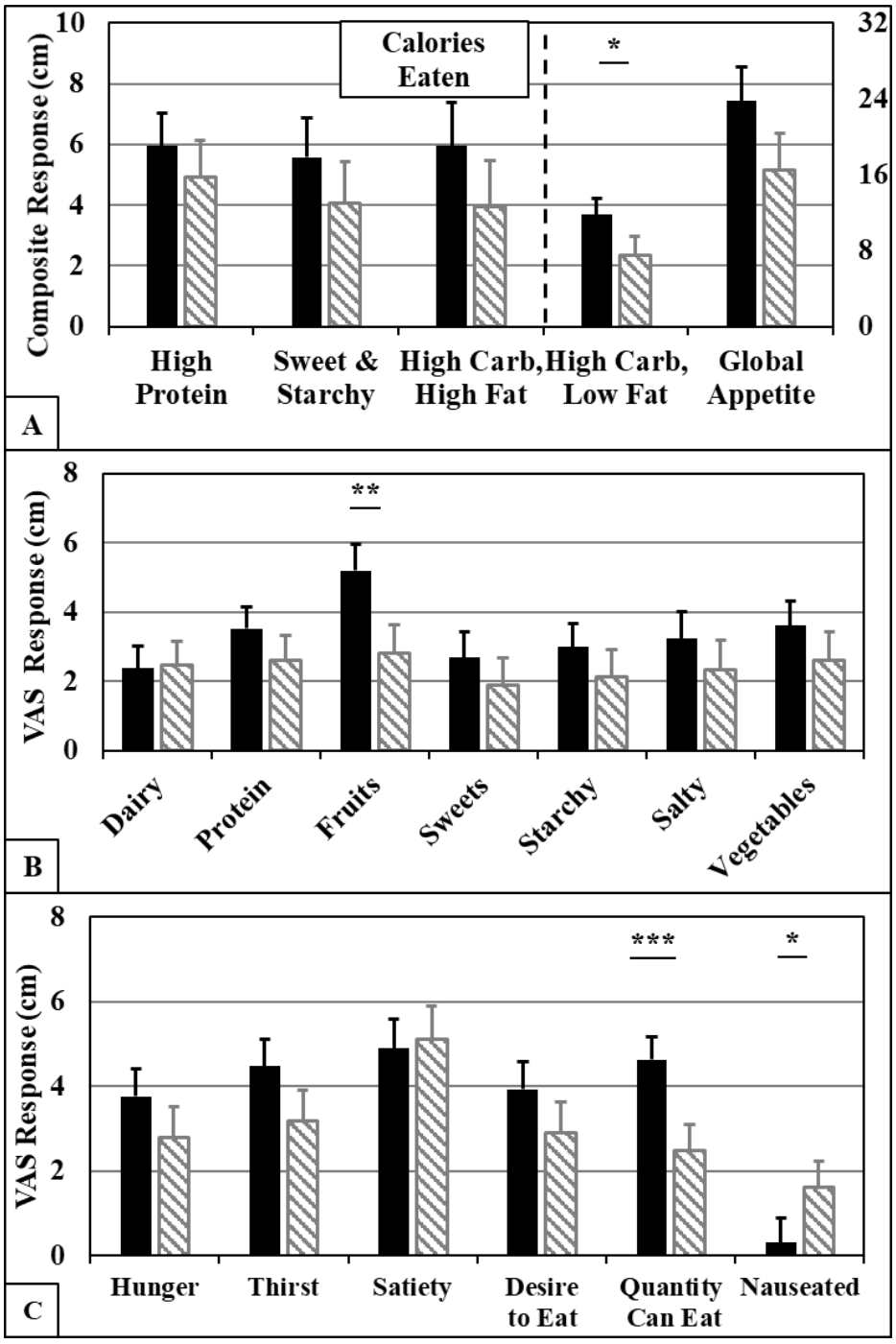

Figure 2.3 Influence of calories eaten on hunger and appetite ratings

Correlations between subjective ratings and calories eaten. Data from eight surveys during first day of identical laboratory sessions at baseline and after surgery. Estimated effects were computed using mixed linear modeling. Models were parameterized with random factor of Subject ID, and a univariate fixed effect based on four-meal average of calories eaten during each laboratory session. Significance determined by two-tailed t-tests with propagated error rates comparing mean VAS responses at baseline (black) to mean VAS at post-surgery (grey stripes). P-values were, respectively: * < 0.05, ** < 0.01 and *** < 0.001.

Survey Responses Across Time

The influence of time across the day on subjective ratings of hunger and appetite was estimated using linear mixed-modeling. Time was revealed to be a significant predictor of changes in ratings of appetite for high protein (HP) foods ($p = 2.9e-4$), sweet & starchy (SS) foods ($p = 0.046$), HCLF foods ($p = 7.9e-4$), and of global appetite ($p = 6.5e-4$; Figure 2.4A). Specifically, later-in-the-day surveys could predict decreases in ratings of appetite for dairy ($p = 0.017$), protein ($p = 5.5e-6$), fruits ($p = 0.023$), starchy ($p = 5.2e-3$), salty ($p = 0.045$), and vegetables ($p = 9.4e-4$; Figure 2.4B). In parallel, time was revealed to predict significant decreases in ratings of hunger ($p = 1.1e-4$), thirst ($p = 0.020$), desire to eat ($p = 7.4e-4$), and quantity can eat ($p = 1.2e-3$). Time across the day was also shown to predict a significant increase in ratings of Satiety ($p = 1.2e-6$), and no significant change in ratings of nausea (Figure 2.4C).

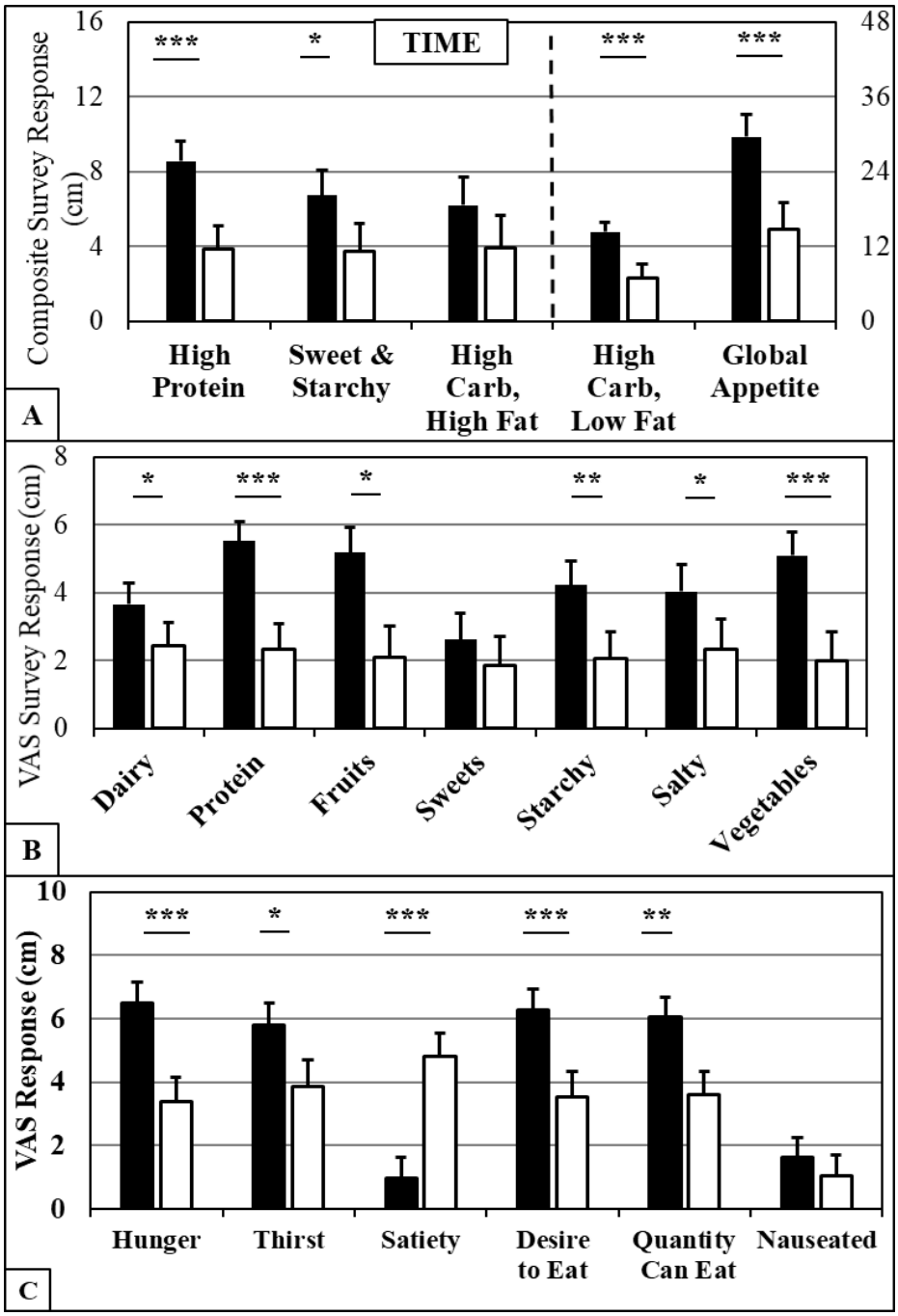

Figure 2.4 Influence of time on hunger and appetite ratings

Correlations between time across day and subjective ratings of (**A**) Composite appetite, (**B**) Appetite for various food groups, and (**C**) Hunger. Data from eight surveys during identical laboratory sessions at baseline and after surgery are represented. Estimated effects were computed using mixed linear modeling. Models were parameterized with random factor of Subject ID, and bivariate fixed effects (meal, prandial). Significance was determined by two-tailed t-tests with propagated error rates comparing responses from before the first meal (i.e., fasting; black) to responses after the last meal (i.e., end of day; white). P-values were, respectively: * < 0.05, ** < 0.01, and *** < 0.001.

Survey Responses at Baseline & Post-Surgery: Hunger

Significant changes in the difference between average ratings at baseline and post-surgery were observed for the hunger categories of hunger ($p = 8.6e-3$), thirst ($p = 0.013$), satiety ($p = 0.041$), and quantity can eat ($p = 7.0e-5$; Figure 2.5A). Specifically, the significant differences between ratings were observed early in the day (i.e., before the first meal; Fasting), with no significant differences observed between ratings after the last meal (Last; Figure 2.5B-G).

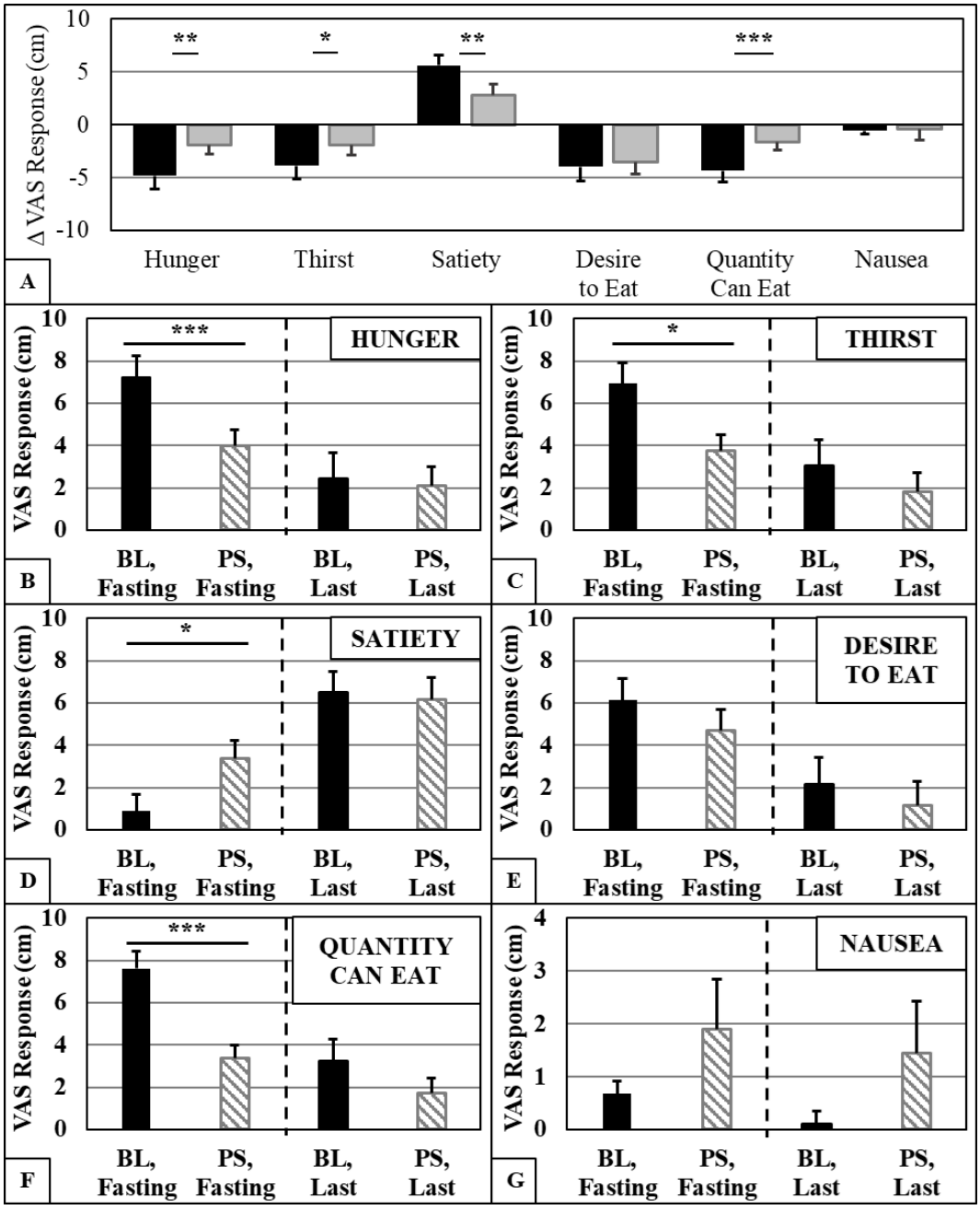

Figure 2.5 Analysis of hunger survey responses across time at baseline or post-surgery
Survey responses were collected before and after each of four isocaloric mini-meals at baseline (black, BL) and twelve weeks following surgery (solid grey or grey stripes, PS). (**A**) The change in ratings of each estimated survey response from before the first meal to after the last meal is plotted. Significance of differences were determined by two-tailed t-test on the absolute change in responses. (**B-G**) Average responses were computed to estimate survey responses from before the first meal (Fasting) compared to after the last meal (Last). Mixed linear modeling was used to analyze each session data separately (n=10). Significance of differences were determined by two-tailed t-test. P-values were, respectively: * < 0.05, ** < 0.01, and *** < 0.001.

