

ABSTRACT

Title of Dissertation: O-GLCNACYLATION IS NOT INCREASED
IN THE HYPOTHALAMUS OF RATS
GIVEN 6 WEEK ACCESS TO SUCROSE
SOLUTION DESPITE MARKERS OF
METABOLIC DYSREGULATION

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The peptide hormone leptin acts globally to maintain various metabolic processes. Impaired response to leptin binding is referred to as leptin resistance and results in metabolic dysregulation. Leptin is essential in the prevention of weight gain through central signals to increase energy expenditure and reduce food intake. A sugar sensitive pathway, the hexosamine biosynthesis pathway (HBP), may be the cause of diet induced leptin resistance. The HBP glycosylates proteins by modifying fructose-6-phosphate molecules from glycolysis. While high sugar diets have been linked to leptin resistance, O-GlcNAcylation of pathway proteins have not been examined. Approximately 8-week-old male rats were assigned to *ad libitum* access to diet and water or 30% sucrose solution, diet and water. On Day 5 rats were surgically fitted with a third ventricle cannula. On Day 41, diet and sugar solutions were removed for an overnight fast. On Day 42 each rat received a central injection of leptin or control

solution and subsequently euthanized 30 minutes post injection. Body weight and body composition were not significantly different between treatment groups after 42 days. However, the Sucrose group exhibited signs of metabolic syndrome, evidenced by increased fasting serum triglycerides and glucose as well as decreased serum HDL. Analysis of hypothalamic O-GlcNAcylation revealed no significant difference between treatment groups. These data may be the result of variability of glucose utilization within the hypothalamus. These data support previous findings that 42-day access to a 30% sucrose solution yields evidence of metabolic syndrome in the absence of obesity as well as the absence of increased hypothalamic O-GlcNAcylation. Future research should examine O-GlcNAcylation regionally within the hypothalamus. Analysis of protein specific O-GlcNAcylation was not achieved; however, a novel O-GlcNAcylation was observed in hypothalamic tissue at the Threonine 1808 residue of prolow-density lipoprotein receptor-related protein 1 isoform X1 (LRP-1), a protein that may play a crucial role in leptin signaling. These data give further evidence to support the use of 30% sucrose solution to model leptin resistance in Sprague Dawley rats, as well as provide a target protein for future analysis.

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RATS GIVEN 6 WEEK ACCESS TO SUCROSE SOLUTION DESPITE
MARKERS OF METABOLIC DYSREGULATION

by

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Dedication

To my husband, this would not be possible without you. Your emotional support picked up my spirits when I could not. Your physical support picked up the pieces of my life that I could not. You pushed me to trust in myself and in God. I love you more than I can express.

To my family, thank you for your patience, your love and your support. To my dad, I wish you could have been here to see it all and for one more hug. I carry you in my heart always.

To my daughter Blair, you are an amazing person. You were here for day one of college and you never once asked me to quit. How one little girl can be so kind, mature, and loving; I don't know. All I know is you are special, and you have given me everything. The first thing I ever knew I was truly good at was being your mom. I still had a lot of growing up to do when you were born but knowing I was a good mom, despite the circumstances, gave me the confidence to try for more. I would have never tried for a Masters or PhD without you. I love you, you're the light of my life.

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List of Abbreviations

Abbreviation	
ABC	ATP Binding Cassette
AGRP	Agouti Related Protein
AKT	Protein Kinase B
apo	Apolipoprotein
ARC	Arcuate
BBB	Blood brain barrier
C/EBPα	CCAAT/Enhancer-Binding Protein α
CID	Collisionally Induced Dissociation
DM	Dorsomedial Hypothalamic Nuclei
DNL	De Novo Lipogenesis
ETD	Electron transfer dissociation
F6P	Fructose-6-Phosphate
FoxO1	Forkhead Box O1
G6P	Glucose-6-Phosphate
GFAT/GFA	Fructose-6-Phosphate Amidotransferase
GlcN-6-P	Glucosamine-6-Phosphate
GLM	General Linear Model
HBP	Hexosamine Biosynthesis Pathway
Hyp	Hypothalamic
ICV	Intracerebroventricular
IRS	Insulin Receptor Substrate
Jak2	Janus Kinase 2
LH	Lateral Hypothalamic Nuclei
LIRKO	Liver Insulin Receptor Knockout
LRP-1	Prolow-density Lipoprotein Receptor-related Isoform X1
MetS	Metabolic Syndrome
MI	Myocardial Infarction
MT	Mammillothalamic Tract
O-GlcNAc	O-linked N Acetylglucosamine
OCh	Optic Chiasm
OGA	B-D-N Acetylglucosamine
OGA-L	Long B-D-N Acetylglucosamine
OGA-S	Short B-D-N Acetylglucosamine
OGT	O-linked N Acetylglucosamine Transferase

pAKT	Phosphorylated Protein Kinase B
PI3K	Phosphoinositide 3-Kinase
POMC	Pre-opiomelanocortin
pSTAT3	Phosphorylated STAT3
PTM	Post Translational Modification
PVN	Paraventricular Nucleus
S727	Serine Residue 727
SHP	Small Heterodimer Partner
SOCS	Suppressors of Cytokine Signaling
STAT	Signal transducer and Activator of Transcription
T317	Threonine Residue 317
UDP-GlcNAc	UDP-N Acetylglucosamine
VH	Ventromedial Hypothalamic Nuclei
VMH	Ventromedial Hypothalamus
Y705	Tyrosine Residue 705

Introduction

Metabolic Syndrome (MetS) is a collection of defined metabolic abnormalities that include elevated markers including fasting triglycerides and fasting glucose as well as anthropometric measures such as waist circumference. Possessing three or more of the parameters used to define MetS has been shown to increase the risk of developing diseases like type 2 diabetes and cardiovascular disease. For example, the risk of developing type 2 diabetes is 5-fold higher and cardiovascular disease is 2-fold higher in MetS patients. (K. G. Alberti et al., 2009) Further, MetS patients are at a 3-4 fold increased risk of a myocardial infarction (MI) with a 2 fold increase in the likelihood of dying from the MI. (K. G. M. M. Alberti, Zimmet, & Shaw, 2005)

Leptin, a hormone primarily secreted by adipocytes, circulates in concentrations that reflect adiposity. The greater the adiposity, the higher the leptin concentration in circulation. (Maffei M, 1995; Tobe K, 1999) Further, leptin acts both peripherally and centrally to regulate functions such as fat deposition and appetite, respectively. Leptin resistance, when tissues no longer respond to circulating leptin, causes hyperphagia, obesity and insulin resistance. Exogenous leptin treatment does not reverse leptin resistance, suggesting the leptin receptor or signal cascade has been disrupted. Leptin resistance phenotypes, obesity and insulin resistance, are diagnostic parameters for MetS. Thus, understanding the cause of leptin resistance is a crucial step in preventing and reducing the risk to other diseases.

Recent evidence suggests that a nutrient sensing pathway involving the posttranslational modification of proteins may be the molecular cause of leptin resistance. The Hexosamine Biosynthesis Pathway (HBP) uses glucose and glucosamine as substrates to produce the end product, UDP-N acetylglucosamine (UDP-GlcNAc). This high-energy sugar is then used as the

protein modifying substrate. O-GlcNAcylation, resulting from the O-linked addition of UDP-GlcNAc to serine and threonine residues, modifies protein activity primarily through influences on phosphoprotein interactions.

High sugar diets can lead to leptin resistance without obesity, presumably by increasing glucose availability for the HBP. (R. B. Harris & Apolzan, 2015) Glucosamine infusion can lead to leptin resistance without obesity, further suggesting the cause of leptin resistance may be related to nutrient flux through the HBP as glucosamine enters the pathway unregulated. (Vasselli, Scarpace, Harris, & Banks, 2013) Additionally, removal of high sucrose and high fat choice diets reverses diet induced leptin resistance in as little as 4 days, further linking nutrient availability to leptin resistance as a function of hexosamine signaling. (Apolzan & Harris, 2013)

Leptin signaling is dependent on activation of two signaling pathways. Signaling is activated in a Janus Kinase 2 (JAK2) -dependent and JAK2-independent manner. One major signaling pathway involves transducing leptin signaling through transcriptional regulation by signal transducer and activator of transcription (STAT) 3. Activation of STAT3 begins by phosphorylation of tyrosine 705 (Y705) residues and the subsequent dimerization between two phosphorylated STAT3 (pSTAT3) molecules. However, selective inhibition of pSTAT3 at serine 727 (S727), a second STAT3 residue possibly essential to leptin signaling, reduced DNA binding of STAT3 even though pSTAT3 (Y705) were unchanged. (Gewinner et al., 2004) A second major signaling pathway, Phosphoinositide 3-Kinase (PI3K), regulates acute responses to leptin on feeding. (Hill et al., 2008) While data suggests there is no contribution of PI3K signaling to the long-term effects of leptin on body weight, a distal target of PI3K signaling, forkhead box O1 (FoxO1), may be an important component as FoxO1 promoter sites are adjacent to STAT3. (Kitamura et al., 2006)

Central but not peripheral leptin signaling is essential to preventing the phenotypes associated with leptin resistance. Specifically, leptin signaling in the arcuate nucleus (ARC) of the hypothalamus is a major target of circulating leptin as the ARC is rich with appetite modulating neurons.(Shutter JR, 1997) Furthermore, the two major neuronal populations in the ARC have opposing effects on appetite and are inversely regulated by STAT3 and FoxO1. Leptin induced PI3K signaling is responsible for regulating ion channels in both populations, inhibiting appetite stimulating neurons and increasing activity of appetite suppressing neurons (see Illustration 1). Transcriptional competition between STAT3 and FoxO1 is also present in both neuronal populations. Furthermore, the transcriptional activity of STAT3 and FoxO1 are opposing which leads to opposite effects on energy balance within the two neuronal populations. (Kitamura et al., 2006) STAT3 acts to reduce appetite by promoting anorexigenic signaling and inhibiting orexigenic signaling whereas the opposite is true for FoxO1. As a result, dysregulation of either STAT3 or FoxO1 could impair leptin signaling that ultimately results in impaired metabolism.