Survey Responses at Baseline & Post-Surgery: Appetite

Significant decreases in ratings of appetite at baseline and post-surgery were observed for the categories of: global appetite ($p = 1.3e-3$), HCLF ($p = 1.6e-4$), HP ($p = 8.1e-5$; Figure 2.6A). The specific food types for which appetite ratings were significant decreased were starchy ($p = 2.4e-3$), dairy ($p = 0.034$), protein ($p = 2.5e-4$), and vegetables ($p = 1.3e-3$; Figure 2.6B). As with hunger survey responses, significant differences between ratings at baseline and post-surgery were observed early in the day, with the addition that ratings of appetite for fruits were also significantly different after the last meal (Figures 2.6C-N).

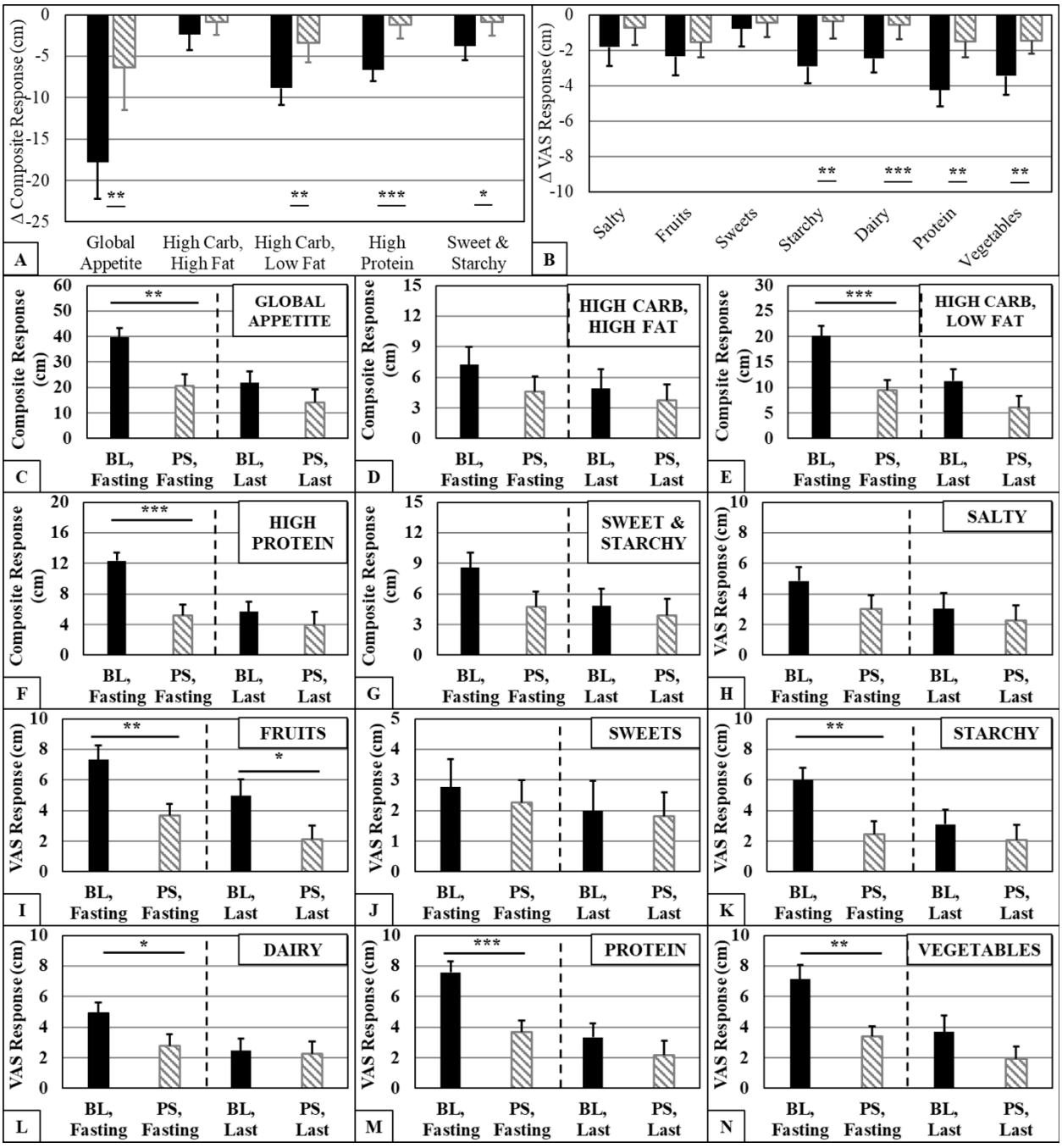

Figure 2.6 Analysis of appetite survey responses across time at baseline or post-surgery
Survey responses were collected before and after each of four isocaloric mini-meals at baseline (black, PS) and twelve weeks following surgery (grey stripes, PS). (**A, B**) The change in each estimated survey response from before the first meal until after the last meal is plotted. Significance of differences were determined by two-sided t-test on the absolute change in either simple or composite responses. (**C-N**) Average responses were computed to estimate fasting responses, before the first meal, and after the last meal. Mixed linear modeling was used to analyze each session data separately (n=10). Significance of differences were determined by two-tailed t-test. P-values: * < 0.05, ** < 0.01, and *** < 0.001.

2.3.3. Hormone Levels

Serum Leptin & Ghrelin

Fasting serum levels of leptin and ghrelin were assessed in 8 of the 10 subjects with subjective ratings analyzed. The percent of fasting acylated ghrelin was significantly increased post-surgery relative to baseline ($p = 0.014$; Figure 2.7A). In parallel, fasting serum leptin was found to be significantly decreased post-surgery ($p = 6.7e\text{-}4$; Figure 2.7B). Linear modeling revealed that post-surgery percent weight-loss was correlated with the changes in: fasting serum leptin ($p = 1.5e\text{-}10$), the fasting percent ratio of acylated to des-acylated ghrelin ($p = 8.3e\text{-}3$), and calories eaten ($p = 5.8e\text{-}4$; Figure 2.7C. The ratio of fasting leptin to total fasting ghrelin was significantly decreased post-surgery relative to baseline ($p = 0.030$; data not shown).

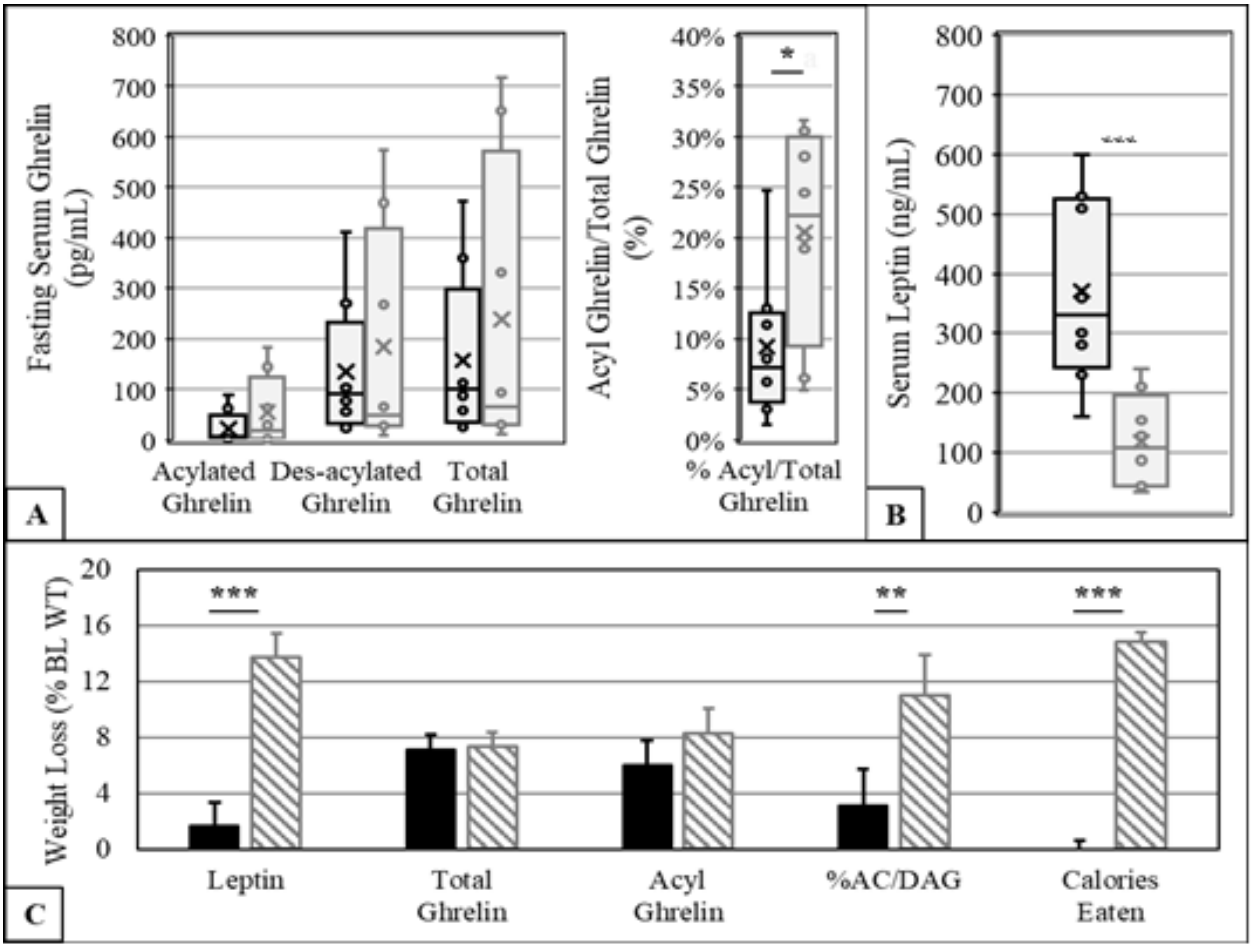

Figure 2.7 Fasting serum levels of ghrelin and leptin

Fasting serum was collected (N = 8) during the morning prior to the first meal at baseline and at twelve weeks post-surgery. Hormones measured were: acylated ghrelin and total ghrelin via ELISA (Millipore Sigma EZGRT-89K, EZGRA-88K), and leptin via radioimmunoassay (Millipore Sigma HL-81K). (**A&B**) fasting serum hormone levels at baseline (black), or post-surgery (grey or grey stripes). Significance of differences was determined by two-tailed paired t-test with propagated error rates comparing hormone levels at baseline and at post-surgery. (**C**) Linear modeling to estimate percent weight lost using respective single fixed factor of respective hormone levels, the ratio of acyl-ghrelin to total ghrelin, or calories eaten across four meals of laboratory session at baseline (black) or post-surgery (grey stripes). Significance of differences was determined by two-tailed paired t-test with propagated error rates comparing estimated weight loss at post-surgery associated with unchanged baseline levels or with the average of actual post-surgery levels of the respective measures. P-values were: * <0.05, **, <0.01, *** <0.001.

Hunger & Appetite

The relationships between serum leptin and ghrelin levels and subjective ratings of hunger and appetite were estimated using linear mixed-modeling. Specifically, given average hormone levels at baseline and post-surgery and the association derived from the trained model, the expected survey ratings were approximated. As predicted, with weight loss and decreased percent body fat mass following VSG surgery, there was a decline in circulating leptin levels 12 weeks after surgery. The only significant difference between average baseline and post-surgery ratings that was associated with the decrease in serum leptin was for Quantity Can Eat (Figure 2.8A-C). Furthermore, no significant differences between average baseline and post-surgery ratings of hunger and appetite were predicted by the increase in the percent ratio of acylated ghrelin to des-acylated ghrelin (%AG/DAG; Figure 2.8D-F).