Given the importance of STAT3 and FoxO1 signaling in the hypothalamus and the link between hexosamine biosynthesis pathway flux and leptin resistance, it's important to consider the potential modification of these factors by hexosamine signaling. Indeed, high sucrose diets have been shown to increase O-linked N-acetylglucosamine (O-GlcNAc), the protein bound form of UDP-GlcNAc, in the hypothalamus of leptin resistant mice.(Zimmerman & Harris, 2015). In the hypothalamus of the same animals, phosphorylation of pSTAT3 (S727) was decreased, even though STAT3 and pSTAT3 (Y705) concentrations were independent of leptin sensitivity. These data suggest O-GlcNAc may inhibit the phosphorylation of the S727 residue; thereby reducing STAT3 activity. Additionally, FoxO1 has been shown to be O-GlcNAcylated in

liver tissue, a modification that did not reduce FoxO1 transcriptional activity. (Housley et al., 2008) Together these data suggest O-GlcNAcylation may reduce STAT3 transcriptional activity and impair the inhibition of FoxO1 transcriptional activity. Further analysis is required to definitively model these effects in relevant leptin resistant tissue rather than correlative data or data from another disease state.

For this reason, we propose exploring O-GlcNAcylation of leptin signaling proteins in the hypothalamus of Sprague Dawley rats given long-term access to sucrose solution. Our first goal is to demonstrate the reproducibility of modeling diet induced leptin resistance using *ad libitum* access to a 30% sucrose solution. We then aim to analyze O-GlcNAcylation in the hypothalamus by quantifying total O-GlcNAc levels of the hypothalamus and examining phosphorylation of Protein Kinase B (AKT) at the serine 473 site as O-GlcNAcylation has been shown to reduce phosphorylation and as a result enzymatic activity of AKT. Further, FoxO1 is marked for degradation by phosphorylated AKT. Finally, we will attempt to analyze O-GlcNAcylation of proteins in the hypothalamus using enzymatic tagging followed by copper click chemistry which biotinylates O-GlcNAcylated proteins. Biotinylation will allow for enrichment of O-GlcNAcylated proteins for mass spectrometric analysis, as the abundance of the post translational modification (PTM) is too low for analysis without enrichment.

Hypothesis 1: Sprague-Dawley rats given *ad libitum* access to 30% (w/v) sucrose solution for 42 days will exhibit markers of metabolic syndrome without the presence of obesity.

Hypothesis 2: O-GlcNAcylation of the hypothalamus will be increased in Sprague Dawley rats given 42 day *ad libitum* access to 30% (w/v) sucrose solution.

Hypothesis 3: Novel O-GlcNAcylation sites will be observed in hypothalamus of Control fed and/or Sucrose fed rats.

Chapter 1: Literature Review

Introduction to Leptin

Leptin is a hormone primarily secreted by adipocytes, though under certain conditions other tissues express leptin in small amounts. In contrast, leptin's signal transducing receptor, ObRb, is ubiquitously expressed and is expressed in higher concentrations in non-adipocytes than adipocytes. The ubiquity of the ObRb receptor suggests leptin has pleiotropic effects. Indeed, leptin is known to play key roles in both energy balance and reproduction; thus, leptin and leptin receptor knockout results in obesity and infertility. The role of leptin in energy balance is particularly interesting given that leptin expression increases with adiposity and central leptin is a potent suppressor of appetite. However, in obese subjects, plasma leptin concentrations are significantly higher than in lean subjects. These data suggest leptin resistance, a physiological state in which tissue no longer responds to endogenous or exogenous leptin, is a function of impaired receptor signaling or signal transduction. Leptin resistance leads to obesity and can lead to diabetes; as a consequence, understanding the molecular basis for leptin resistance is important in order to develop preventative measures and treatments.

Leptin

Leptin is a 16 kDa peptide hormone best known for its potent effect on energy homeostasis. The discovery of leptin and its crucial role in energy balance occurred well before the hormone itself was identified. Jackson Laboratories developed a strain of mice with an unknown genetic mutation that resulted in a hyperphagic, obese and sterile rodent, now known to be the leptin knockout model or ob/ob mouse.(Ingalls AM, 1996) Through parabiosis, the

surgical pairing of two animals' circulatory systems, a wild-type mouse paired to an *ob/ob* mouse could restore normal food intake and body weight. This experiment clearly demonstrated the *ob/ob* phenotype was caused by the deletion of a circulating factor and with the incidence of obesity increasing researchers were particularly interested in discovering the deleted molecule. Once the advancement of positional cloning became available the *ob* gene and its product, leptin, were identified. Upon discovery, leptin was identified as a member of the cytokine family. Cytokines are small proteins that are expressed from one type of cell and have an effect on the behavior of another type of cell. Conforming to characteristics of cytokine family peptides, leptin is expressed by adipocytes and affects the behavior of many other cell types. As a result, leptin is often referred to as an adipokine.

As an adipokine, factors that regulate adipose are often regulators of leptin expression, including food intake, insulin and corticosteroids to name a few. CCAAT/Enhancer-Binding Protein α (C/EBP α) is a unique regulator of adipose tissue as it is expressed in high concentrations during adipocyte development and remains high throughout maturity. Upstream DNA of the *ob* gene contain sequences specific to C/EBP α binding. (Miller et al., 1996) C/EBP α significantly increases leptin gene expression in primary adipocytes. Through integrating pathways, insulin and corticosteroids modulate C/EBP α activity to regulate leptin expression. Other factors like adiposity and adipocyte size regulate leptin expression. The relationship of leptin expression and adiposity are important components in obesity research given leptin's effect on satiety.

Leptin has been referred to as a "satiety hormone" due to its potent effect on decreasing food intake and increasing energy expenditure, whether centrally or peripherally administered. Given the relationship between leptin and adiposity, it comes as no surprise that during fasting

serum leptin levels decrease while refeeding returns serum leptin levels. Interestingly, the decreases in leptin concentrations during fasting are greater than the decreases in body weight. Obese humans and rodents have high circulating leptin concentrations. (Maffei M, 1995) High circulating leptin should cause hypophagia when in fact the opposite is observed. These data strongly support the theory that leptin related obesity is most likely a factor of signaling rather than expression. In fact, leptin receptor knockout mice or db/db mice are known to share a similar phenotype to ob/ob mice except db/db mice are diabetic, hence the abbreviation “db”.

Leptin Receptors

Leptin receptor or ObR is present in six isoforms, ObRa- ObRf. Each isoform is created via alternative splicing. As a consequence, domains are mostly homologous between the isoforms though functions are not. For example, the extracellular domains of the 6 isoforms are uniform. Apart from ObRe, transmembrane domains are uniform as well. However, of the six isoforms only one, ObRb, is known to transduce leptin signaling. Also known as the long form, ObRb, is the only isoform with a large intracellular domain capable of transducing leptin signaling (see Illustration 2).

However, ObRb is not the only isoform relevant to leptin activity. ObRa and ObRc transport leptin across the blood brain barrier (BBB). The two receptors are saturated during leptin resistance and may contribute to obesity by reducing central leptin. However, saturation of ObRa and ObRc are not considered to be the sole cause of leptin resistance as central leptin administration is not sufficient to reestablish metabolic homeostasis. (R. B. Harris & Apolzan, 2015) Further, the ObRe isoform is secreted into circulation and modulates bioavailability of

leptin.(Ge, Huang, Pourbahrami, & Li, 2002) Together these receptors are responsible for the movement and signaling of leptin; but are not considered to be the cause of leptin resistance.

Receptor Signaling

Upon leptin binding to ObRb, the receptor dimerizes to initiate leptin signaling. JAK2 is constitutively bound to the intracellular domain of the receptor and is activated upon leptin binding. Additionally, JAK2 independent activation of the pathway occurs via the Src family of kinases.(Jiang, Li, & Rui, 2008) JAK2-dependent and independent pathways work in concert to activate target pathways. Neither deletion of JAK2 nor blocking Src kinases like c-Src or Fyn completely ablates leptin signaling. (Jiang et al., 2008)

Leptin signaling is integrated into several pathways. STAT3 is the primary regulator of transcriptional activity by leptin. The JAK/STAT pathway is a basic pathway for cytokines. Phosphorylation of ObRb tyrosine 1138 residue recruits STAT3 to bind to the receptor. Once recruited, JAK2 will phosphorylate STAT3 (Y705) residues, which activates STAT3 and promotes dimerization. Upon dimerization, STAT3 is translocated to the nucleus by the Importin-a-Importin-b1 heterodimer pathway, where STAT3 binds TTC/AT/CG/TGGAA sequence to regulate transcription. Among the factors regulated by STAT3, suppressors of cytokine signaling (SOCS) 3 transcription is upregulated by STAT3 as a negative feedback loop. By binding tyrosine 985 residue on the leptin receptor, SOCS3 inhibits leptin signaling. While overexpression of SOCS3 can inhibit STAT3 activation, SOCS3 is unlikely to cause leptin resistance as SOCS3 expression has been shown to decrease in diet induced leptin resistance compared to control. (Reed et al., 2010; Zimmerman & Harris, 2015)

Central STAT3, specifically within the hypothalamus, regulates the expression of orexigenic and anorexigenic proteins. Pro-opiomelanocortin (POMC) is a strong inhibitor of food intake.(Yaswen L, 1999) Targeted ablation of POMC neurons leads to obesity and hyperphagia.(Xu, Kaelin, Morton, et al., 2005) The expression of POMC is increased during leptin signaling, as STAT3 binds the promoter region to increase transcription. Further, leptin also acts to reduce orexigenic signals like Agouti Related Protein (AGRP) by transcriptional repression via STAT3 binding in the promoter region. STAT3 conditional knockout mice exhibit leptin resistance phenotypes like obesity, diabetes and infertility.(Gao Q, 2004) Thus, STAT3 signaling plays an essential role in the effect of leptin on energy balance.

STAT3 phosphorylation and subsequent dimerization occurs at the Y705 residue. This step is essential for STAT3 translocation and transcription in the nucleus. However, recent evidence has emerged that a second residue on STAT3, S727, may be a required component to STAT3 action. (Gewinner et al., 2004) As demonstrated in macrophages, leptin increases phosphorylation of Y705 and S727 residues of STAT3. Inhibition of S727 phosphorylation significantly inhibits DNA binding by STAT3.(Gewinner et al., 2004) Furthermore, it is still unknown if phosphorylation of S727 is essential in all cell types. Interestingly, hypothalamic tissue and HepG2 cells show reduced phosphorylation of S727 but not Y705 during leptin resistance, suggesting the role of S727 in leptin signaling as well as leptin resistance. (Zimmerman & Harris, 2015)

Some of the effects of leptin signaling are rapid; therefore, STAT3 cannot be solely responsible for the effect of leptin due to the time required for transcription. Another pathway, most likely involving phosphorylation, would be required to modify proteins rapidly to transduce acute signals. In fact, PI3K signaling is required for the acute effects of leptin in the brain by

acting on ion channels. (Hill et al., 2008) PI3K is a key component in the PI3K/AKT/MTOR pathway known to be responsive to hormones and growth factors. PI3K is activated upon leptin binding through the formation of a tertiary complex of JAK2, Small Heterodimer Partner (SHP)-B and Insulin Receptor Substrate (IRS)-1 or IRS-2.(Duan, Li, & Rui, 2004) SHP-B binds JAK2 at Tyrosine 813 to enhance JAK2 autophosphorylation.(Duan et al., 2004) While SHP-B is bound to JAK2, SHP-B binds and activates IRS-1 or IRS-2, the rate-limiting step for PI3K activation. The p85 regulatory subunit of PI3K can then bind to the tertiary complex containing activated IRS1/2. Upon binding, conformational changes to the subunit activate the catalytic p110 subunit of PI3K. In turn PI3K activates target molecules like cation channels in POMC neurons to depolarize and increase firing rate.