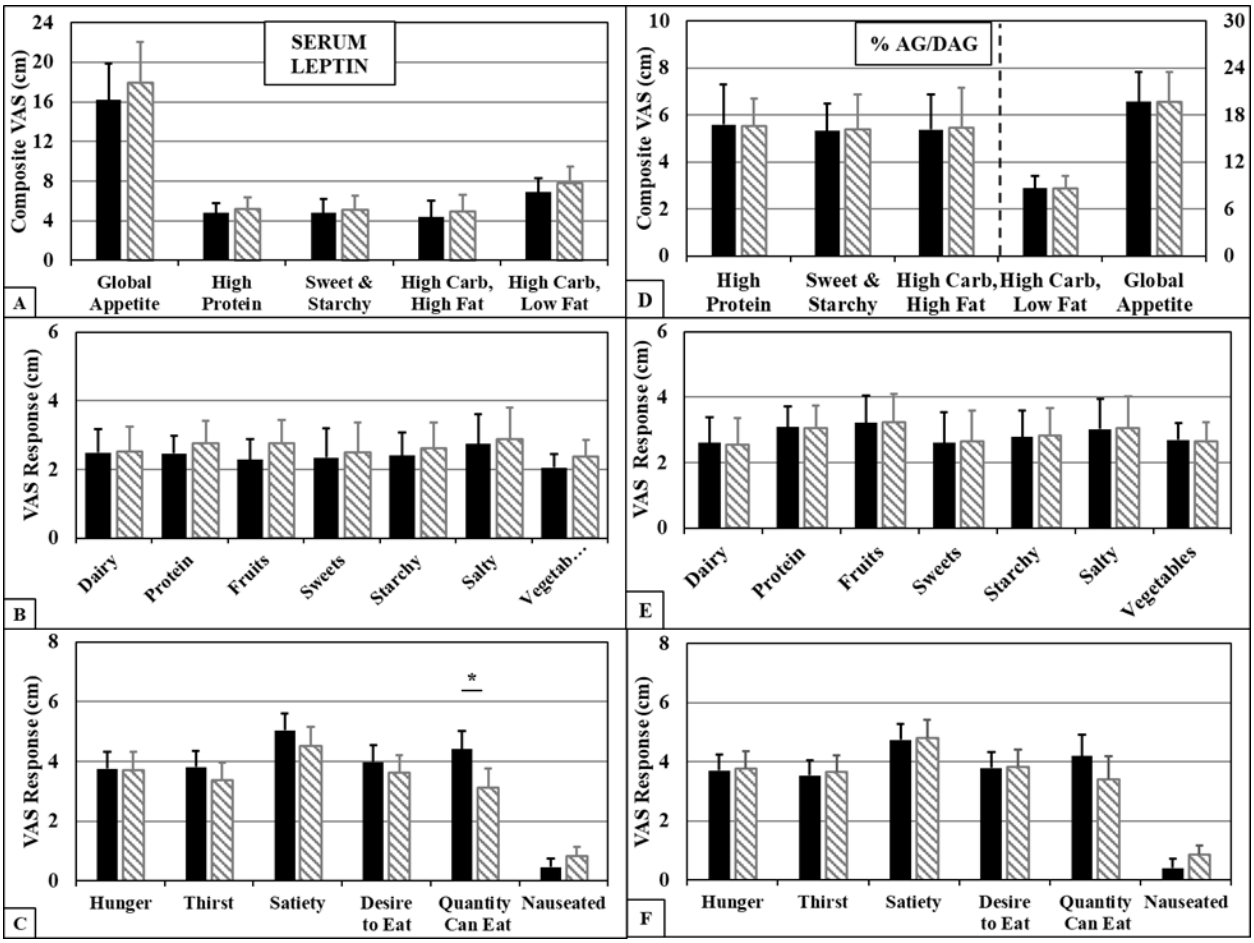

Figure 2.8 Influence of serum leptin and active ghrelin activity on hunger and appetite
Correlations between subjective ratings and serum leptin or serum active ghrelin activity. Data from eight surveys during the first day of identical laboratory sessions at baseline and after surgery. Estimated effects were computed using mixed linear modeling. Models were parameterized with random factor of Subject ID, and a univariate fixed effect based on: (**A-C**) fasting serum leptin and (**D-F)** percent ratio of acyl-ghrelin to total ghrelin in fasting serum. Significance determined by two-tailed t-tests with propagated error rates comparing mean VAS responses at baseline (black) to mean VAS responses at post-surgery (grey stripes). P-values were, respectively: * < 0.05, ** < 0.01 and *** < 0.001.

2.4 Discussion

The current study measured changes in subjective ratings of hunger and appetite in women with obesity undergoing VSG surgery in parallel with endocrine hormones involved in the homeostatic control of hunger. As expected, subjects lost significant weight, BMI, and body fat mass in the twelve weeks following VSG surgery, and these changes were strongly

correlated with reduced caloric intake as participants consumed an average of only 26.5% of the average calories consumed at baseline. However, all subjects still had obesity at twelve weeks post-surgery. In addition, while total macronutrient intake fell, protein consumption as percent of total calories was higher post-surgery, whereas carbohydrate and fat consumption as percent of total calories decreased. It is recommended for bariatric surgery patients to consume more protein relative to carbohydrate and fat in order spare lean body mass during caloric deficit, thus this finding was expected. Relative to baseline, post-surgery fasting serum levels of the hormones leptin and ghrelin were altered in favor of increased food intake, despite subjective ratings of hunger and appetite being reduced overall. As expected, fasting leptin was found to be significantly reduced post-surgery, and this change was significantly associated with a decrease in ratings for Quantity Can Eat. With regard to fasting ghrelin activity, as expressed by the percent-ratio of acylated-ghrelin to des-acylated ghrelin, %AC/DAG was significantly increased post-surgery, but this increase was not significantly associated with any changes in subjective ratings. In addition, a majority of baseline ratings of hunger and appetite were found to decline over the course of the laboratory session, but twelve weeks after surgery most ratings of hunger and appetite were found to be relatively constant over the course of the laboratory session.

We also found that, relative to baseline values, greater weight loss was associated with decreases in particular ratings of hunger and appetite. This trend was notably significant for ratings of appetite for Global Appetite, High Carb-High Fat (HCHF), High Carb-Low Fat (HCLF), Fruits, Sweets, and high carb foods. A significant trend was also seen for hunger

ratings of Quantity Can Eat and Nausea, but not for Hunger, Thirst, Satiety, or Desire to Eat. Similarly, the drop in Calories Eaten was significantly associated with decreases in ratings for HCLF, Fruits, and Quantity Can Eat, and with an increase in ratings of Nausea. Taken together, these results suggest that VSG surgery may have a greater impact on subjective appetite than on subjective hunger, and that lesser Quantity Can Eat and greater Nausea may contribute more to weight-loss and caloric intake after VSG surgery than does suppression of Hunger and increased Satiety. Although in line with previous studies observed alterations in hunger and appetite [293], these findings are somewhat surprising since, under normal conditions, it is expected that caloric restriction associated with rapid and dramatic weight-loss leads to increased ratings of hunger and appetite concomitant with changes in leptin and ghrelin levels favoring food intake. This suggests that VSG surgery may mask the effects of homeostatic endocrine hormones through mechanisms not explored in the current study. Such mechanisms may involve the impact of bariatric surgery on gut-brain communication, the sympathetic nervous system, and neuroendocrine signaling.

In support of our hypothesis that subjective ratings after surgery would be overall reduced relative to baseline, we found that at twelve weeks post-VSG surgery, subjective ratings of certain hunger and appetite categories were significantly reduced when compared to ratings obtained two weeks before surgery. Most ratings of hunger and appetite indeed trended down (except for Satiety, Nausea, and Dairy), but only ratings of Thirst, Quantity Can Eat, Global Appetite, and appetite for HCLF foods were significantly lower than at baseline. Specifically, ratings of appetite for non-dairy Protein, Fruits, and Vegetables were

significantly different post-surgery compared to baseline. These findings are in line with the significant reduction in caloric intake, yet are observed in spite of fasting serum leptin and ghrelin levels altered in favor of food intake.

To our knowledge, the current study is the first to examine pre- and post-prandial subjective ratings of hunger and appetite across the day in subjects before and after bariatric surgery. To evaluate the overall impact of time, we combined baseline and post-surgery survey responses and compared responses from before the first meal (fasting) to responses from after the last meal (end-of-day) of laboratory sessions. We found prior to VSG surgery that ratings in nearly all categories of hunger and appetite, with the exception of HCHF, Sweets, and Nausea, were significantly different after the last meal than before the first meal, and indicated an overall decrease in hunger and appetite across the day. In contrast, post-surgery ratings were comparatively low throughout the day, with little difference between morning fasting and end-of-day ratings. Post-surgery, there was also little difference between pre- and post-prandial ratings throughout the day (data not shown). These findings support our hypothesis that post-surgery ratings would be flat across the day, and may be attributed to ratings already being low at the start of the day. It is again notable that this is observed in spite of post-surgery fasting serum levels of leptin and ghrelin having changed in favor of food intake.

While the roles of leptin and ghrelin on feeding behavior are mostly well-understood, the effects of bariatric surgery on circulating levels of leptin and ghrelin are less established but have been reported by a number of studies [296, 302-306]. In the current study, we

showed that mean fasting serum leptin twelve weeks after VSG surgery was significantly less than it was two weeks before surgery. This result can be attributed to the parallel decrease in body fat mass and weight. Interestingly, and in agreement with other reports [307-309], linear modeling revealed that percent weight lost correlates positively with the degree of change in fasting serum leptin due to a loss of energy storage in adipose tissue. The post-surgery induced decrease in hunger despite the observed decline in leptin levels could also point to a reduction in leptin resistance in these individuals. In conjunction with excess body fat mass, individuals with obesity frequently have high circulating levels of leptin that do not seemingly exert normal anorexigenic effects, thus suggesting that these individuals have become leptin resistant [310-312]. In our current study, the decrease in leptin concentration concomitant with the decrease in hunger scores may suggest that these individuals were previously leptin resistant pre-surgery, but are more leptin sensitive 12 weeks post-surgery. Perhaps post-surgery circulating leptin levels are now more effectively or adequately exerting anorexigenic effects, resulting in decreased levels of hunger. In contrast, while serum levels of acyl, des-acyl, and total ghrelin appeared to be higher post-surgery, only the percent-ratio %AC/DAG was significantly increased. Interestingly, when we evaluated the influence of ghrelin activity on hunger and appetite ratings, we found that the increase in the acylated-total ghrelin percent-ratio, was not correlated with any significant changes in subjective ratings between baseline and post-surgery sessions. These findings are particularly unusual because it would be expected that total ghrelin levels would decrease due to stomach resection, and may suggest a compensatory response during active weight loss occurring

12-weeks after surgery. Yet, given the roles of leptin and ghrelin in regulating feeding behavior, these findings would suggest that VSG surgery would subsequently promote food intake, as under normal conditions, declines in leptin and elevations in ghrelin are homeostatic responses to weight-loss and food restriction [130, 309, 313-315]. However, as we and others have shown caloric intake to indeed be significantly reduced after surgery, the impact of leptin and ghrelin on ratings and food intake may be masked or otherwise modulated by additional effects brought upon by VSG surgery. Such effects include changes in the levels of other gastrointestinal and pancreatic peptide hormones not investigated in the current study. While measurements of peptide YY (PYY), gastric inhibitory polypeptide (GIP), and glucagon-like-peptide-1 (GLP-1) would certainly be interesting to examine pre and post bariatric surgery, particularly interesting in the context of the Roux-en-Y procedure when part of the small intestine is bypassed, these measurements were outside of the scope of the current study.

Our study had some limitations: The follow-up time point was chosen at twelve weeks after VSG surgery, when subjects were in the midst of significant weight loss but before their final post-surgical weights has been reached. As such, our study provides insights on short-term physiological mechanisms by which bariatric surgery decreases food intake, but our results may not be generalizable to longer-term maintenance of caloric restriction and weight-loss. Future research should conduct longitudinal assessments on bariatric surgery subjects at regular intervals over the course of several years to track meal patterns. In addition, the current study only evaluated fasting serum hormone levels. The results from ratings taken

over the course of a day may be more striking if they were paired with serum hormone levels from the same time of day as the surveys. Future research should assess serum hormone levels in parallel with hunger and appetite over a 24-hour period. Lastly, the current study was part of a larger interventional study, in which recruitment and participant retention was challenging. Ten participants completed assessments at baseline and 12 weeks post-surgery that allowed for sufficiently powered analysis, for which we observed significant findings. To that end, fasting hormone levels were assessed pre- and post-surgery in only 8 of the participants. Even though we did observe significant differences in both leptin and ghrelin serum concentrations with this within-subject design, future experiments would benefit from obtaining a larger sample size.

Our findings suggest that homeostatic regulation of food intake is perturbed by VSG surgery. By the twelfth week after VSG surgery, subjective ratings of hunger and appetite were found to be reduced overall despite fasting serum levels of leptin and ghrelin having changed in favor of increased food intake. Notably, at the post-surgery session, the majority of fasting hunger and appetite ratings from before breakfast were not different from end-of-day ratings obtained after dinner, suggesting that the physical restriction of food intake, perhaps in conjunction with increased nausea, imparted by VSG overcomes the elevated homeostatic drive for food intake. These findings support the notion that bariatric surgery leads to comparable levels of hunger and appetite as observed prior to surgery, even with the intake of fewer calories, and highlights the effectiveness of bariatric surgery in curbing food-seeking behavior despite a very steep negative calorie balance.

CHAPTER 3:

ACUTE IMPACT OF SYNTHETIC CANNABINOID WIN 55,212-2 ON MURINE VISCERAL ADIPOSE TISSUE METABOLISM AND GENE EXPRESSION

3.1 Introduction

The behavioral and psychoactive effects of cannabis are well-understood to occur through central activation of the endocannabinoid system (ECS). Since the discovery of the cannabinoid receptors, research has made significant progress in uncovering the mechanisms by which cannabinoids exert their effects on pain and stress, mood and motivation, immune function, and, importantly, food intake and metabolism. However, these investigations have largely focused on central cannabinoid action despite the clear presence of cannabinoid receptors throughout the body. Besides the central nervous system, the CB1 receptor is significantly expressed in metabolic tissues including the liver, pancreas, GI tract, and adipose tissues, which points to a role in regulating energy metabolism. Indeed, the complete knockout of CB1 in mice imparts resistance to diet-induced obesity and improved cardiometabolic outcomes [157, 316]. Similarly, administration of the global CB1 inverse agonist rimonabant leads to a reduction of food intake and significant weight-loss [24, 25]. However, the success of rimonabant was short-lived as the results of long-term trials revealed serious risks of depression and suicide [24]. Thus, it has been an active research goal to determine whether peripheral CB1 antagonism is both safe and sufficient to curb the rising rates of obesity. Indeed, peripherally restricted CB1 antagonists have been shown to alleviate obesity, though the safety and the exact sites and mechanisms of action leading to the

observed effects remain poorly characterized though highly investigated [144, 317, 318]. The adipose tissue-specific mechanisms by which the ECS mediates metabolism are similarly not fully understood despite the existence of ECS machinery in adipose tissue being well established [157, 319-324]. Interestingly, cannabinoid agonists and antagonists have been shown to exert cannabinoid receptor-independent effects, suggesting a diversity of functions for the endocannabinoids aside from their relationships with known GPCR signaling pathways [274, 275, 325-327].

The current study aims to explore the impact of cannabinoid action on adipose tissue through functional enrichment analysis and pathway analysis of mRNA-sequencing data, and through interrogating the metabolic and molecular changes resulting from acute exposure to the synthetic cannabinoid WIN 55,212-2. We hypothesized that gene sets and pathways directly related to the regulation of energy metabolism would be significantly modulated, and that exposure of primary white adipose tissue to WIN 55,212-2 would potentiate insulin action and push the adipose tissue metabolic state towards anabolism. In addition, the majority of previous ECS studies in adipocytes have utilized cultured adipocytes differentiated *in vitro,* the characteristics of which do not faithfully reflect the phenotype of mature adipocytes *in vivo.* Thus, the current study utilizes primary adipose tissue cultured *ex vivo,* and aims to yield a more physiologically relevant picture of the metabolic and molecular changes resulting from cannabinoid action in adipose tissue.

3.2 Materials & Methods

3.2.1 Murine Adipose Tissue

All animal studies were approved by the University of Chicago Institutional Animal Care and Use Committee (IACUC; Protocol 71184). Wild-type male C57BL/6 mice were fed a regular chow diet *ad libitum.* Mice were sacrificed at 18-24 weeks of age for sufficient adiposity. The processing of adipose tissue was a modification of the Fried protocol [328]. For each experimental cohort of mice, the perigonadal adipose tissue of all mice was extracted, pooled together, and minced into ~2mm pieces, then washed 3x with M199 media (no Phenol-Red; Gibco) supplemented with 1% bovine serum albumin (BSA; Gemini Bio).

3.2.2 Adipose Tissue Explant Culture

Freshly minced adipose tissue was cultured in fresh M199+1% BSA media (100mg tissue/mL media) for 2 hours in a humidified tissue culture incubator at 37°C with 5% CO_2. This resting incubation serves as a serum-starve and allows for any acute-phase response and endogenous hormone activity to equilibrate. Rested adipose tissue was then washed with fresh M199+1% BSA media 2x before allocation to subsequent experiments. All experiments were performed in duplicate.

3.2.3 Acute Cannabinoid Treatment of Adipose Tissue

Rested adipose tissue was cultured in M199-1% BSA with vehicle or 10μM WIN (50mg tissue/mL media/sample). Tissue samples were collected at 0.5-, 1-, 2-, 4-, and 8-hour timepoints. At each timepoint, adipose tissues were transferred to bead lysis tubes and flash frozen in liquid nitrogen for mRNA and protein analysis.

3.2.4 RNA Isolation

Cannabinoid time-course samples were homogenized in ice-cold RNA-Solv (containing phenol and guanidinium isothiocyanate; Omega Bio-Tek) using a Bullet Blender. Total RNA was isolated via cold phenol-chloroform extraction and spin column purification. RNA was evaluated by UV-vis spectrophotometry (NanoDrop).

3.2.5 Transcriptomics

RNA from vehicle- and WIN-treated samples of the 0.5hr, 1hr, and 2hr timepoints (six conditions) were submitted in two batches to the University of Chicago Genomics Facility for QC (Agilent Bioanalyzer), library prep (Oligo-dT mRNA directional) and sequencing (100bp, ~30M paired-end reads/sample; Illumina NovaSEQ6000). Each batch had two biological replicates for each of the six conditions to give a combined total of 24 samples. Raw data were provided as FASTQ.

FASTQ files were uploaded to on Illumina BaseSpace Sequence Hub. The RNA-Seq Alignment app was used to perform read alignment using STAR (Spliced Transcripts Alignment to a Reference; RefSeq mm10) and transcript abundance quantification using *Salmon* [329, 330]. Count data were downloaded from Illumina BaseSpace Sequence Hub for differential gene expression analysis using the R software package *DESeq2* (v1.32.0) in RStudio (v1.4.1717) [331]. *DESeq2* fits a generalized linear model of the negative binomial family. Batch effect was accounted for in the model design code: design = ~ batch + time + treatment + time:treatment, and Wald tests were used to generate differential expression results tables for the treatment:time interaction effect at each timepoint separately. A

log_2(Fold-Change) threshold of 1 (i.e., a fold-change > 2 or < 0.5) in either direction was used for significance testing and generation of p-values. *DESeq2* uses the Benjamini-Hochberg procedure to control the false-discovery rate (FDR) and report an adjusted p-value (padj). Genes with a padj ≤ 0.05 were considered differentially expressed. Log_2FoldChange LFC estimates were shrunk in *DESeq2* for ranking of genes by calling the adaptive shrinkage estimator function from the R package *ashr* (v2.2-47) [332]. The average effect of treatment across all three timepoints was determined by extracting and summating 1/3 of the treatment:time interaction from each timepoint.

3.2.6 Functional Enrichment Analysis & Topology-Based Pathway Analysis

Gene Set Enrichment Analysis (GSEA) is a widely-used tool in RNAseq analysis to explore differential gene expression data. Results from DE analysis were formatted for input into GSEA software to identify significantly enriched gene sets (UCSD-Broad Institute's Molecular Signatures Database, MSigDB) [333-335]. Specifically, data was formatted for Pre-Ranked GSEA. The rank metric used to order the list of genes of each DE results table was the product of the sign of the log_2(FoldChange) value and the log_{10} of the nominal p-value. GSEA considers a gene set an unordered, unstructured collection of genes and does not assess whether a pathway is activated or inhibited. GSEA analyzes all genes from the DE results table regardless of significance, and assigns lower p-values more weight, and higher p-values are assigned less weight, if any. Data from each timepoint were separately analyzed using the KEGG (Kyoto Encyclopedia of Genes and Genomes) or Reactome gene set collections from MSigDB as reference databases. GSEA reports FDR < 0.25 as significant enrichment.

Data on the DE genes (padj ≤ 0.05) from DE analysis *(DESeq2)* were also used in the R software package *SPIA* (*Signaling Pathway Impact Analysis,* v2.44.0) to identify significantly affected signaling pathways [336]. *SPIA* uses the KEGG database as reference. A total of 40 relevant KEGG signaling pathways were evaluated (Table 3.1). The SPIA algorithm takes as input the names (as Entrez IDs) and LFCs of DEGs to produces a table of pathways ranked from the most to the least significant. Each SPIA table reports whether the queried pathways are activated or inhibited, and a combined global p-value based on both over-representation evidence and perturbations-based evidence. These perturbations depend on the position of genes in a pathway and whether they are known as activators or inhibitors.

Table 3.1 KEGG Signaling Pathways Assessed By SPIA

Calcium	Insulin	PI3K-Akt	cAMP
MAPK	FoxO	Hedgehog	IL-17
ErbB	Phosphatidylinositol	TGF-beta	TNF
Ras	Sphingolipid	VEGF	Retrograde endocannabinoid
Rap1	Phospholipase D	Apelin	HIF-1
PPAR	p53	Hippo	Thyroid hormone
cGMP-PKG	mTOR	Toll-like receptor	Adipocytokine
JAK-STAT	Notch	NOD-like receptor	Glucagon
Chemokine	AMPK	RIG-I-like receptor	Relaxin
NF-kappa B	Wnt	C-type lectin receptor	AGE-RAGE

3.2.7 Anti-Lipolysis Assay

Rested adipose tissue was pretreated in M199 media supplemented with 3% fatty acid-free bovine serum albumin (FA-free BSA; Sigma-Aldrich) with vehicle or with 10μM of the synthetic cannabinoid WIN 55,212-2 (WIN; Tocris) for 1 hr (50mg tissue/2mL/sample) at 37°C with periodic gentle agitation. Lipolysis was then stimulated by replacing media with 2mL fresh M199+3% FA-free BSA media containing the following experimental treatments:

Basal (media only, untreated), 100nM isoproterenol (Iso), Iso + 10μM WIN (Iso-WIN), Iso + 10nM insulin (Iso-Ins), or Iso + Ins + WIN (+++). Samples were incubated for 1 hour at 37°C with periodic gentle agitation, after which 1mL of media was removed, syringe-filtered, then stored at -20°C. Lipolytic activity was assessed by measuring media glycerol concentration (Glycerol Colorimetric Assay Kit, Cayman Chemical). Each experiment produced a total of 20 media samples (10 parallel conditions in duplicate).

3.2.8 Insulin Sensitivity Assay

A portion of rested adipose tissue was cultured in M199+1% BSA media with vehicle control (DMSO) or with 10μM WIN for 1 hour at 37°C (50mg tissue/1mL media/sample). Tissue samples were then transferred to 1.5mL microtubes containing M199+1% BSA media (1ml/sample) with or without 10nM insulin and incubated for 10 minutes at 37°C with gentle agitation. Samples were then immediately placed on ice to halt signaling, and media was replaced 2x with ice-cold tris-buffered saline to wash out insulin, BSA, and free lipids. Tissue samples were then transferred to bead lysis tubes containing 125μL of ice-cold 2x Laemmli Sample Buffer supplemented with 10% beta-mercaptoethanol for homogenization using a Bullet Blender (Next Advance). After homogenization, samples were placed in a 95°C heating block for 5 minutes, briefly vortexed, then put on ice for 10 minutes. Cooled samples were centrifuged for 5 minutes to separate excess lipid (top, clear layer). Protein fractions (lower, blue layer) were then transferred to new microtubes and stored at -20°C for subsequent western blot analysis.

Levels of total-Akt protein and phospho-Akt protein were quantified by two-color

near-IR fluorescent immunoblot (LI-COR Odyssey CLx Imaging System). Protein samples were resolved in 10% SDS-PAGE gels (Mini-PROTEAN® TGX™ Precast Gels, Bio-Rad) and blotted onto PVDF membranes (Immobilon-FL, Millipore; Trans-Blot, Bio-Rad) overnight at 4°C. Membranes were blocked in 5% PhosphoBlocker for 1 hour before incubating overnight with primary antibodies recognizing phospho-Akt (S473) and total-Akt. Appropriate secondary antibodies recognizing the primary antibody animal backgrounds were used for detection. Total-Akt served as the lane loading control for phospho-Akt. Band signals quantified by Image Studio™ Lite Quantification Software (LI-COR). The normalized signal ratio of phospho-Akt to total-Akt was used to quantify insulin-stimulated Akt phosphorylation, and thus served as a readout of adipose tissue insulin sensitivity. For each individual blot, the total-Akt signal/area of each lane was normalized to the maximum total-Akt signal/area of the whole blot to calculate the lane normalization factor for that lane (as per LI-COR documentation). The phospho-Akt signal/area of each lane was divided by the lane normalization factor for that lane to calculate the normalized phospho-Akt signal. Phospho-Akt was visualized on the 700nm channel and total-Akt was visualized on the 800nm channel. Antibodies were purchased from Cell Signaling Technology: Phospho-Akt (Ser473) (Clone D9E) XP Rabbit mAb #4060; Akt (pan) (Clone 40D4) Mouse mAb #2920.

3.2.9 Quantitative Reverse Transcription PCR

500ng of RNA extracted from time course samples were reverse-transcribed into cDNA (qScript cDNA SuperMix, Quantabio). All primers (except for 18S rRNA used as reference gene) were purchased pre-validated (RT^2 qPCR Primer Assay, QIAGEN).

The 18S rRNA primer sequence was obtained from published literature, and was purchased from IDT (Integrated DNA Technologies).

3.2.10 Statistical Analyses

Dr. Sang Mee Lee, PhD and the University of Chicago biostatistics clinic were consulted for guidance on non-transcriptomics data analyses. In order to explicitly account for the batch effect of mouse cohort, all non-transcriptomics data were analyzed by fitting a linear mixed-effects model as implemented in GraphPad Prism 9.1. Data for all treatment groups were matched by mouse cohort, and the random effects associated with "cohort" were included in the mixed models to account for variability between cohorts of mice. This mixed-effects model uses a compound symmetry covariance matrix, and is fit using Restricted Maximum Likelihood (REML). In the absence of missing values, this method gives the same p-values and multiple comparisons tests as repeated measures ANOVA. Sphericity was not assumed, and Prism was instructed to use the Greenhouse-Geisser correction (GGε) to estimate sphericity as necessary. P-values reported by post-hoc analyses were either Bonferroni corrected to control for the family-wise error rate (FWER), or corrected using the Benjamini-Hochberg procedure to control for the false-discovery rate (FDR).

3.3 Results

3.3.1 Adipose Tissue Gene Expression

mRNA Sequencing

In order to investigate the potential mechanisms involved in WIN-mediated effects on adipose tissue metabolism, we began our study with an unbiased mRNA-sequencing approach. Specifically, mRNA from adipose tissue treated with vehicle or WIN for 0.5-, 1-, and 2-hours were analyzed. Differential gene expression analysis was conducted in *DESeq2* using a log2(Fold-Change) threshold of 1 to assess the main effect of treatment (WIN vs vehicle) on gene expression. Differentially expressed genes (DEGs) were determined for each timepoint separately by coding for the specific contrast argument associated with the WIN- vs. vehicle-treated pairwise comparison for each timepoint. The quantity of DEGs identified at each timepoint is visualized in Figure 3.1. Furthermore, there were 604 DEGs common to all timepoints: 37 were induced at all timepoints, 107 were induced or repressed at one or two timepoints, and 460 were repressed at all timepoints (Figure 3.2).