Interestingly, there appears to be no effect of PI3K signaling on the long-term effects of leptin. However, a distal target of PI3K signaling, FoxO1, does play a role in the effects of leptin signaling. FoxO1 is a transcription factor that regulates the transcription of many targets including POMC and AGRP. Furthermore, FoxO1 competes with STAT3 on POMC and AGRP promoter regions and acts to oppose STAT3. Under insulin and leptin signaling, FoxO1 is phosphorylated by PI3K target AKT to cause nuclear exclusion and degradation.

Central vs. Peripheral Signaling

Given the ubiquity of leptin receptor expression, leptin signaling is important in many tissues. However, the regulation of food intake and metabolism is controlled centrally. Peripheral knockouts of the leptin receptor exhibit normal energy balance and insulin sensitivity. (Guo et al., 2007) Clinically, peripheral leptin injections are used to treat lipodystrophy, a condition of impaired adipose depots. As a result, lipodystrophic patients exhibit insulin resistance due to

ectopic fat deposition. Increasing beta-oxidation via leptin administration is a common treatment for lipodystrophy. However, lipodystrophy is a disease of impaired leptin expression, rather than leptin signaling. As previously mentioned, leptin injections do not reverse leptin resistance, because leptin signaling is impaired. Therefore, exploring central tissues rather than peripheral tissues is pertinent to understanding leptin resistance.

Leptin receptors are expressed throughout the brain, including the hindbrain and hypothalamus.(Tartaglia et al., 1995) All nuclei within the hypothalamus express ObRb. Leptin binding increases projections between hypothalamic nuclei. Hypothalamic control of energy balance is complex though it remains clear that the ARC is a major contributor of leptin's effect on satiety. Located at the base of the hypothalamus, the ARC is rich with both POMC and AGRP neurons, both of which co-express ObRb. Leptin regulates POMC and AGRP neurons in two ways. First, leptin depolarizes both POMC and AGRP neurons in a PI3K dependent manner. AGRP depolarizes from leptin withdrawal where as POMC depolarizes from leptin addition. (Xu, Kaelin, Takeda, et al., 2005) Secondly, leptin regulates the expression of POMC and AGRP by promoter binding of STAT3 and FoxO1.(Kitamura et al., 2006) Deletion of ObRb in either or both neuronal populations of young rats results in hyperphagia, reduced energy expenditure and increased weight gain. Additionally, hyperinsulinemia occurs in the same knockout model and is partially restored upon unilateral ARC ObRb recovery.(Coppari et al., 2005) Further, the ARC projects to all other hypothalamic nuclei. Therefore, the ARC is a prime region for impaired leptin regulation.

PI3K is essential to the acute effects of leptin signaling in both POMC and AGRP neurons. Leptin induced activation of PI3K phosphorylates mediators that increase the firing rate of POMC neurons. (Hill et al., 2008) Additionally, activation of PI3K is essential for insulin and

leptin induced reduction of FoxO1. (Kim et al., 2006) Phosphorylation of FoxO1 by AKT marks FoxO1 for degradation in the cytoplasm, a process known as nuclear exclusion. However, without phosphorylation, FoxO1 will remain in the nucleus to regulate transcription, specifically repressing POMC transcription and enhancing AGRP transcription to increase appetite (see Illustration 3). Furthermore, leptin signaling reduces the expression of FoxO1, while constitutively active FoxO1 inhibits the effect of leptin on food intake.(Kim et al., 2006) Furthermore, STAT3 competes with FoxO1 to increase POMC transcription and repress AGRP transcription. Coexpression of STAT3 and FoxO1 in AtT20 cells reduces FoxO1 induced AGRP promoter activity whereas the opposite effects were seen in POMC promoter activity. (Kitamura et al., 2006) Further, POMC and AGRP promoter regions contain adjacent binding sites for STAT3 and FoxO1 where the two transcription factors inhibit the others activity through transcriptional squelching. Given the effect of leptin on ARC AGRP and POMC expression as well as the regulatory effects of STAT3 and FoxO1 on those peptides, dysregulation between these two transcription factors in the ARC are potentially major players in leptin resistance. However, it remains unclear what causes the dysregulation of these factors, though diet induced leptin resistance suggests a potential pathway.

Introduction to Hexosamine Pathways

The HBP is designed to use nutrients to produce protein modifying O-linked hexosamines. O-GlcNAcylation is unique compared to other protein glycosylations, which are typically added to proteins to form long glycan chains and are often membrane associated. Similar to phosphorylation, O-GlcNAcylation of proteins acts to modulate protein activity

through the addition of a single modifier. Interestingly, the HBP has approximately 1,000 target proteins but only uses two enzymes to add and remove the glycosylate. In contrast, phosphorylation requires several specific kinases and phosphatases to add and remove phosphate groups, respectively. While overall metabolism produces the ATP required for phosphorylation, the substrate for HBP is supplied by nutrient flux, specifically glucose. As a result, the HBP is strongly linked to disorders related to nutrient excess.

Hexosamine Biosynthesis Pathway

HBP is a nutrient-sensing pathway used to monitor nutrient excess of lipids, sugars and glucosamine. Free fatty acids, glucose or glucosamine flux increases HBP activity.(Cooksey & McClain, 2011; Marshall S, 1991) The pathway senses nutrient excess by integrating with glycolysis. As with all pathways there are regulatory measures to supply substrate to the relevant pathway as well as feedback mechanisms to slow substrate conversion when product concentrations become too high. Typically, HBP will utilize about 1-3% of glucose to produce the end product UDP-GlcNAc.(Marshall S, 1991) Under conditions in which cellular glucose concentrations are increased, the pathway will use even more. Furthermore, high free fatty acid concentrations increase Acetyl-CoA, which increases activity of the TCA cycle, in turn reducing glycolysis. As a result, more glucose is available to shuttle into the pathway. For these reasons, HBP is considered a nutrient sensing pathway and a target for disorders of metabolism.

UDP-GlcNAc is formed through a series of enzymatic reactions involving glucose or glucosamine. The two substrates share similar pathways except glucosamine enters the pathway later, bypassing the rate-limiting step. When glucose enters the cell the conversion to glucose-6-phosphate (G6P) immediately takes place by hexokinase, requiring an ATP as a phosphate

donor. Next, G6P is converted into fructose-6-phosphate (F6P), the integrating point between glycolysis and the biosynthesis pathway. As previously mentioned, the pathway uses 1-3% of F6P but more can be used depending on nutrient status. F6P is catalyzed by the rate limiting enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT) into glucosamine-6-phosphate (GlcN-6-P) (see Illustration 4). The product of GFAT catalysis is joined by available glucosamine and quickly converted to the end product, UDP-GlcNAc. The high-energy substrate UDP-GlcNAc then covalently modifies proteins.

Protein Modification Through Hexosamine Biosynthesis Pathway

The HBP uses UDP-GlcNAc and two enzymes to catalyze the removal and addition of O-GlcNAc to residues. O-linked N-acetylglucosamine transferase (OGT) is responsible for catalyzing the addition of O-GlcNAc to proteins. Similarly, only B-D-N acetylglucosamine (OGA) is responsible for the removal of O-GlcNAc. Since there are only two enzymes involved in O-GlcNAc signaling there must be a mechanism to allow for the dynamic action of O-GlcNAcylation. Indeed, one such mechanism is that variant isoforms of each enzyme act in different locations or on different systems. OGT and OGA both exist in variable isoforms; 3 splice variants for OGT and 2 splice variants for OGA (see Illustration 5). N-Terminal repeats differentiate the structure of the OGT isoforms as well as their locations and action. Two isoforms, mOGT and sOGT, are associated with apoptosis, whereas ncOGT is present in the cytoplasm and nucleus, and acts as the major enzyme for O-GlcNAcylation. The OGA isoforms vary by structure and location. Long OGA (OGA-L) contains a pseudo HAT domain at the c terminus, whereas short OGA (OGA-S) does not have the extra C-terminal domain and is strongly associated with lipid droplets. OGA-L is responsible for two thirds of OGA activity,

made evident by OGA-L expression reverses O-GlcNAcylation by 72% whereas OGA-S reversed O-GlcNAcylation by only 35%. (Keembiyehetty, Krzeslak, Love, & Hanover, 2011)

HBP is a seemingly simple regulatory mechanism given it has only three components for signaling, whereas phosphorylation has numerous kinases and phosphatases that work specifically within their respective pathway. HBP uses only UDP-GlcNAc, OGA and OGT to regulate the same pathways regulated by phosphorylation. An additional layer of complexity to hexosamine signaling is the competition between O-GlcNAcylation and phosphates, which is divided into 3 categories. First, both posttranslational modifications can bind serine and threonine residues; therefore, O-GlcNAc can compete with phosphates to bind a specific residue. Secondly, the modifiers can compete for steric hindrance. As a result, the binding of one modifier creates steric hindrance to prevent the binding of the other at an adjacent site. Finally, both modifiers can simultaneously bind to a protein but the effect of one of the two modifiers will predominate. For example, a phosphorylated protein can become O-GlcNAcylated at which point the phosphoprotein activity will reduce while the effect of the glycosylation will take precedence.