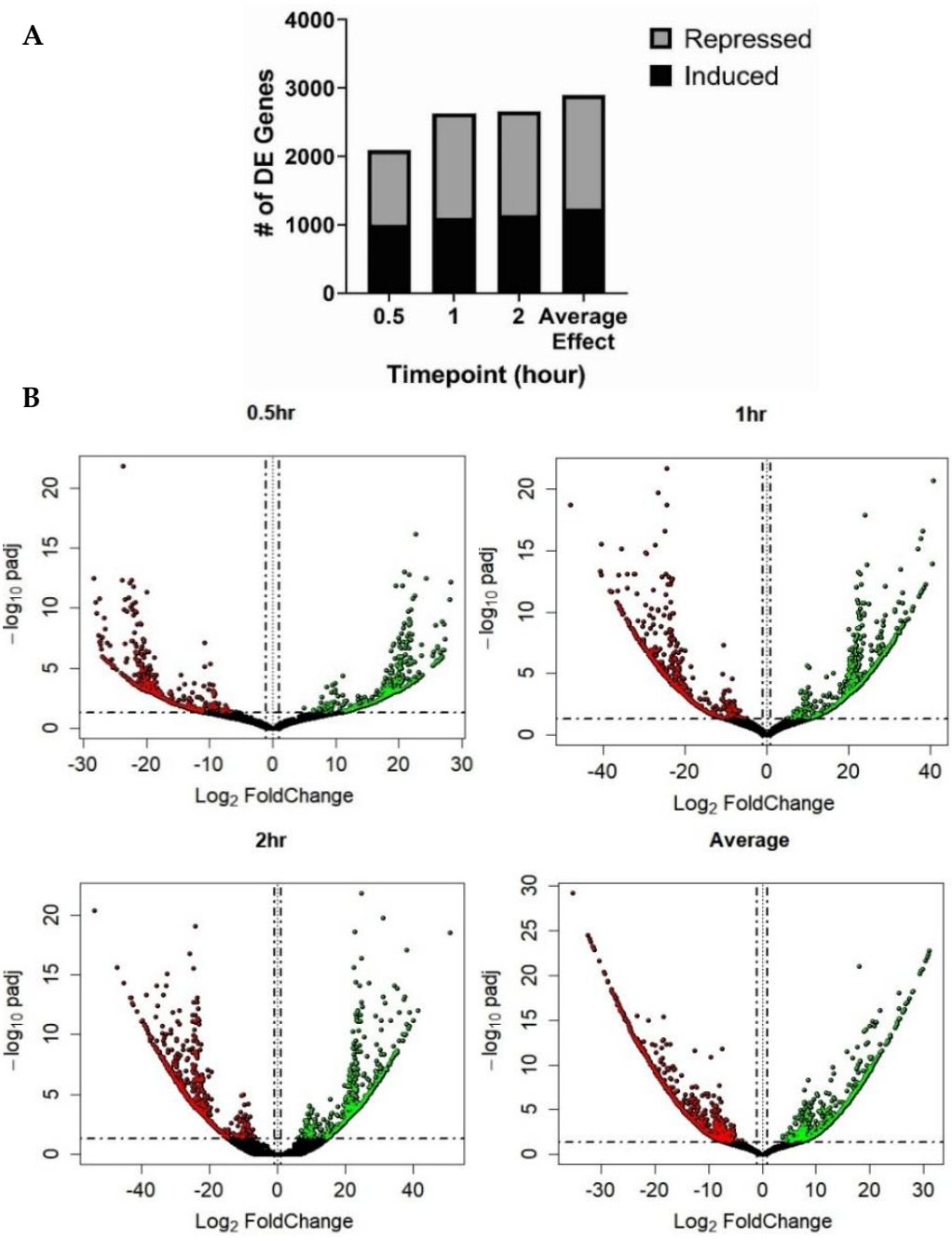

Figure 3.1. WIN 55,212-2 caused differential gene expression in murine adipose tissue
(A) Stacked bar chart depicting the number of genes at each treatment time point and for the average effect across all timepoints that were considered differentially expressed by meeting the significance criteria: log2FoldChange > 1, padj ≤ 0.05. Induction/repression by WIN relative to vehicle **(B)** Volcano plots of the log2FoldChange vs. -logpadj of all genes at each treatment timepoint and for the average effect across all timepoints. Green: significantly induced genes. Red: significantly repressed genes. Black dots: not differentially expressed (i.e., did not meet significance criteria). N = 4 (2 batches of 2), padj reflects p-values adjusted by controlling for the false-discovery rate.

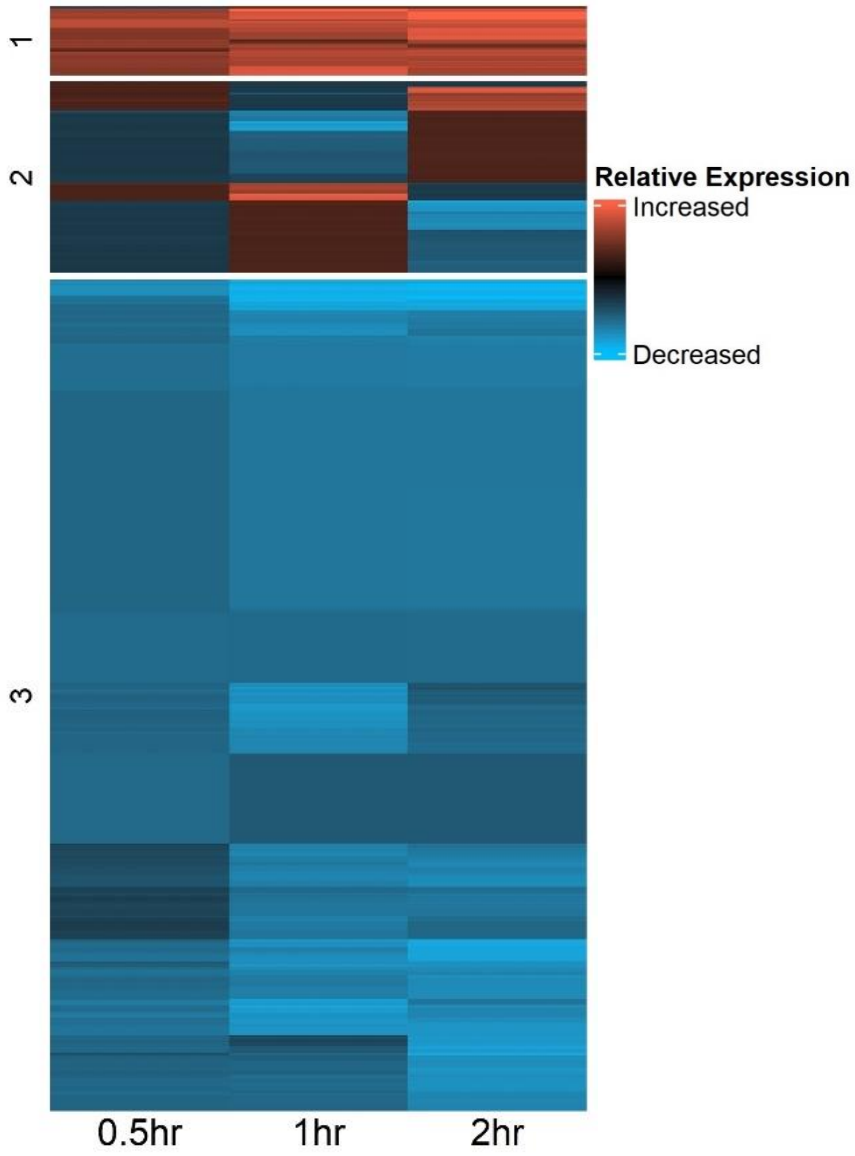

Figure 3.2. WIN significantly modulated genes in the same direction at all timepoints
Heatmap depicting the 604 differentially expressed genes (i.e., met significance criteria: log2FoldChange > 1, padj ≤ 0.05) in adipose tissue due to WIN (relative to vehicle) that were common at all timepoints. Groups 1-3: 1) 37 genes up at each timepoint (red). 2) 107 genes up or down (blue) at one or two timepoints. 3) 460 genes down at each timepoint. N = 4 (2 batches of 2)

Gene Set Enrichment Analysis

Data from each timepoint were separately analyzed by Preranked-GSEA using the KEGG or Reactome gene set collections on MSigDB as reference databases. Conducting GSEA using the full KEGG collection (186 gene sets) did not report significant (i.e., FDR < 25% [333])

enrichment of any gene sets (data not shown). However, instructing GSEA to use only gene sets denoted as "signaling pathways" (25 gene sets) yielded significant results for the 0.5-hour and 1-hour timepoints, as well as for the Average Effect across all times (Table 3.2).

Table 3.2 WIN-mediated gene set enrichment in primary murine adipose tissue

Timepoint (hour)	MSigDB Collection	Gene Set	NES	FDR
0.5	KEGG signaling	B cell receptor signaling pathway	1.43	0.244
	Reactome signaling	Negative regulation of FGFR3 signaling	-1.66	0.122
		Negative regulation of FGFR2 signaling	-1.60	0.210
		FGFRL1 modulation of FGFR1 signaling	-1.57	0.232
		Downstream signaling of FGFR3	-1.56	0.212
		Signaling by FGFR3	-1.55	0.191
1	KEGG signaling	JAK-STAT signaling pathway	1.51	0.065
		p53 signaling pathway	-1.51	0.162
2	n.s.	n.s.	n.s	n.s
Average Effect	KEGG signaling	p53 signaling pathway	-1.48	0.163
	Reactome (full)	Signaling by IGF1R	-1.62	0.104
		Insulin receptor signaling cascade	-1.60	0.083
		IRS-mediated signaling	-1.59	0.080
	Reactome signaling	Signaling by IGF1R	-1.62	0.015
		Insulin receptor signaling cascade	-1.62	0.009
		IRS-mediated signaling	-1.61	0.011
		Signaling by insulin receptor	-1.53	0.096

NES: Normalized Enrichment Score; FDR: False-Discovery Rate; n.s. = not significant

GSEA using the full Reactome annotation database (1604 gene sets) yielded significant results only for the average effect across timepoints, in which three gene sets directly related to insulin signaling were negatively enriched (Table 3.3). Limiting the Reactome annotations to only "signaling pathways" (278 gene sets) yielded significant enrichment results for the

0.5-hour timepoint and average effect across time: 5 gene sets directly related to Fibroblast Growth Factor (FGF) receptor signaling were negatively enriched at 0.5-hour, and 4 gene sets directly related to insulin signaling were negatively enriched for the average effect across all timepoints (Table 3.2).

Signaling Pathway Impact Analysis

A major weakness of GSEA is that it does not assess whether a pathway is activated or inhibited. To address this, we further analyzed the differential expression data through SPIA. Significantly perturbed signaling pathways were assessed by SPIA for each timepoint separately. We first queried only the most metabolically relevant pathways in adipose tissue, namely the calcium-, cAMP-, insulin-, and PI3k-Akt signaling pathways. When limited to these four pathways, SPIA reported the PI3k-Akt signaling pathway to be significantly inhibited at 0.5-hour, and the cAMP signaling pathway to be significantly inhibited at 1-hour (Figure 3.3A). The calcium signaling pathway was also significantly inhibited at 0.5- and 1-hour, and also under the average effect across all timepoints (Figure 3.3A). Increasing the number of relevant pathways queried to 40 yielded only one significantly impacted pathway at any timepoint: the calcium signaling pathway. Specifically, the calcium signaling pathway was significantly inhibited at 1-hour and in the average effect across all timepoints (Figure 3.3B). Taken together, the SPIA data suggest that acute WIN exposure leads to suppressed calcium signaling pathway activity in adipose tissue, perhaps in conjunction with decreased PI3k-Akt signaling activity very early on, followed by decreased cAMP signaling activity a short while later.

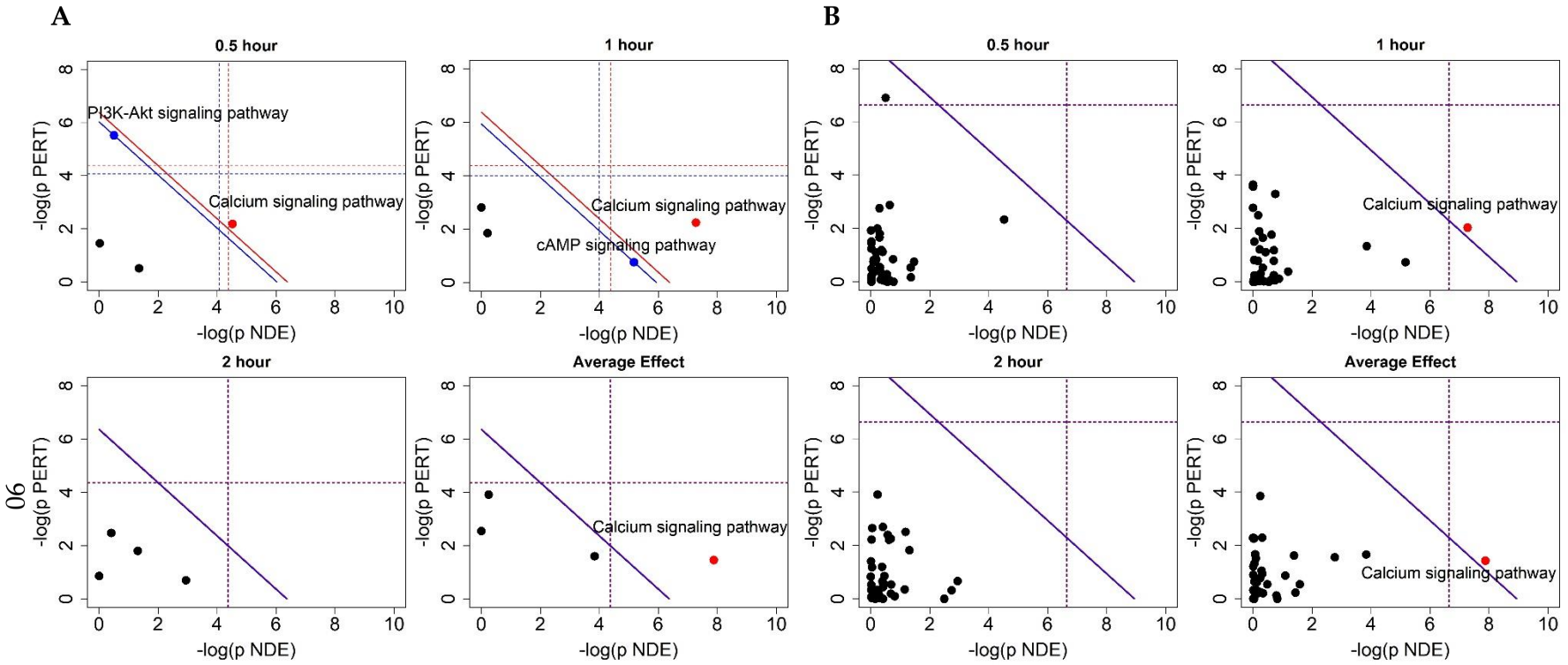

Figure 3.3. Signaling Pathway Impact Analysis of genes differentially expressed in murine adipose tissue due to WIN

SPIA two-way evidence plots for each treatment timepoint and of the average effect. Each pathway is represented by one dot. The pathways at the right of the red or blue oblique line are significant after Bonferroni (red, FWER ≤ 0.05) or Benjamini-Hochberg (blue, FDR ≤ 0.05) correction of the global p-values, pG, which *SPIA* obtained by combining the pPERT and pNDE values using Fisher's method. Red dot: pG-FWER ≤ 0.05, Blue dot: pG-FDR ≤ 0.05, Black dot: not significant. pPERT reflects the significance of pathway perturbation. pNDE reflects the significance of pathway representation by the number of differentially expressed genes (i.e., genes meeting the significance criteria: log2FoldChange > 1, padj ≤ 0.05 **(A)** Plots after SPIA queried 4 KEGG pathways chosen for metabolic relevance **(B)** Plots after SPIA queried a selection of 40 KEGG signaling pathways relevant to adipose tissue (i.e., excluding signaling pathways with a non-adipose tissue cell type in their title).

qRT-PCR

Given the acute length of WIN treatment in addition to GSEA and SPIA pointing to insulin and calcium signaling, we theorized that calcium channels may be involved in mediating insulin signaling at least in the presence of WIN. Indeed, cannabinoids have been shown to interact with the TRP family of channels, some of which were differentially expressed for at least one timepoint. Therefore, to further explore the interactions between the calcium and cAMP signaling pathways and cannabinoid action, we investigated the effect of WIN on gene expression levels of Trpv1, Trpv2, Trpv6, Trpa1, Calm1, and Camkk2, as well as the CREB genes Atf3 and Atf4, which have been reported to be directly involved in mediating glucose tolerance and insulin sensitivity [267-269, 271-273]. Vehicle- and WIN-treated adipose tissue samples at 0.5-, 1-, 2-, 4-, and 8-hour timepoints were processed for qRT-PCR. The initial mixed-effects model analyses of these data did not indicate a significant interaction between time and treatment, so the interaction terms were removed and the mixed-effects analysis focused on the main effect of treatment for each gene independently. Post-hoc analyses comparing WIN treatment to vehicle reported significantly (FDR ≤ 0.05) greater relative mRNA expression of Trpv1 and Trpv2, and significantly lower relative mRNA expression of Atf4 and Calm1 (Figure 3.4). Relative mRNA expression of Trpa1 was also increased, though this did not quite meet statistical significance (FDR = 0.0509). Expression of Trpv4, Trpv6, Camkk2, and Fasn was not significantly different between treatments (Figure 3.4). These results show that the expression of some but not all Trp channels or Ca^{2+}/cAMP related genes were modulated by WIN treatment, suggesting that

WIN may be selective in its effects on adipose tissue thus differentially regulating these interrelated pathways. In addition, the decrease in Atf4 expression may indicate a reduction of anabolic signaling in line with insulin resistance [271, 273, 337].