Nearly one thousand proteins have been identified as being GlcNAcylated; including proteins of cytoskeletal networks, stress responses and transcriptional regulators. Furthermore, potential contributors to leptin resistance, STAT3 and FoxO1, are included in O-GlcNAc targets. In fact, O-GlcNAcylation of several STAT proteins was previously demonstrated in HC11 mammary epithelial cells. (Gewinner et al., 2004) In HC11 cells STAT3 was identified as a target of O-GlcNAcylation through hormonal stimulation. However, the only STAT protein thoroughly examined was STAT5. Specific data regarding target residues is unknown as well as the effect of O-GlcNAcylation on STAT3 activity. Further, FoxO1 O-GlcNAcylation is well

characterized through the use of several liver cell culture models. In addition to being O-GlcNAcylated at several sites, FoxO1 O-GlcNAcylation and phosphorylation do not inhibit the occurrence of one another. (Housley et al., 2008) O-GlcNAcylation increases FoxO1 DNA binding in FAO hepatoma cells to increase expression of gluconeogenic and stress related genes. (Housley et al., 2008) Insulin signaling or mutation of threonine 317 (T317) inhibits the effects of O-GlcNAcylation on FoxO1. The dynamics of O-GlcNAcylation in hypothalamic tissue needs to be established, as the ARC is the major target of leptin dysregulation. However, current knowledge of both STAT3 and FoxO1 O-GlcNAcylation in non-hypothalamic tissues shows great promise as a potential target of diet induced leptin resistance through impaired POMC and AGRP binding.

Diet Induced Leptin Resistance

Leptin resistance is achieved through dietary interventions like high fat, high sugar and choice diets. Sprague Dawley rats given ad libitum access to a choice diet of chow, lard and 30% sucrose solution causes peripheral and central leptin resistance by day 16. (Apolzan & Harris, 2013) Removal of choice diet returns peripheral and central leptin sensitivity within 4 days. (Apolzan & Harris, 2013) These data suggest leptin resistance is sensitive to nutrient flux, which further supports the theory that O-GlcNAcylation of leptin signaling molecules impairs leptin signal transduction. High fat diets and high sucrose diets have been shown to cause leptin resistance in 19 weeks and 4 weeks, respectively. (R. B. Harris & Apolzan, 2015; Lin S, 2000)

Detecting O-GlcNAcylation

O-GlcNAcylation is a PTM to proteins, which requires mass spectrometry techniques conducive to identifying proteins as well as site modifications. Two important factors in identifying PTMs are enriching for the modifier of interest and analyzing the sample using tandem mass spectrometry.

Enzymatic tagging and copper click chemistry facilitate enrichment of O-GlcNAcylated proteins. Copper click chemistry facilitates the reaction between highly reactive azides and alkynes. Azides are added to O-GlcNAcylated proteins through the enzymatic addition of azide containing sugar GalNAz. Once azide groups are added present the click reaction can take place through the addition of the chosen tag containing an alkyne group. Copper catalysts like CuSO₄ are used along with reducing agents like sodium ascorbate to catalyze the cyclic reaction. Additionally, polyligands like THPTA are added to accelerate the reaction. Selecting a tag that can be enriched with affinity columns, primarily biotin conjugates, allow the researcher to select for the modification and remove unmodified proteins from a complex sample. Once enriched, mass spectrometric analysis of PTMs are analyzed using tandem mass spectrometry. Collisionally induced dissociation (CID) and electron transfer dissociation (ETD) are used in conjunction to identify fragmented peptide sequences and the site of modification, respectively.

Chapter 2: Methods

Treatment Protocol

Diet

All animals were given *ad libitum* access to their respective treatment diets. A standard diet was provided to all treatment groups. The diet was a nutritionally complete blend [Teklad Global 18% Protein Rodent Diet] prepared by Envigo (Madison, WI) containing 3.1 kcal/g of diet (see Table 1). All rodents were provided with *ad libitum* access to water, while the sucrose group were also given *ad libitum* access to a 30% (w/v) sucrose solution. A 30% sucrose solution was used because a large body of data has demonstrated a 30% sucrose solution consistently induces leptin resistance as well as being the preferred model for diet induced obesities compared to other sugars, modes of delivery and percent solute.(Apolzan & Harris, 2012, 2013; R. B. Harris & Apolzan, 2012, 2015; London et al., 2007) Access to a sucrose solution results in a displacement of diet intake of approximately 60%, which helps mirror the increase of dietary sugar intake in humans. (Castonguay, Hirsch, & Collier, 1981; London & Castonguay, 2009) Further, the use of sucrose is theorized to best model human consumption of dietary sugars as the composition of dietary sugars are rarely pure glucose or fructose.

Animal Model

Adult male Sprague Dawley (CD strain) rats (Charles River Laboratories, Wilmington, MA) weighing approximately 300 g were used. All animals were individually housed under a 12-hour light/dark cycle in a temperature-controlled room ($22 \pm 1^{\circ}\text{C}$). Upon arrival, the animals

were given a 3-day acclimation period of *ad libitum* access to diet and water. On Day 1, animals were randomly assigned to two dietary treatment groups, Sucrose or Control. Rats assigned to the Sucrose group were given *ad libitum* access to sucrose solution, water and diet; rats assigned to the Control group were given *ad libitum* access to water and diet only. On Day 5, each rat was fitted with a lateral ventricle cannula via stereotaxic placement. On Day 7, cannula placement was tested using an angiotensin II (150 ng/5 ul) challenge.(Bellinger, 1997) On Day 41, food was removed at the start of the light cycle for an overnight fast. On Day 42, each rat was lightly anesthetized using Isoflurane and was then administered an intracerebroventricular (ICV) injection of either leptin (1.5 ug/2 ul) or saline (2 ul). This protocol timeline is represented in Illustration 6. Each rat was sacrificed 30 minutes post injection. All methods were approved for use by the Panel on Euthanasia of the American Veterinary Medical Association as well as the University of Maryland Institutional Animal Care and Use Committee (approved protocol # R-16-01). All procedures described herein are in compliance with and were approved by the University of Maryland's Animal Care and Use Committee guidelines.

Surgery

Rats were anesthetized via intraperitoneal injection of ketamine (100 mg/kg) xylazine (10 mg/kg) cocktail (0.2 ml/100 g). Once an animal lost motor control, the surgical site was prepped, and the rat was placed in the stereotaxic. The coordinates for placement of the cannula were lateral – 1.4 mm, AP -0.8 mm and ventral -3.3mm of bregma, using the Watson and Paxinos Rat Brain Atlas. The cannula was fixed into place using dental acrylic and post-surgical recovery was aided with a subcutaneous injection of saline immediately post-op and a subcutaneous injection of buprenorphine (0.05 mg/kg) upon return of motor control.

Sacrifice and Tissue Collection

Post injection on Day 42, each rat was returned to its cage for 30 minutes before sacrifice using a CO₂ O₂ exchange chamber. Immediately after noting its time of death each rat was decapitated and truncal blood was collected. Serum samples were collected from truncal blood after clotting and centrifugation at 1,400 x g at 4°C for 15 minutes. Collected serum was stored at -80°C. Brain and liver were dissected and flash frozen in dry ice chilled isopentane then stored at -80°C. Carcasses were collected and stored at -20°C. Brains were later thawed to -15°C for macrodissection following a protocol by E. London, personal communication (see Illustration 7).

Metabolic Profiling

Body Composition

Rat carcasses were thawed and total body composition of each was measured using an EchoMRI-900 quantitative NMR analyzer (Echo Medical Systems, Houston, TX). Output included measurement of fat mass and lean tissue mass.

Leptin and Insulin

Serum leptin and insulin concentrations were determined using the Mouse/Rat Insulin Leptin Multiplex kit (Meso Scale Discovery #K15158C; Rockville, MD) from sera of Sprague Dawley rats from each treatment group.

Glucose and Lipid Profiling

Plasma glucose concentrations were measured enzymatically (Smith-Kline Beecham Laboratories, Brentford, London). A 36 Dimension clinical chemistry system Flex reagent cartridge (Siemens Healthcare Diagnostics, Newark, DE) was used with a Dade Behring Dimension Xpand automated system to quantify plasma triglyceride. Profiling of lipids and glucose was performed using a Vitros Clinical Analyzer to measure glucose, HDL, LDL, cholesterol and triglycerides.

Protein Extraction and Digestion

Protein extracts from left hypothalamic tissue were obtained using a modified method of Ma and Hart (2016). Briefly, tissue extract was weighed and lysis buffer (Tris-HCl pH 7.5, NaCl, Triton-X, NaF, β -Glycerophosphate, PUGNAc, 1 x Halt Protease Inhibitor Cocktail) was added. Tissue was homogenized by passing sample through 18 gauge needle and subsequently sonicated twice for 10 seconds with 10 second rest in between. Samples were then incubated on ice for 30 minutes with vortexing every 5 minutes. Finally, samples were centrifuged at 10,400 x g for 10 minutes at 4°C. The supernatant was collected, protein quantification was analyzed via BCA quantitation (Pierce™ BCA Cat# 23225; Waltham, MA). Lysate was separated into aliquots and stored at -80°C.

O-GlcNAc Quantitation

Total hypothalamic O-GlcNAc was quantified using the Simple Wes system by ProteinSimple™ (San Jose, CA). Briefly, hypothalamic lysate was diluted to 0.7 ug/ul with 10mM Tris-HCl pH 7.5 and 0.1x sample buffer; O-GlcNAc antibody CTD 110.6 (Cell Signaling

Technology, Danvers, MA) was diluted 1:75 in Antibody Diluent 2 provided in ProteinSimple™ Mouse Diluent pack . Samples and reagents were loaded in duplicate onto 25 capillary plates. Plates were run using Simple Wes Total Protein protocol for all parameters including separation time, incubation times and washes. Samples were normalized to total protein in the same run by applying the adjusted total protein factor to the total chemiluminescence from the O-GlcNAc capillaries of corresponding samples. Between plate normalization was adjusted by applying total protein factor of an internal standard adjusted for between plates variations.

Statistical Analysis

Quantitative Analyses

Due to difficulties arising during surgery or post-surgery, some animals had incomplete data sets. To keep sample sizes as large as possible incomplete data sets were not immediately omitted; sample sizes are noted for all analyses. Further, rats were compared by Sucrose fed and Control fed treatments only as cannula placement could not be accurately verified. For data related to injection (leptin vs saline) please refer to the Appendix for cannula verification data via angiotensin II and pSTAT3 (Y705), hypothalamic protein analyses and enrichment of O-GlcNAcylation data.

Statistical assumptions were met for all analyses and were analyzed using the general linear model (GLM) for normality of the residuals and the Levene's test for homogeneity of the variances. Body weight and composition (Control n=26, Sucrose n=21) were analyzed using the GLM (SAS, Cary, N.C.), as this model best handles unbalanced data. Analysis for repeated measures was used to analyze the effect of time on body weight. Circulating factors and O-

GlcNAC blot (Control n=16 Sucrose n=16) were analyzed using a two sample t-test.