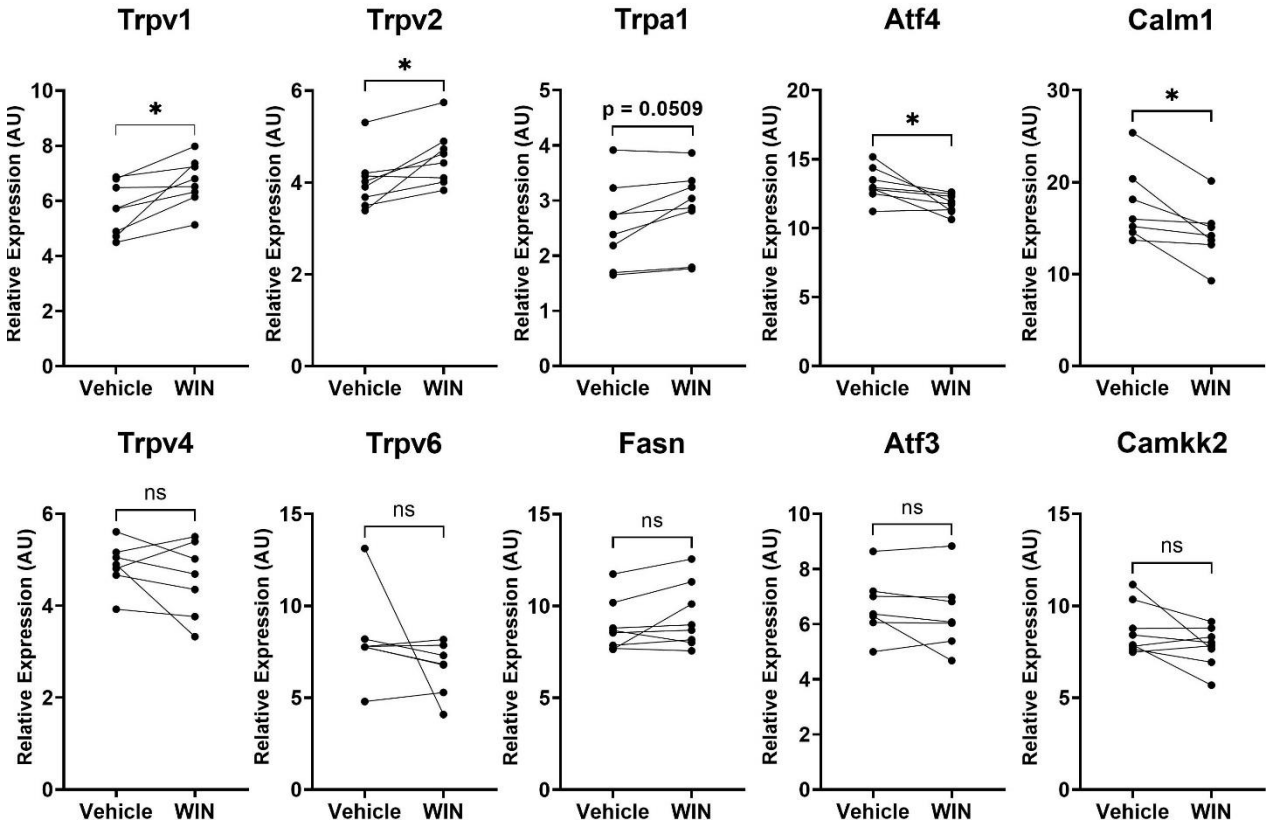

Figure 3.4. Modulation of Ca^{2+}- and cAMP-related genes in adipose tissue by WIN
qRT-PCR results depicting the main effect of WIN treatment in adipose tissue. Exponentiated dCt values are shown to illustrate the relative change in expression for each mouse cohort. Analyses were conducted on the dCt data, which were matched by mouse cohort and analyzed using a mixed-effects model fitting only the main effects of treatment and time without their interaction. Random effects associated with "cohort" were included in the model. Data for each gene was independently fitted to a mixed-effects model. P-values reflect the main effect of treatment and were adjusted by controlling for the False-Discovery Rate. N = 8. * : $p < 0.05$; ns: not significant.

3.3.2 Impact of WIN on adipose tissue metabolism

Rested adipose tissue was pretreated for 1 hour with vehicle or 10μM WIN before replacing the media to stimulate lipolysis under five conditions: Basal, Iso, Iso-WIN, Iso-Ins,

and Iso-Ins-WIN (+++). The degree of lipolysis was determined by comparing the media glycerol concentrations under each condition.

A mixed-effects model was used to compare the fixed effects of pretreatment, lipolysis condition, and the interaction between them on adipose tissue glycerol release. Mixed-effects analysis reported a significant interaction between pretreatment and lipolysis condition ($N = 9$; $F[2.027, 16.22] = 9.044$, $p < 0.01$, $GG\varepsilon = 0.6756$). Post-hoc analyses reported that, as expected, isoproterenol indeed stimulated lipolysis in all treated samples as indicated by significantly greater media glycerol concentrations compared to Basal ($p < 0.001$, Figure 3.5, comparisons not shown). Importantly, Basal and Iso-only media glycerol levels were not different between the pretreatment groups, indicating that WIN-pretreatment alone (i.e., without continued WIN exposure during lipolysis) did not affect basal or isoproterenol-induced lipolysis (Figure 3.5A, first and second comparisons). In addition, the Iso-WIN glycerol levels were not significantly different between pretreatment groups (Figure 3.1A, third comparison), nor were they significantly different from the Iso-only glycerol levels in their respective pretreatment groups (Figure 3.5B, red vs. purple comparisons). These findings show that neither WIN-pretreatment alone nor the continued exposure to WIN alone during lipolysis affected isoproterenol-induced lipolysis.

The effect of WIN on the insulin-dependent inhibition of lipolysis was also assessed. Media glycerol concentrations in the Iso-Ins condition were not significantly different between pretreatment groups (Figure 3.5A, fourth comparison). However, as expected, the Iso-Ins glycerol levels were significantly less than the Iso-only glycerol levels within both

pretreatment groups (both $p < 0.001$, Figure 3.5B, red vs. green comparisons). Taken together, these data show that insulin indeed significantly inhibited isoproterenol-induced lipolysis, and that this effect was not impacted by WIN-pretreatment alone. Interestingly, when the effects of continued WIN exposure on the insulin-dependent inhibition of lipolysis were assessed, we found that the +++ glycerol levels were consistently higher after WIN-pretreatment than after vehicle-pretreatment (mean difference: 2.10 mg/mL, 95% CI of difference: 0.45 to 3.74 mg/mL, $p < 0.001$; Figure 3.5A, fifth comparison). Moreover, the +++ glycerol levels were also consistently higher than the Iso-Ins glycerol levels within the WIN-pretreatment group (mean difference: 2.15 mg/mL, 95% CI of difference: 0.01 to 4.30 mg/mL, $p < 0.05$, Figure 3.5B, green vs orange comparisons), but not within the vehicle-pretreatment group. Taken together, these data indicate that insulin-dependent inhibition of isoproterenol-induced lipolysis in adipose tissue was attenuated only with continued exposure to WIN after 1-hour WIN-pretreatment.

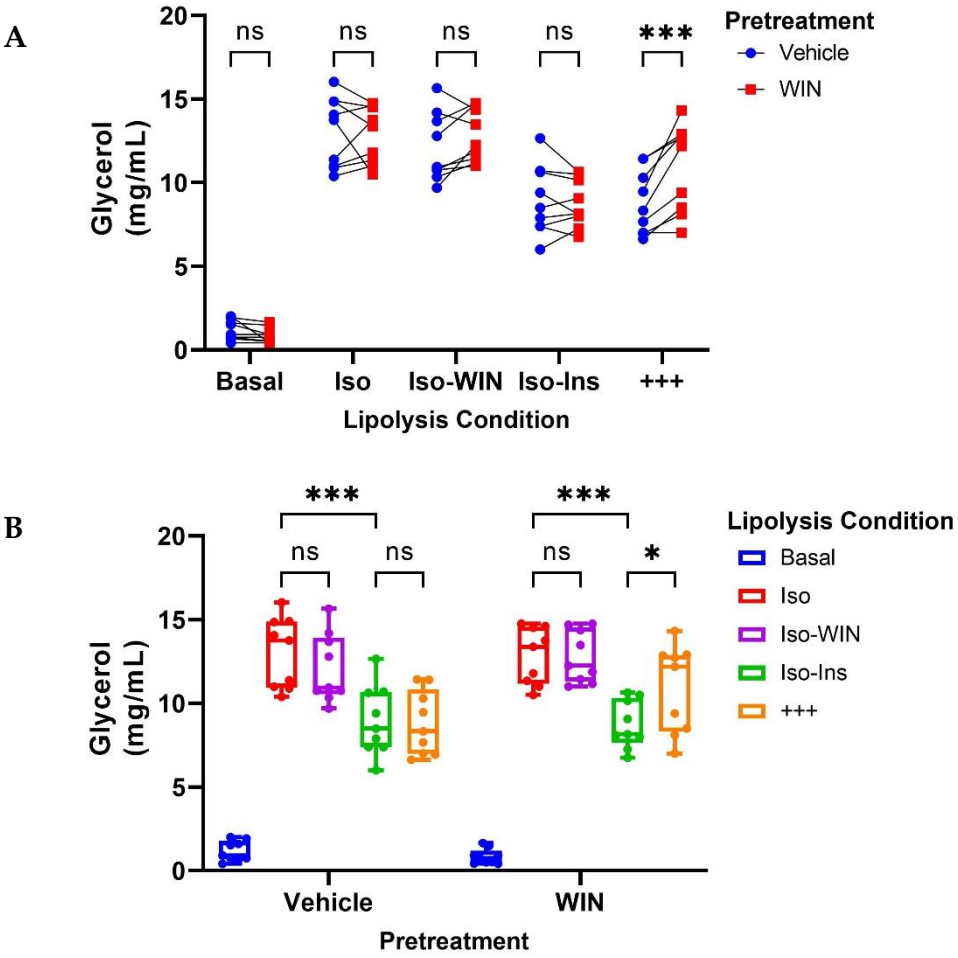

Figure 3.5. WIN 55,212-2 inhibits the antilipolytic action of insulin

Minced, rested epididymal adipose tissue was pretreated for 1 hour with vehicle or WIN. Then, media was replaced to stimulate lipolysis for 1 hour under 5 conditions: Basal (media only), Iso (100nM isoproterenol alone), Iso-WIN (Iso & 10μM WIN 55,212-2), Iso-Ins (Iso & 10nM insulin), and +++ (Iso + WIN + Ins). Media glycerol concentrations were quantified by colorimetric assay. **(A)** Comparisons between pretreatments across lipolysis conditions. **(B)** Comparisons between lipolysis conditions across pretreatments. Glycerol concentration data were matched by mouse cohort and fitted to a mixed-effects model to compare the fixed effects of pretreatment, lipolysis condition, and the interaction between them. Random effects associated with "cohort" were included in the model. Model fitting was followed by Bonferroni post hoc test. N = 9. * : $p < 0.05$, *** : $p < 0.001$, ns: not significant.

3.3.3 Impact on Insulin Signaling

The impact of WIN on lipolysis may be due to altered insulin signaling. To test this, rested adipose tissue was pretreated for 1 hour with vehicle or 10μM WIN before a 10-minute stimulation without insulin (-Insulin) or with 10nM insulin (+Insulin). Adipose tissue insulin sensitivity was determined by comparing the normalized signal ratios of phospho-Akt (Ser473) to total-Akt of each condition.

A mixed-effects model was used to compare the fixed effects of 1-hour WIN pretreatment, 10-minute insulin treatment, and the interaction between the two on Akt-phosphorylation in adipose tissue. Mixed-effects analysis reported a significant interaction between pretreatment and insulin treatment ($N = 9$, $F[1, 8] = 35.92$, $p < 0.001$). Post-hoc analysis reported no significant differences in the normalized phospho-Akt signals of the -Insulin condition between pretreatment groups, indicating that the 1-hour WIN-pretreatment alone did not affect Akt phosphorylation. As expected, insulin treatment significantly increased Akt phosphorylation in both pretreatment groups (both $p \leq 0.05$, Figure 3.6). However, we found that the normalized phospho-Akt signal of the +Insulin condition was consistently higher with WIN-pretreatment than with vehicle-pretreatment (mean difference: 5.47, 95% CI: 2.90 to 8.03, $p < 0.05$). Consistent with the lipolysis findings, these data indicate that WIN-pretreatment reduced the insulin-stimulated phosphorylation of Akt, and suggests an induction of insulin resistance due to continued WIN exposure.