Correlations were analyzed using the same experimental units for analyzing circulating factors.

Pearson's correlation coefficients were calculated to determine if x and y factor were

significantly correlated in all samples (n=32) or by treatment group (n=16). Comparison of

Control vs Sucrose groups correlations were performed using Fisher's z transformation of the

Pearson correlation coefficient and defined variance of $1/(n_1-3) + 1/(n_2-3)$, where n1 and n2 are

the sample size of each treatment group.

Chapter 3: Results

Body Composition

Neither body weight nor body composition differed significantly between feeding groups during the 6-week treatment period. Body weights taken at the start of the dietary treatment were not different between treatment groups (see Table 2). Mean final body weights that were measured on Day 42 did not differ between treatment groups. The changes in body weight from Day 1 to Day 42 were not different. Mean weight gain over the 42-day experiment was 178 g (± 7.2 SEM) in Control fed rats vs 182.7 g (± 9.6 SEM) change in Sucrose fed rats (p -value=0.6891). However, there was a significant effect of time on body weight during the 6-week feeding period (see Figure 1). Further analysis showed no group differences in body weight measurements at any time during the 6-week period.

On Day 41 chow and sucrose solutions were removed from cages for a 12 hour overnight fast. The difference in body weight was measured between day 41 and day 42. The data show no difference in weight loss between these two days. Control fed rats lost an average of 2.3 g (± 1.7 SEM) whereas the Sucrose fed group lost an average of 4.6 g (± 2.0 SEM) (see Figure 2). Body composition analysis showed little difference between treatment groups as well. Both groups on average had approximately 20% fat mass (see Table 1). To analyze the effect of diet on body composition, a ratio was used of fat to lean mass (FTL ratio). The difference between FTL ratios was not significant between Sucrose and Control fed animals (see Figure 3). Together these data suggest that 42-day ad libitum access to a 30% sucrose solution did not significantly affect growth rate or body composition in male Sprague Dawley rats when compared to Controls.

Circulating factor profiling

While body composition showed little differences between treatment groups, several circulating factors that are hallmarks of metabolic syndrome were found. In particular, the lipid profile of sucrose fed rats revealed that serum triglycerides were significantly higher when compared to controls ($p=0.002$) (see Figure 4). Further, serum HDL cholesterol was significantly ($p=0.0278$) lower by approximately 14% in sucrose fed vs control fed rats. Interestingly, serum cholesterol and LDL of sucrose fed rats did not differ significantly from that of controls.

Despite the lack of difference in body fat composition, circulating leptin was significantly higher in sucrose fed animals vs control fed animals ($p=0.0362$) (see Figure 5a). Finally, plasma insulin and glucose profiling revealed no significant difference in serum insulin between treatment groups ($p=0.9451$) but revealed significantly increased serum glucose in sucrose fed animals compared to control fed animals ($p=0.0207$) (see Figure 5).

Correlating metabolic data

The correlations between metabolic factors (like body composition) and circulating factors were statistically evaluated. The FTL ratio was positively correlated with both serum triglyceride levels and leptin levels despite the lack of treatment effect on body composition as previously discussed (see Figure 6). Further, cholesterol and HDL were strongly and positively correlated with one another. However, the effect of diet on HDL dampened the correlation, resulting in a reduced correlation between the two in sucrose fed animals (see Figure 7). Similarly, HDL and glucose were significantly and positively correlated in sucrose fed rats ($r=0.636$) but not control fed rats ($r=0.097$). While the differences between groups approached significance the difference in the correlation of HDL and glucose between treatment groups was

not significantly different (see Figure 8). Finally, a moderately positive correlation between triglycerides and glucose was found in the control group (see Figure 9).

Global O-GlcNAcylation of Hypothalamus

Sucrose access results in increased activity in the hexosamine biosynthesis pathway by providing the pathway more substrate. Total O-GlcNAc chemiluminescence signal was averaged between replicates and normalized to a factor based on total protein chemiluminescence. The data was then normalized between blots using a factor between membranes. Contrary to our expectations, there was no significant difference in total hypothalamic O-GlcNAcylation between treatment groups (see Figure 10).

Chapter 4: Discussion

Diet Induced Metabolic Dysregulation

Body Composition

Our dietary treatment is modeled from *Harris and Apolzan (2015)*, where they reported that body weights were not significantly different after 44 days between rats given a control chow diet and sucrose fed rats given *ad libitum* access to a 30% sucrose solution and chow. Our results replicate the Harris findings, showing no significant difference in body weight after 42 days of *ad libitum* access to 30% sucrose solution compared to control. Other investigators have reported that sucrose solutions can induce significant weight differences in as little as two weeks.(Castonguay et al., 1981; Lindqvist, Baelemans, & Erlanson-Albertsson, 2008; Rattigan & Clark, 1984) However, those experiments may have been influenced by other factors like species, composition of chow, age and social housing. A previous study in our laboratory revealed no significant difference in body weight of male rats fed 16% or 32% sucrose solution when compared to controls for 72 days.(London et al., 2007) Our model has consistently demonstrated that long term access, greater than 72 days, to sucrose solution is required to see significant differences in body weight.(Apolzan & Harris, 2012; R. B. S. Harris, 2018; Kanarek & Orthen Gambill, 1982; Zimmerman & Harris, 2015)

Contrary to our observations that no differences in body composition at day 42 were statistically significant, Harris reported that at Day 44 the sucrose fed rats had increased body fat. We believe that these differences are a function of method rather than differing results.

Measuring body composition via echo MRI allows for the measurement of total body fat mass,

both subcutaneous and visceral. Harris' method of dissecting fat pads measures only distinct collections of fat and fails to include an accurate estimate of subcutaneous fat. Echo MRI replicates the body composition methods most like what human subjects and patients would undergo to determine excess adiposity, such as dual-energy X-ray absorptiometry as well as displacement of air or water. This includes developing an estimate for subcutaneous fat. Note that our findings do not suggest that Harris et al were in error. Fat pad analysis can be used to more effectively estimate increases in harmful depots like visceral adipose tissue. As a major contributor to metabolism, adipose tissue plays an essential role in preventing metabolic diseases such as insulin resistance. However, not all adipose tissue is metabolically equivalent. Subcutaneous and visceral adipose tissue are believed to have opposite influences on the risk of developing insulin resistance. Removal of visceral adipose tissue as well as transplanting subcutaneous adipose tissue into visceral depots have been shown to improve insulin sensitivity.(Barzilai et al., 1999; Cox-York, Wei, Wang, Pagliassotti, & Foster, 2015; Foster et al., 2013) For a more thorough review of adipose depot comparisons please refer to *Bjorndal et. Al. 2011*.

Initially we had predicted that fasting weight loss (performed after 42 days of access to sucrose) would be stunted in sucrose fed rats due to the reduction in metabolic rate that accompanies leptin resistance. However, fasting weight loss was not significantly different between treatment groups. Fasted weight loss can be used to measure differences in metabolic rate when more sophisticated equipment is not available. (Rixon & Stevenson, 1956) However, the goal of the overnight fast was to sacrifice the animals in a fasted metabolic state rather in than estimate metabolic rate. What is more, the procedure that we used was not designed for such analysis. Weights were measured early on day 41 rather than just before the start of the fasting

period, thereby increasing variability. Interestingly, during the light cycle, when rats are mostly dormant but still regularly consume calories, caloric intake is not different between control and sugar fed rats. (Castonguay et al., 1981) Rather, it is likely that the observed differences in intake occurred during the dark cycle. A more accurate protocol would better capture fasted weight loss without the variability of intake and may have captured any differences in metabolic rates.

Sucrose Solution Promotes Metabolic Syndrome

Three of the diagnostic parameters for MetS were observed in the fasting serum of our sucrose fed rats, despite no differences in body weight. Firstly, we observed an increase in serum glucose of sucrose fed rats compared to their controls. The increase in circulating glucose is potentially a result of the body's inability to properly metabolize the high supply of sugar from the diet. Rats will consume ~60% of their total daily calories from a sucrose solution. (Castonguay et al., 1981; London et al., 2007) Hyperglycemia can be attributed to different etiologies depending on the conditions. Our data suggests that impaired glucose uptake could be an indication of insulin resistance. Further, we failed to observe hyperinsulinemia despite increased glucose levels in circulation; this pattern is associated with a later stage of insulin resistance in which insulin secretions cannot keep up with the demand and are no longer elevated. The result of excess glucose, as well as fructose, is indirect stimulation of de novo lipogenesis (DNL), a process in which sugar is converted into triglycerides. (McDevitt et al., 2001) Due to the lack of regulatory steps in fructose metabolism, intake of fructose solution has been shown to induce hypertriglyceridemia in as little as 24 hours. (Colley & Castonguay, 2015) Lipids are produced in the liver as well as adipocytes as a means to store excess energy in the form of fat. We believe that sucrose access may have stimulated hepatic DNL as fasting

triglycerides of sucrose fed rats were almost doubled that of the control counterparts. It should also be noted that, serum HDL levels were reduced in sucrose fed rats. This decrease in HDL is most likely a function of the downregulation of ATP-binding cassette transporter (ABC) A1 and apolipoprotein (apo) AI by excess glucose in the diet.(Hussein et al., 2015) Together these data have allowed us to suggest that sucrose access initiates a cyclical disruption in metabolism that can lead to MetS associated diseases. To start, HDL has been shown to regulate glucose metabolism by increasing insulin secretion, glucose uptake and tissue utilization.(Drew, Rye, Duffy, Barter, & Kingwell, 2012) However, this overabundance of glucose reduces HDL production thereby impairing glucose usage to some degree. The impaired utilization of glucose as well as dietary fructose results in increased triglycerides as a means to deal with the excess substrate. Under acute conditions these effects are not pathogenic, as adipose tissue is designed to store fat. However, combined with the loss of leptin sensitivity (as evidenced by high leptin concentrations), lipids are deposited into non-adipocyte tissue such as liver and pancreas. These tissues are insufficient to store excess lipids found in circulation. As a result, glucose uptake is further impaired leading to increases in circulating glucose which continues to supply the cycle.