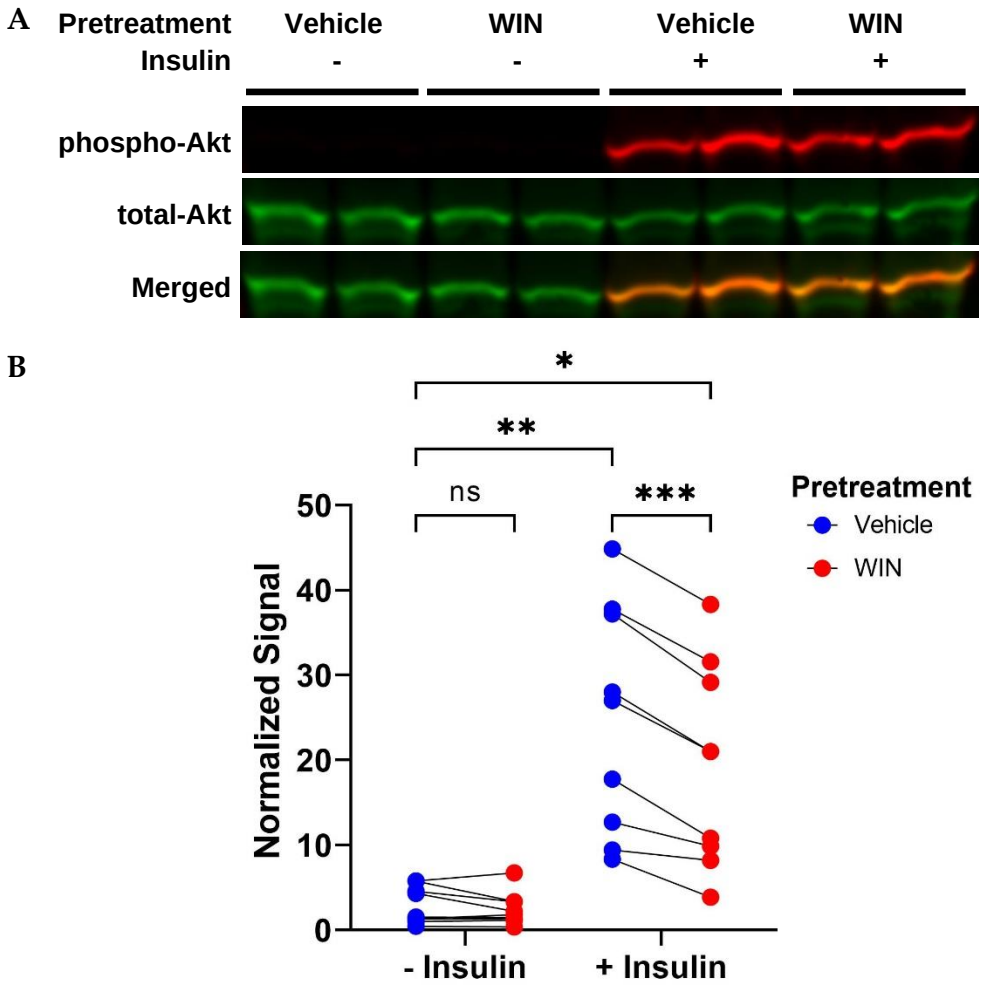

Figure 3.6. WIN 55,212-2 attenuates insulin-dependent Akt phosphorylation
Minced, rested epididymal adipose tissue was pretreated for 1 hour with WIN or vehicle. Then, media or insulin (final concentration 10nM) was added to stimulate the insulin signaling pathway. Insulin-induced phosphorylation of Akt was detected by near-IR immunoblot for phospho-Akt (Ser 473) and total-Akt. Total-Akt served as the loading control for phospho-Akt signal normalization. **(A)** Representative western blot image depicting phospho-Akt, total-Akt, and merged signals under the treatment conditions. **(B)** Comparisons of insulin conditions between pretreatment groups. Normalized signal data were matched by mouse cohort and fitted to a mixed-effects model to compare the fixed effects of pretreatment, insulin condition, and the interaction between them. Random effects associated with "cohort" were included in the model. Model fitting followed by Bonferroni post hoc test. N = 9. * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$, ns: not significant

3.4 Discussion

The current study assessed the physiological relevance of cannabinoid action in primary murine adipose tissue. The impact of the synthetic cannabinoid WIN 55,212-2 on adipose tissue gene expression, insulin signaling, and lipid metabolism was investigated. At the time of writing, to our knowledge, there were no published reports of next-generation sequencing being utilized to compare vehicle- and cannabinoid-treated primary adipose tissue. In addition, we report a significant effect of WIN on adipose tissue lipid metabolism paralleled by a reduction in Akt phosphorylation by insulin. Moreover, the findings from gene set enrichment and pathway analyses are consistent with the lipolysis and insulin signaling results.

Differential expression analysis reported that, as expected, a great number of significant DEGs were identified at each timepoint. Interestingly, a few hundred more genes were reported as differentially expressed when extracting the average effect of WIN-vs-vehicle treatment across all timepoints relative to the number of DEGs reported at each individual timepoint. Given that extraction of the average effect in *DESeq2* takes into account 1/3 of the treatment effect at each timepoint, it can be interpreted as an assessment of the main effect over three replicate WIN-vs-vehicle comparisons. Furthermore, there were 604 genes that were significant differential expression across all three timepoints, which reflects the previously unreported significant impact and relevance of cannabinoid action in primary murine white adipose tissue. Of these 604 DEGs, 37 were upregulated and 460 were downregulated at all timepoints. Simple over-representation analysis (ORA; reference

database: Reactome) reported that 6 gene sets related to insulin signaling (specifically: PI3K cascade, IRS-mediated signaling, IRS-related events triggered by IGF1R, IGF1R signaling cascade, Signaling by IGF1R, and Insulin receptor signaling cascade), were represented within the 460 repressed genes with an FDR of 0.141 (data not shown). While this FDR does not reflect statistical significance at the 95% confidence level, it does suggest that the result is likely to be valid 85.9% of the time, which is reasonable to for this exploratory investigation. Moreover, the pathways implicated are informative as they directly relate to adipose tissue metabolism. Significance could perhaps be improved with additional replicates or a longer length of WIN treatment. Interestingly, most of the gene sets identified by ORA were also reported by GSEA to be negatively enriched in the average effect across all timepoints, which is notable since GSEA uses a greater number of genes as input. This suggests that the 604 DEGs common to all timepoints may be the primary drivers of the significant GSEA findings. Taken together, the ORA and GSEA results point to a role for cannabinoids in modulating adipose tissue metabolism via insulin signaling.

GSEA reported that other gene sets of interest were represented in our data, though they did not meet the significance threshold. Such gene sets include: Gαs signaling events, Phosphatidylinositol signaling, Calcium signaling, and Adipokine signaling. These gene sets are relevant to metabolic function, and with additional replicates or longer WIN treatment may more significantly contribute to the larger picture of potential mechanisms by which cannabinoids mediate peripheral tissue metabolism. GSEA also reported that gene sets related to FGF receptor signaling were significantly enriched at the 0.5hr timepoint but were

absent under the average effect. Little is known regarding the relationship between FGF signaling and the ECS, though one study suggests that FGFR-CB1 receptor interactions may be coupled to axonal growth [338].

A weakness of GSEA, however, is that it does not specifically assess whether a pathway is activated or inhibited. To address this, topology-based network analysis, such as by SPIA, is typically utilized. Surprisingly, and in contrast to ORA and GSEA findings, SPIA did not report the insulin signaling pathway per se to be significantly impacted. Interestingly, however, the cAMP signaling, PI3k-Akt signaling, and calcium signaling pathways were reported to be significantly inhibited (Figure 3.3). Given that the PI3k-Akt pathway is almost directly downstream of the insulin receptor and in all regards is a part of the insulin signaling cascade, it is reasonable to interpret this result as an inhibition of downstream insulin signaling pathway signaling. Thus, taking together the results of both GSEA and SPIA suggests a potential link between cannabinoid action and negative regulation of the calcium and insulin signaling pathways in adipose tissue.

Our follow-up gene expression analysis of selected calcium pathway genes, namely some Trp channels, calmodulin, and Camkk2, indicated that WIN may exert selective effects on calcium signaling. Interestingly, expression of Trpv1, Trpv2, and Trpa1 was increased, which may indicate an activation of calcium-mediated signaling. However, these increases in expression may also be a compensatory response to restore calcium-mediated signaling after desensitization by WIN. Indeed, WIN and the endocannabinoid anandamide have been shown to bind to and desensitize Trpv1 and Trpa1 [275, 278, 319, 325]. Moreover, the

increases in these genes may appear small, but depending on baseline levels, substrate availability, or post-translational modifications, relative changes in transcription do not necessarily translate into a proportional change in protein expression or pathway activity. Thus, the increase in the expression of these channels may be sufficient to significantly impact signaling activity. It should be noted here that GSEA reported the Reactome "Trp Channels" gene set (note: not a signaling pathway) to be represented in the data for the average effect across timepoints, though it was not positively enriched at a significant FDR (data not shown). This highlights another weakness of GSEA in our study in that it may be underestimating effects or ignoring possible selectivity of WIN on the expression of particular genes such that the enrichment of a gene set or pathway is not significant.

qRT-PCR analysis also reported a significant reduction in Calm1 expression by WIN treatment, which is consistent with the SPIA-reported inhibition of the calcium signaling pathway. This presents further evidence for the role of WIN in regulating metabolism via calcium-mediated effects. However, the lack of change in Fasn expression, which has been shown to be upregulated by high dietary calcium and insulin action [177, 178], suggests that cannabinoids may not appreciably impact metabolism on their own, but rather modulate the signaling of other hormones and growth factors. This is evident in our finding that WIN only had a significant impact on lipolysis and Akt phosphorylation in the presence of insulin.

To assess the physiological impact of cannabinoid action in adipose tissue, we investigated the effects of acute WIN exposure on catecholamine-stimulated lipolysis, and how it might interact with the antilipolytic action of insulin. Contrary to the hypothesis that

acute cannabinoid exposure potentiates insulin action, we found that WIN had a significant inhibitory effect on the ability of insulin to suppress isoproterenol-stimulated lipolysis, but it was only observed under continued exposure to WIN after 1hr pretreatment. Importantly, WIN alone did not affect basal or isoproterenol-stimulated lipolysis. This finding suggests that the observed effect of WIN may be specific for insulin-mediated signaling. Moreover, the observed repression of Atf4 gene expression, is consistent with previous reports of Atf4-deficient mice exhibiting increased lipolysis [337, 339]. This also indicates that Atf4 may not be regulated by the GPCR activity of the CB1 receptor, which is coupled to Gαi, and suggests a non-CB1 receptor mediated effect of WIN.

To link the metabolic findings to a molecular mechanism, we assessed the ability of insulin to stimulate Akt phosphorylation in the presence of WIN. Consistent with our lipolysis results, we observed significantly less insulin-dependent Akt phosphorylation when WIN was present before and during insulin treatment. These findings are in line with the well-known associations between high ECS activity, obesity, and insulin resistance, as well as with the results of a recent study investigating the effects of anandamide administration in mice and *ex vivo* treatment of rat adipose tissue [324]. Moreover, that study also demonstrated that the negative effects of AEA on Akt phosphorylation were dependent on the presence of insulin, which was consistent with our own findings using WIN. Notably, the impact of WIN on lipolysis and Akt phosphorylation were observed after only one or two hours of WIN exposure and only apparent in the presence of insulin. This is likely because basal Akt phosphorylation is negligible such that inhibition by WIN is near undetectable,

which suggests that WIN may be acting upstream of Akt. Therefore, follow-up studies should evaluate insulin receptor and Irs1/2 phosphorylation, as well as PI3k activity to identify the potential site(s) of WIN action. Furthermore, if WIN is indeed acting on Insr/Irs, then the activity of other signaling cascades, such as that of mitogen-activated protein kinase (MAPK), may be affected. Indeed, GSEA reported significant negative enrichment of gene sets related to FGF receptor signaling. Thus, assessment of insulin- or FGF-dependent MAPK phosphorylation in the presence of WIN should be conducted.

Akt is a critical member of the insulin signaling cascade, and is largely responsible for the amplification of the insulin signal to promote its anabolic effects. One such effect is the activation of Pde3b, which is known to be a major negative regulator of lipolysis through its hydrolysis of cAMP and subsequent suppression of protein-kinase-A (PKA) activity [105]. Importantly, it has been shown that that reductions in either intracellular or extracellular calcium can modulate insulin signaling pathway activity largely by attenuating Akt phosphorylation [340, 341]. Thus, the results of our mRNA-seq analyses are in agreement with our functional analyses with regard to the role of calcium in regulating insulin signaling, and presents a potentially novel role for cannabinoids in modulating this interaction perhaps through the phosphatase activity of calcineurin (CaN; Figure 3.7). The mechanism by which cannabinoids may modulate Calm1 expression remains unclear, but the observed increase in Trpv expression suggests a potential feedback mechanism. Specifically, cannabinoids may initially activate TrpV/A channels and increase Ca^{2+} influx to promote CaN-mediated inhibition of Akt phosphorylation in the presence of insulin. After these initial rapid events,

ongoing cannabinoid action may desensitize TrpV/A channels in an attempt to reduce cytosolic Ca^{2+} and attenuate ongoing CaM/CaN activity. Trp channel-mediated cannabinoid action in the absence of insulin may also inhibit lipolysis through CaN-mediated deactivation of Pka or adenylyl cyclase (Figure 3.7). Future studies should evaluate the temporal activation of the various signaling cascade events and interactions.

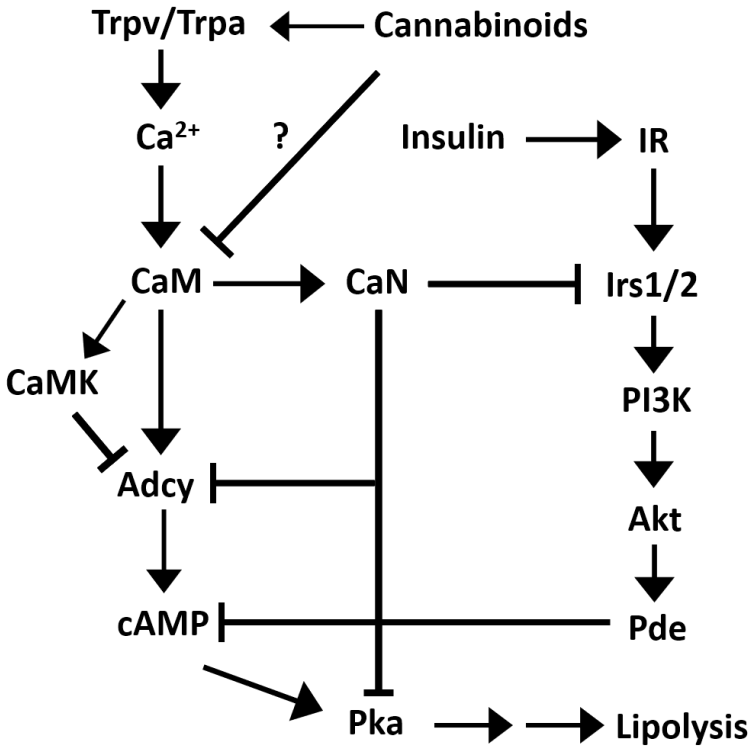

Figure 3.7 Hypothesized mechanisms by which acute cannabinoid action impacts lipolysis Graphical representation of the potential interactions between the calcium and insulin signaling pathways. Cannabinoids may desensitize Trp channels to reduce Ca^{2+} influx. How cannabinoids may reduce calmodulin expression remains unclear. Calcineurin may inactivate Irs1/2 to attenuate the phosphorylation of Akt. IR, insulin receptor; Irs1/2, insulin receptor substrate 1/2; PI3K, phosphoinositide 3-kinase; Pde, phosphodiesterase; CaM, calmodulin; CaN, calcineurin; CaMK, calmodulin-dependent protein kinase; Adcy, adenylyl cyclase; cAMP, cyclic-adenosine monophosphate; Trpv/Trpa, transient receptor potential channel type V/A, Pka, protein kinase A

The current study has some limitations. Firstly, despite accounting for batch effect computationally in *DESeq2*, its existence remains as a potential confounder. This limitation

was difficult to avoid due to the relatively small sample size. Analyzing each batch separately would half the power. Moreover, the relative uncertainty of the WIN response in primary adipose tissue was a concern. Most studies have typically utilized adipocytes differentiated *in vitro*, or WIN was administered to mice *in vivo*, so conditions needed to be optimized while keeping sequencing costs reasonable. The variability between experimental mouse cohorts represents another source of batch effect in our study limitation. Though statistical significance was reached in our analyses, the magnitude of the effect of WIN on adipose tissue was not consistent between cohorts of mice. This may be explained by the presence of multiple cell types in adipose tissue, which may not be homogenously distributed throughout the tissue or even between samples despite fine mincing and the use of primary adipose tissue as opposed to cultured clonal cell lines. Another limitation is that the adipose tissue of female mice was not investigated. It is reasonable to suspect that WIN may impact female adipose tissue similarly as observed in male adipose tissue, but the differences in hormonal signaling and the local microenvironment may lead to different outcomes. Lastly, the study did not make any direct measurements of adipose tissue cAMP or calcium levels with WIN treatment. Measurements of these second messengers would provide a snapshot of the activity of their respective pathways, and would be vital for understanding their potential contributions and associations with the observed effects on lipolysis and Akt phosphorylation. Indeed, WIN has been shown to increase intracellular Ca^{2+} in differentiated human adipocytes [322]. Furthermore, the direct involvement of calcium in the observed WIN-dependent reduction of insulin sensitivity should be investigated. This could be tested

by depleting intracellular calcium levels with thapsigargin and/or eliminating calcium from culture media before WIN treatment. However, thapsigargin would also cause endoplasmic reticulum stress, which would be an additional confounder. Alternatively, calmodulin or CaMK inhibitors could also be utilized to more specifically target calcium signaling enzymes.

This investigation has demonstrated that acute WIN treatment of primary murine white adipose tissue affects lipid metabolism in the presence of insulin, and that these changes are driven by altered Akt phosphorylation and perhaps by negative regulation of the calcium signaling pathway. Besides directly measuring adipose tissue cAMP and Ca^{2+} levels with WIN treatment, further investigation should assess the phosphorylation/activity of effector proteins of the respective pathways, for example PKA or CaMKs, as well as the enzymes that mediate lipolysis, such as ATGL and HSL. Further investigation should also determine whether Trpv1, Trpv2, Trpa1, Calm1, or Atf4 are necessary for WIN to inhibit insulin action. These studies should also be extended to the various adipose tissue depots and even other tissues to identify any tissue-specific effects. Given the widely-held understanding that subcutaneous adipose tissue (SAT) expansion is associated with improved glucose and insulin tolerance compared to visceral adipose tissue (VAT), cannabinoids may play a role in the development of obesity by negatively affecting the insulin sensitivity of SAT in addition to VAT. The potential involvement of other Trp channels should also be investigated. Finally, follow-up studies should investigate the degree to which CB1-dependent signaling is involved in the observed effects. This could be done through adipocyte-specific CB1 knockout models.

The results reported in the current study highlight the relevance of peripheral cannabinoid action in the regulation of energy metabolism. mRNA sequencing analyses highlight the calcium, insulin, and cAMP signaling pathways as targets of cannabinoid action in adipose tissue. These findings were supported by the inhibitive effect of WIN on the antilipolytic action of insulin observed in parallel with an inhibition of insulin-stimulated Akt phosphorylation. These results also suggest that cannabinoid action can reduce adipose tissue insulin sensitivity in as little as 1 hour, perhaps through calcium-mediated signaling and/or non-CB1 receptor-mediated effects. With the growing popularity and consumption of medicinal and recreational cannabis worldwide, it will be increasingly important to understand the mechanisms by which peripheral cannabinoid action impacts physiology alongside the well-known centrally-mediated psychological and behavioral effects.

CHAPTER 4:

CONCLUSIONS AND FUTURE DIRECTIONS

The research presented in this dissertation reflects the work of two projects that have produced novel findings across the fields of endocrinology, metabolism, and nutrition. First discussed was the impact of vertical sleeve gastrectomy on the way hunger and appetite are experienced throughout the day of a laboratory session 2-weeks before and 12-weeks after surgery. To our knowledge, no data gathered from such a design has been published. Secondly, the role of cannabinoid action in adipose tissue was explored through mRNA-sequencing, qRT-PCR, and functional assays in primary murine white adipose tissue treated with the synthetic cannabinoid WIN 55,212-2. This chapter serves to summarize these studies and to discuss potential avenues of future research to build upon the foundations laid here.

4.1 Weight-loss Surgery, Hunger, and Appetite

One of the biggest challenges in the management of obesity is the prevention of weight-regain after successful weight loss. To this day, the most effective intervention for severe obesity is bariatric surgery, which can include a food-restrictive component, a malabsorptive component, or both. Our study investigated the effect of vertical sleeve gastrectomy (VSG) on subjective ratings of hunger and appetite, as well as on the hunger-mediating hormones leptin and ghrelin. This study clearly demonstrated that compared to before surgery, VSG resulted in a marked reduction in overall hunger and appetite, as expected. One of the most interesting and novel findings was that, relative to baseline, twelve weeks after surgery fasting serum levels of leptin and ghrelin had changed in favor of

increased food intake, despite subjective ratings of hunger and appetite being reduced overall. This was puzzling as it is not quite clear how reduced leptin and elevated ghrelin would not be associated with increased hunger and appetite. One explanation may be that the persistent nausea commonly associated with bariatric surgery leads to aversive conditioning and suppressed appetite [342]. Another hypothesis is that sensitivity to leptin is significantly enhanced by such a proportion that the observed leptin levels are sufficient to overcome the hunger drive, which further implies that subjects were leptin resistant before surgery. Hyperleptinemia is the key marker of leptin resistance, though there are no other criteria for its diagnosis. Moreover, the mechanisms underlying leptin resistance are not completely understood, though it is thought to involve suppressed JAK-STAT signaling in the hypothalamus, and evidence for altered leptin transport across the blood-brain-barrier, ER stress, and hypothalamic inflammation have been reported [343, 344].

Yet another hypothesis is that levels of other hunger- and appetite-mediating hormones such as peptide-YY (PYY), gastric inhibitory polypeptide (GIP), and glucagon-like-peptide-1 (GLP-1) are changed to oppose food intake. Indeed, a recent meta-analysis investigating the effect of sleeve gastrectomy on ghrelin, GLP-1, PYY, and GIP found that among VSG subjects with an average follow-up time of 11.70 ± 11.38 months, fasting ghrelin levels decreased, in contrast to our findings, whereas GLP-1 and PYY were increased post-surgery, and GIP was not significantly different [345]. These findings are evidence in support of the notion that bariatric surgery not only restricts food intake and nutrient absorption, but also results in a radical change in GI hormone secretion to oppose food intake that persists

even after significant weight-loss. Besides ghrelin, our study did not measure the levels of these hormones, however. We also found fasting ghrelin to be increased post-surgery, which may be due in part to our relatively early follow-up time of only 12-weeks, when subjects still had obesity and thus were likely to be in a state of defending against weight-loss.

A particular limitation of this study that relates to the third chapter of this dissertation was that no measurements of circulating endocannabinoids were made. Data on these mediators of appetite, plus measurements of GLP-1, PYY, and GIP would inform on the effects of bariatric surgery on both homeostatic and hedonic food motivation. Further, if combined with assessments of systemic and tissue-specific insulin sensitivity and expression levels of ECS machinery, the data would provide a more holistic representation of the central and peripheral mechanisms underlying the effects of bariatric surgery on hunger and appetite. Additionally, levels of inflammatory markers and adipokines could inform on relationship between endocannabinoid levels and the low-grade inflammation associated with obesity. Identifying the central and peripheral mechanisms that support and challenge the maintenance of weight-loss following bariatric surgery may inform the research and development of more specifically targeted therapies that support the beneficial effects of bariatric surgery while limiting the negative side effects. Further discovery and innovation may even leverage our knowledge of the underlying mechanisms to eliminate the necessity of surgical intervention altogether.

Lastly, obesity and diabetes are a concern throughout the developed world. Just as the burden of these diseases varies by individual, so it does by ethnicity. Interestingly, there is

clear evidence for variation in weight-loss for different ethnic groups, but the improvements to insulin sensitivity following bariatric surgery are significant across all groups [346]. Future studies should examine the influence of ethnicity, culture, and socioeconomic background on bariatric surgery outcomes.

4.2 Cannabinoid Action in Adipose Tissue

This investigation follows in the footsteps of over three decades of research that has largely focused on the central mechanisms of action by which cannabinoids affect health and behavior. Only in the last decade—after the failure of rimonabant—has research into the peripheral endocannabinoid system made significant strides in our understanding of the potential mechanisms by which whole-body energy homeostasis might be modulated by peripheral cannabinoid action.

Through an analysis of the differentially expressed genes in primary murine white adipose tissue, we reported that the insulin, calcium, and cAMP signaling pathways were all modulated by the synthetic cannabinoid WIN 55,212-2 (WIN). Specifically, we found that WIN inhibited each of these pathways, which was in line with our observations of attenuated antilipolytic action of insulin and reduced insulin-dependent Akt phosphorylation. A clear follow-up to these results would be to investigate the relative mRNA and protein expression of various anabolic and catabolic genes in adipose tissue under the same conditions as our anti-lipolysis assays. A rigorous transcriptomics and proteomics approach would generate more than enough data to explore the impact of WIN or other cannabinoids. This experiment could also be expanded to other metabolic tissues in order to better understand the collective

role of peripheral cannabinoid action in regulating energy metabolism.

The results of our study leave a number of questions unanswered, primarily the CB1-dependence of the observed effects. This might be addressed by similarly assessing the effect of WIN in the adipose tissue of CB1-deficient mice, or in the adipose tissue of adipose tissue-specific CB1-knockout mice. This could reveal whether endocannabinoids play a much larger role in energy homeostasis than previously thought. Indeed, a recent report demonstrated that adipocyte-specific inducible deletion of the CB1 receptor protects against diet-induced obesity (DIO) in mice on a high-fat diet (HFD), and even reverses the phenotype in already obese mice, ostensibly through increased energy expenditure and adipocyte browning [156]. Further investigation is required to corroborate these findings, which did not investigate the insulin sensitivity or lipid metabolism of the CB1-deficient adipose-tissue in these mice. Thus, a clear follow-up to our study would be to repeat our experiments in CB1-deficient primary adipose tissue.

We would be remiss to not consider the impact of diet, food intake, and sleep on cannabinoid action. Future research could repeat our experiments on the adipose tissue of mice that are fasted prior to sacrifice. The negligible basal Akt phosphorylation in our study may be due to the *ad libitum* feeding of the mice, thus fasting the mice would potentially enhance the insulin sensitivity of the adipose tissue, and may lead to an enhanced WIN response. Alternatively, a DIO mouse model might attenuate the insulin sensitivity of adipose tissue, theoretically suppressing the potential WIN response. Furthermore, a sleep-restriction model would likely lead to greater ECS activity, increased food intake without

obesity, and insulin resistance [166]. Given that many people report consuming cannabis to cope with anxiety or sleeplessness, understanding the response of sleep-restricted adipose tissue to cannabinoids could yield physiologically relevant results. Within each of these potential models, tissue-specific gene expression and signaling would demonstrate how various tissues respond to cannabinoids, and contribute to a holistic understanding of central and peripheral cannabinoid action.

The results of this study are far from a complete illustration of the impact of peripheral cannabinoid action. Moreover, whether the effects of a potent synthetic cannabinoid like WIN can be extrapolated to the physiological impact of phytocannabinoids like THC is unclear. While our study demonstrated that acute *ex vivo* treatment of primary adipose tissue with WIN caused insulin resistance in the adipose tissue, it is unknown if a analogous result would be seen in humans following acute cannabis use. Future studies should compare the systemic and tissue-specific insulin sensitivity in lean and obese humans following acute and chronic administration of THC. Such a study should result in more physiologically relevant findings, which in turn could lead to clearer public health guidance with regard to medicinal and recreational cannabis use.

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