Changes in metabolism due to diet can be observed by examining the correlations between factors like serum markers with anthropometric measurements. An important factor in the development of cardiovascular disease is a disruption in the balance of lipids (such as cholesterol) and transport molecules (such as HDL). An analysis of our data revealed that serum cholesterol is positively correlated with serum HDL, as seen in Figure 8. As the lipoprotein responsible for reverse cholesterol transport, HDL is important in the removal of damaging cholesterol concentrations from tissue. As a consequence, a strong positive correlation is expected. However, we observed that the correlation of cholesterol with HDL is reduced in

sucrose fed rats. We believe that the difference between treatment groups is due to the reduction in HDL without a concomitant reduction in cholesterol in sucrose fed rats. When determining risk for cardiovascular disease, HDL levels must be considered in conjunction with cholesterol levels as high cholesterol levels are not always harmful if HDL levels are proportionately increased.(Taskinen et al., 2010) As previously mentioned, HDL and glucose have been shown to regulate one another.(Drew et al., 2012; Hussein et al., 2015) However, HDL is not correlated with glucose in control fed rats. Interestingly, we observed a strong correlation between the two amongst the sucrose fed rats (a correlation coefficient of 0.636). Both HDL and glucose have been shown to downregulate one another; thus, the correlation we would expect to be negative. On average, serum glucose levels increased, and serum HDL levels decreased in sucrose fed rats. This unexpected directionality of the correlate may be due to the limited down regulation of HDL by glucose, which under acute conditions the negative correlation may be observed. However, because the glucose concentrations continue to rise under the feeding conditions the correlation becomes positive. A similar response was observed in the correlation of glucose with triglycerides. Under control conditions the two factors are positively correlated. However, the correlation drops to near zero in sucrose fed rats despite triglycerides and glucose increasing on average as seen in Figure 9. The changes in these relationships suggest that glucose no longer proportionately increases triglycerides. Rather, the glucose levels suggest a phenomenon known as selective insulin resistance. Under these conditions liver tissue is insulin resistant which prevents insulin from downregulating gluconeogenic proteins. However, insulin sensitivity to insulin induced lipogenesis is maintained.(Shimomura et al., 2000) As a result, the liver contributes to both hyperglycemia and hyperlipidemia. This paradox is best illustrated by Brown and Goldstein (2008) in which liver metabolism is modeled 3 ways (see Illustration 8). First in a

normal liver, in which insulin can phosphorylate FoxO1 to inhibit gluconeogenesis as well as increase triglycerides through the upregulation of sterol regulatory element-binding protein (Srebp) -1c. As a result, circulating glucose and triglycerides are taken up by tissue appropriately. The second model is type 2 diabetes in which the liver is selectively insulin resistant. Under these conditions, insulin cannot inhibit gluconeogenesis in the liver due to insulin resistance in the liver as well as other tissue. As a result, insulin upregulates lipogenesis in addition to rising glucose levels. The final model, which supports the theory of selective insulin resistance uses a liver-specific insulin receptor knockout (LIRKO) model in which the liver is completely insulin resistant. As a result, gluconeogenesis is increased without increased triglycerides. Our model deviates from selective insulin resistant model in that our rodents were not hyperinsulinemic; however, the disproportion of glucose to insulin suggests impaired insulin output, a sign of insulin resistance. Further, the insulin that is present in our sucrose fed rats would still be unable to downregulate gluconeogenesis leading to the simultaneous increase in glucose and triglycerides.

Body composition is only a small piece of metabolic dysregulation of diet induced obesities. Changes in circulating factors in combination with composition data better characterize the changes high sugar diets can induce. For example, leptin circulates in concentrations reflecting adiposity. Leptin is positively correlated with FTL (fat mass:lean mass) ratios, the larger the ratio the higher adiposity, However, we observed no significant differences in FTL ratios between treatment groups. Further, triglycerides are positively correlated with FTL ratio. These data suggest the likelihood that body composition is actually different between treatment groups given the consistent correlation between body composition and factors that reflect

adiposity. Collectively, these data demonstrate the promotion of MetS in rats given ad libitum access to a 30 % sucrose solution for 42 days.

Diet Induced O-GlcNAc Protein Modifications

We failed to find a significant increase in protein O-GlcNAcylation in the hypothalamus of sucrose fed rats. Analysis of O-GlcNAcylation in the hypothalamus is fairly new. Previously, mice given 44 day access to 30% sucrose solution had increased O-GlcNAcylation in the hypothalamus. However, a similar study design, using rats, (by the same research group) showed no significant difference in hypothalamic O-GlcNAc. While it is certain that the hypothalamus overall regulates energy balance, within the hypothalamus are specific nuclei that differentially contribute to energy balance. We believe that different nuclei respond to glucose increases differently. For example, AGRP neurons of the arcuate nucleus have higher O-GlcNAc concentrations during fasted versus fed states.(Y. Liu et al., 2012) The increase in O-GlcNAc observed in mice that was not observed in rats may be a result of more robust increases and minimal decreases in O-GlcNAc throughout the hypothalamus generating an overall increase. Further analysis is required to characterize O-GlcNAcylation in the hypothalamus under control and sucrose stimulated conditions.

Future Directions

Hypothalamic Nuclei Specific Changes

As noted above, future investigations into diet induced O-GlcNAc modifications should be focused on specific changes within each nuclei of the hypothalamus. These future

experiments would not only give insight to diet induced changes in specific regions of the hypothalamus but would also generate data regarding uptake of nutrients by the specific regions as increases in O-GlcNAc require glucose or glucosamine. To analyze hypothalamic O-GlcNAc on a nuclei specific basis, pooling would be required between samples in order to have enough material. Further, each region would have to be verified by site specific expression of mRNA or protein to avoid sampling from adjacent nuclei. A previous report using a punch technique for sampling hypothalamus examined 3 nuclei for site specific expression.(Zhao, 2015) As a result, apart from growth hormone, no expression levels were undetectable but varied expression patterns could aid in site verification. The three regions examined were the paraventricular nucleus (PVN), ventromedial hypothalamus (VMH) and the lateral hypothalamus (LH). Expression of 4 factors were robust in the PVN compared to the VMH and LH. The VMH expressed high levels of AGRP while the expression of growth hormone in the PVN was undetectable. Therefore, the VMH can be identified by AGRP expression and the LH can be identified by growth hormone expression in conjunction with low AGRP expression. These regions maintain energy balance as a whole but contribute to energy balance in different ways. The VMH and PVN are believed to be satiety centers as their ablation leads to obesity; the opposite is true of the LH. Previously, O-GlcNAcylation was shown to be increased in fasted AGRP neurons of the ARC.(Y. Liu et al., 2012) Knockout models of AGRP exhibit significant weight reduction as AGRP is an orexigenic signal. The most interesting region to examine would be the VMH given its high expression of AGRP and opposing contribution to energy balance. These data would demonstrate patterns of O-GlcNAcylation as being neuronally specific or regionally specific.

Silent Destruction of Metabolism

The results outlined in this dissertation add to the body of work that identifies high sucrose diets as a means to promote MetS in rats. One of the most important observations noted here is the lack of anthropometric evidence of metabolic syndrome. We believe that under dietary challenges like high sucrose diets, the damage that results from metabolic syndrome is already occurring long before there is gross visual evidence of a change in body composition. Regardless, the reversibility of these changes has not been well established. Leptin resistance induced by 16 days of choice diet can be reversed with 4 days of chow diet.(Apolzan & Harris, 2013) Further, complete reversal of hypothalamic pSTAT3 (Y705) response was not achieved after 45 day access to 30% sucrose solution in rats.(R. B. S. Harris, 2018) The point at which sucrose induced leptin resistance is no longer reversible remains unknown as does the contribution of obesity to the reversibility of sucrose induced leptin resistance. These data could be important in understanding the timeline of diet induced metabolic syndrome in terms of prevention and treatment.

Final Thoughts

The goal of our project was to replicate previous work demonstrating the ability of sucrose solutions to induce leptin resistance while further analyzing the contribution of sucrose solutions to leptin resistance. Indeed, our data support the use of 30% sucrose solution as a means to model metabolic syndrome, though our achievement of leptin resistance is speculative. Regardless, our data clearly demonstrates the ability of sucrose in the diet to elicit metabolic abnormalities without anthropometric evidence, a dangerous response with major implications to

public health. The role of O-GlcNAcylation in these changes remains to be seen. It is important to note that O-GlcNAcylation is dynamic and we as researchers have only cracked the surface of protein regulation by O-GlcNAcylation. A major limitation to this body of research is the difficulty in analyzing proteins for the O-GlcNAcylation. Further complicating the analysis is the simplicity of the pathway components. Because the pathway only utilizes two enzymes for the addition and removal of the O-GlcNAcylation manipulation of the enzymes by different methods of inhibition would result in global changes rather than protein specific changes. Manipulation of kinases is commonly used to characterize phosphorylated proteins; such methods would not be effective in this field.

In conclusion, effective and reproducible techniques need to be established for O-GlcNAc research to continue to grow. Further, our dietary data suggests the need for better characterization of the long-term effects of a high sucrose diet both in regard to continual use as well as seceded use. Growth in both areas of research would support the current need for prevention and treatment of metabolic based diseases.

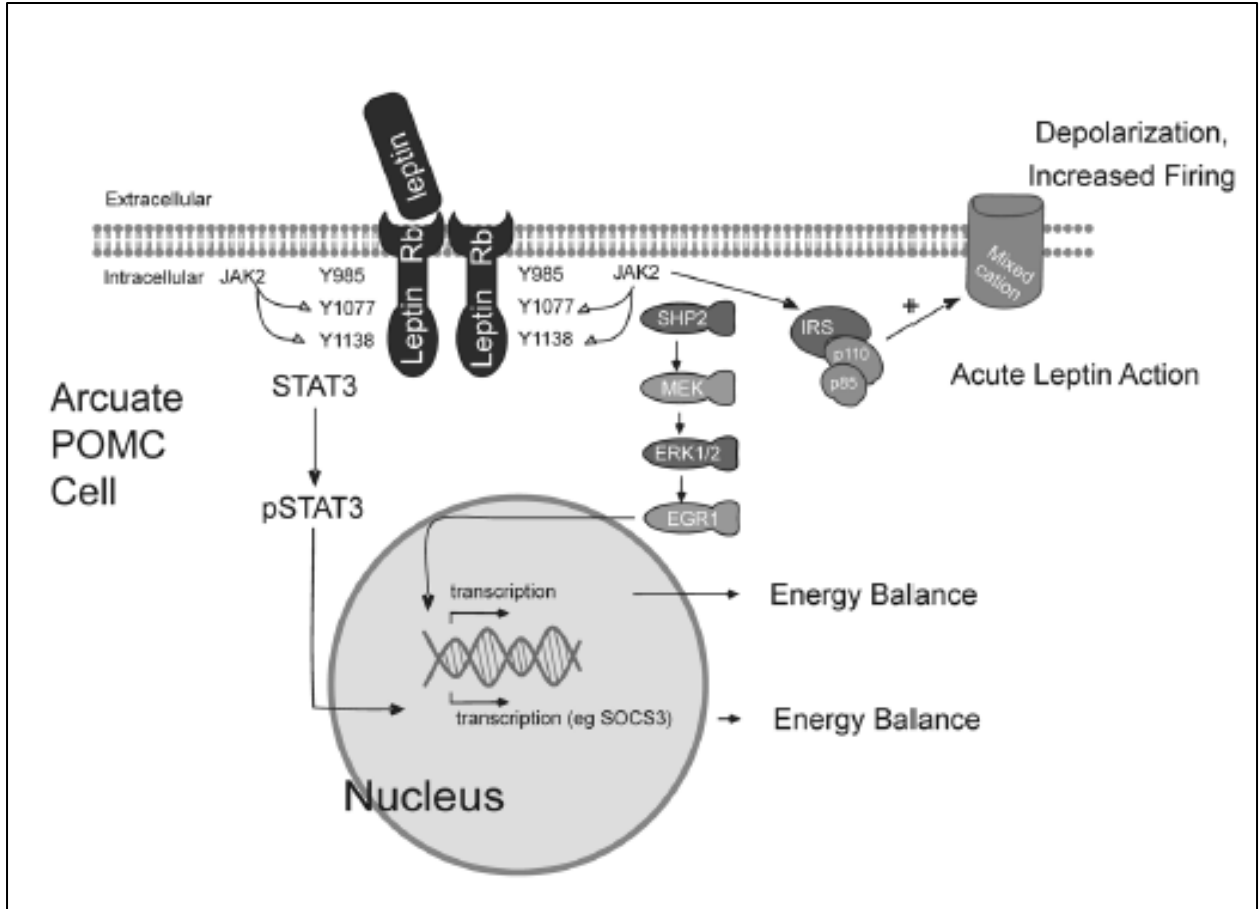


Illustration 1 Acute Effects of Leptin

Pictured above is the leptin signaling pathway within a POMC neuron of the ARC. The acute effects of leptin are diagrammed as a function of PI3K on mixed cation channels. (Hill et al., 2008)

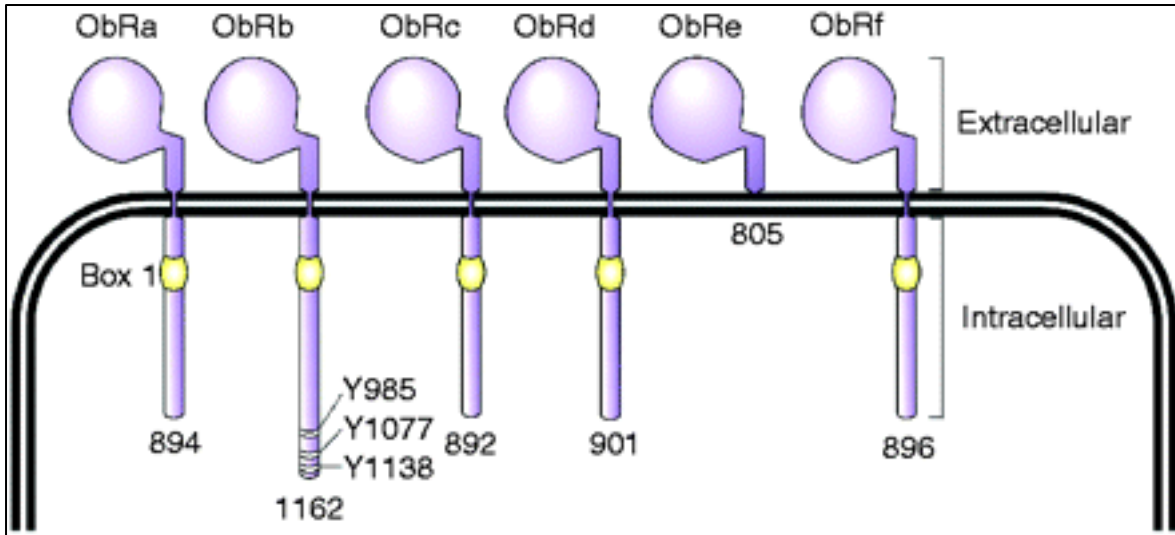


Illustration 2 Leptin Receptor Isoforms

Pictured above are the leptin receptor isoforms. Through alternative slicing the gene ObR expresses six functionally distinct receptors. (Marroqui et al., 2012)

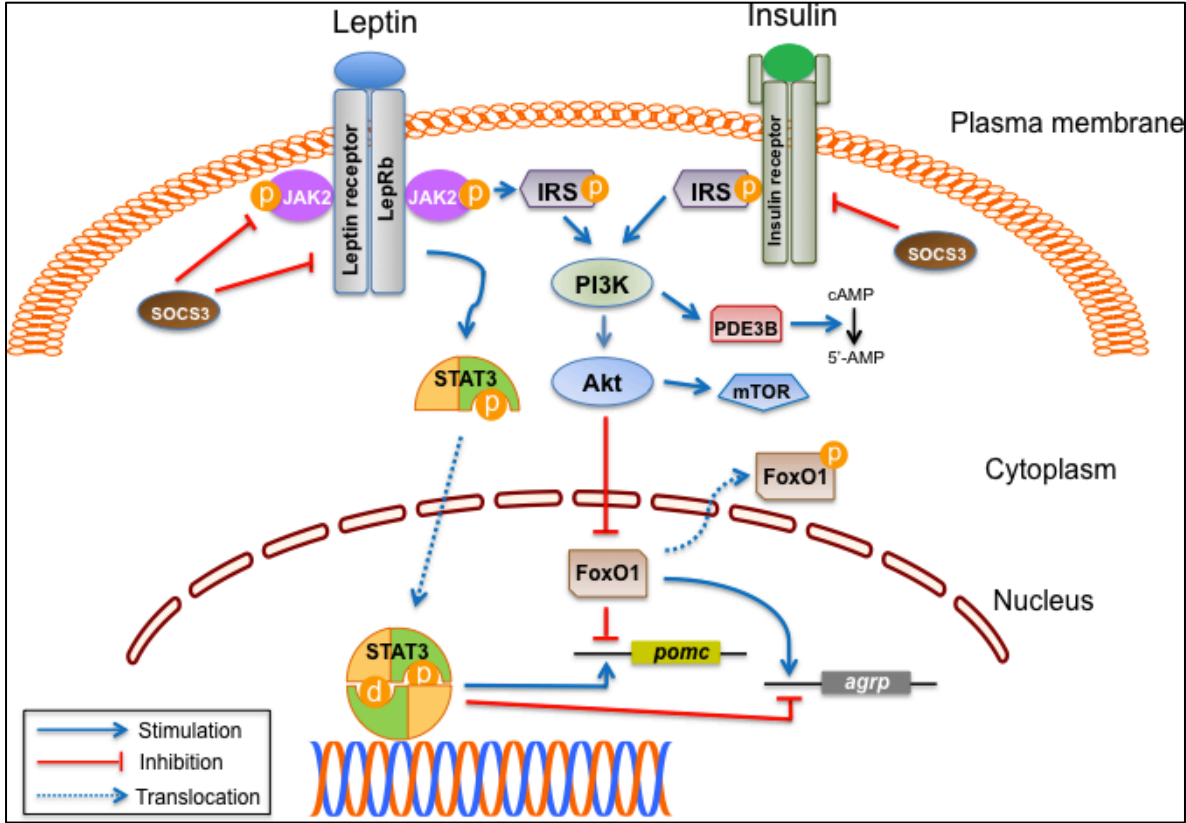


Illustration 3 Leptin Signaling

Pictured above is the signal transduction pathway of leptin signaling in the hypothalamus to regulate Pre-*opiomelanocortin* (POMC) and *Agouti Related Protein* (AGRP) expression. This diagram specifically illustrates the transcriptional competition between STAT3 and FoxO1 in leptin sensitive tissue. (Park & Ahima, 2014)

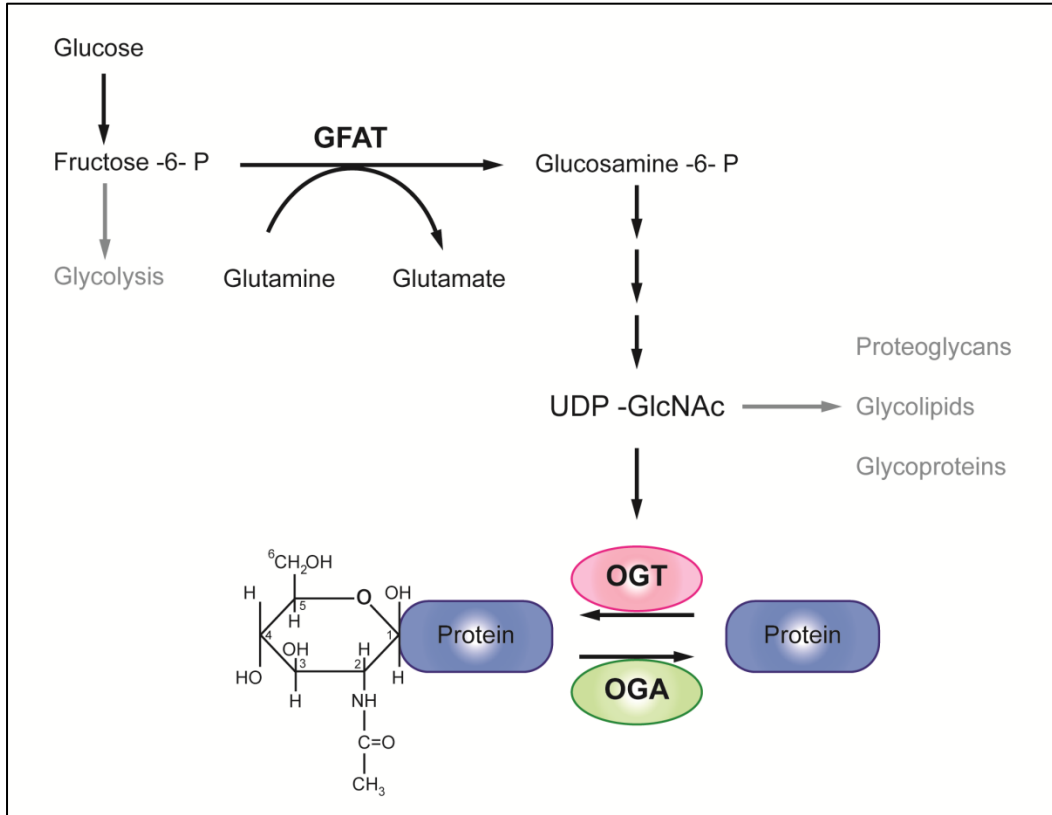


Illustration 4 Hexosamine Pathway

Pictured above is the Hexosamine biosynthesis pathway. Through a series of enzymatic reactions, the biosynthesis pathway converts glucose into the high energy substrate UDP-GlcNAc. Enzymes OGT and OGA can modify proteins with UDP-GlcNAc by adding and removing, respectively. (Gronning-Wang, Bindsboll, & Nebb, 2013)

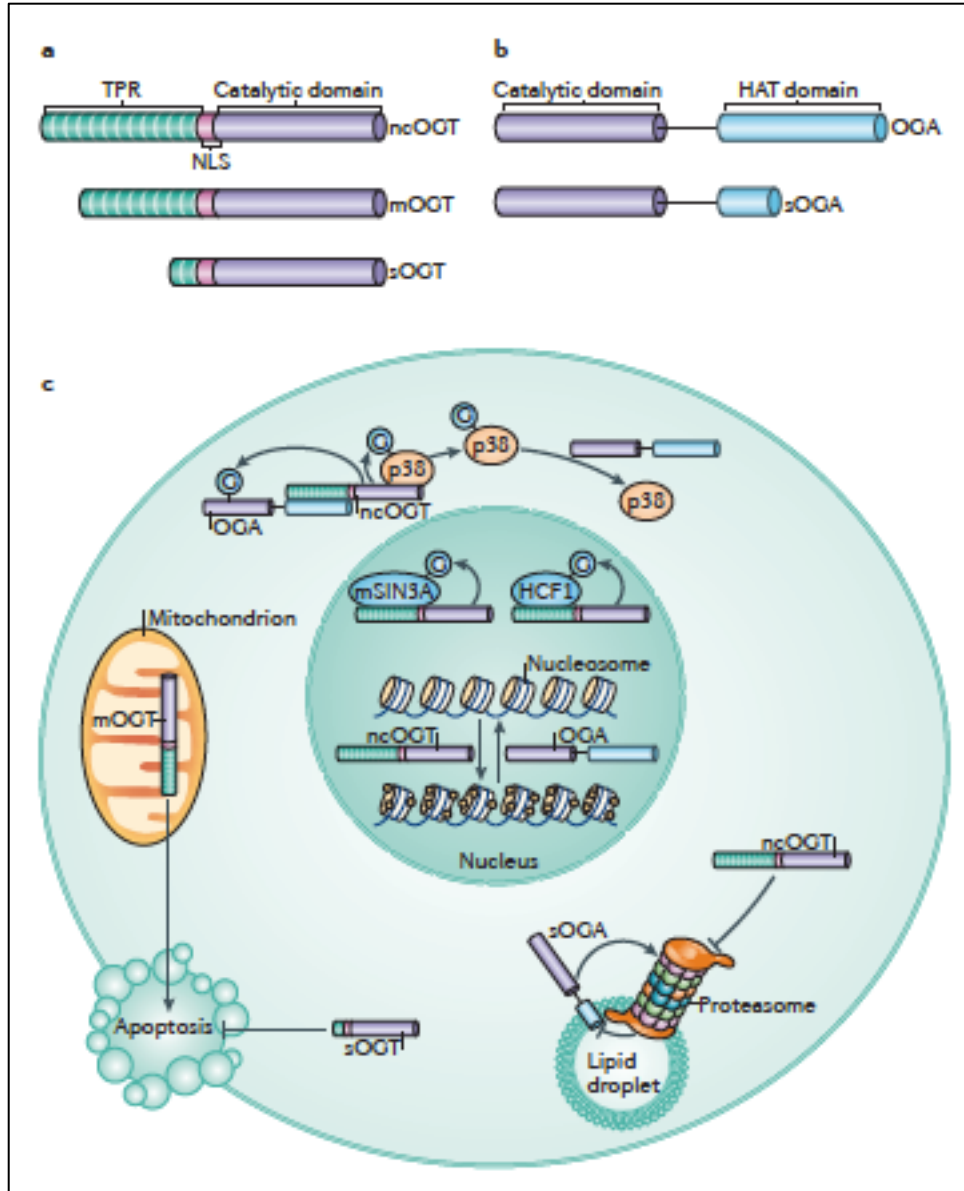


Illustration 5 OGT and OGA Isoforms

Pictured above are the various isoforms of OGT and OGA and their respective contributions to cell O-GlcNAcylation. A) Three isoforms of OGT differentiated by their N terminal repeats. B) Two OGA isoforms differentiated by a pseudo HAT domain. C) Localization of the enzymes. (Hanover, Krause, & Love, 2012)

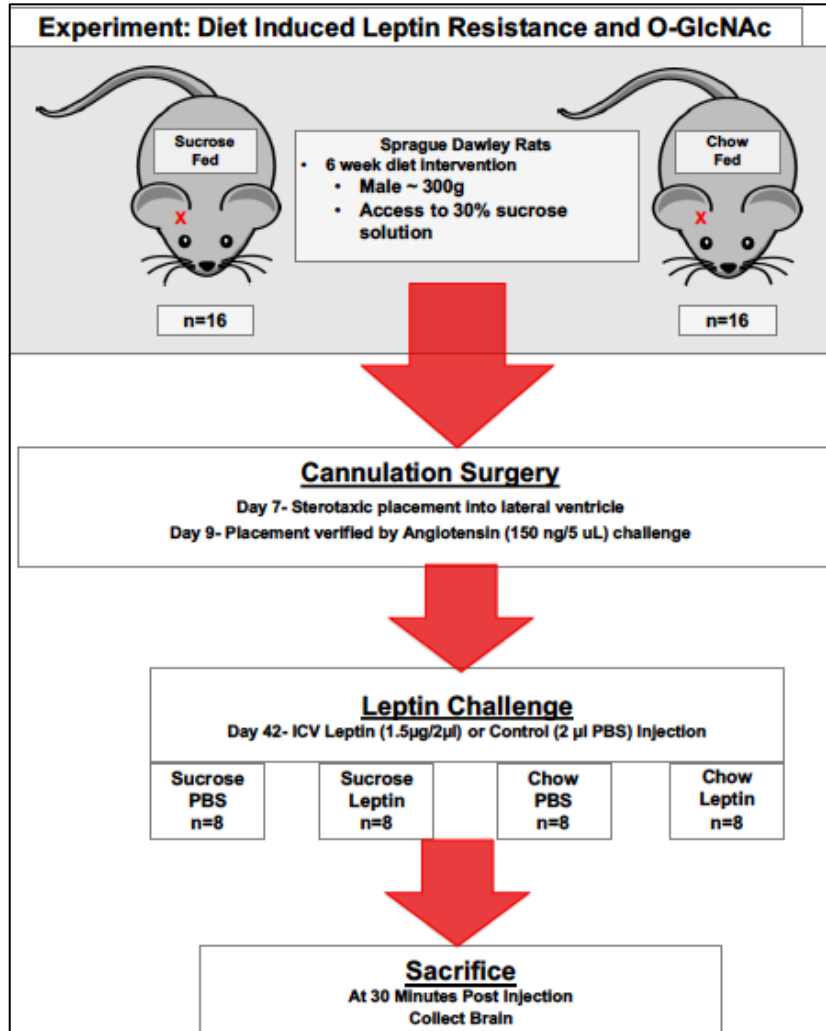


Illustration 6 Protocol for Modeling Diet Induced Leptin Resistance

Flow diagram of the treatment protocol for dietary intervention and surgical intervention

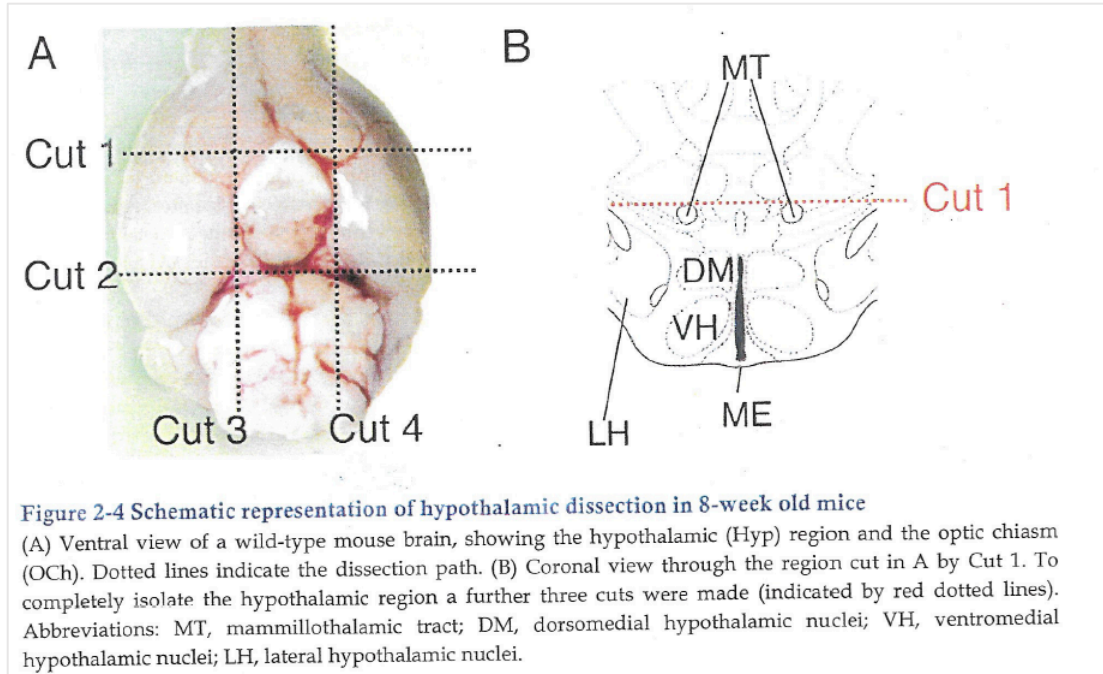


Illustration 7 Hypothalamic Macrodissection

Pictured above is the process through which the hypothalamus can be macrodissected using only a razor blade. The process is described above for mice but can also be applied to rats. Protocol from E. London, personal communication.

Teklad Global 18% Protein Rodent Diet		
Energy Density	kcal/g	3.1
Calories from Protein	%	24
Calories from Fat	%	18
Calories from Carbohydrate	%	58
Ingredients (in descending order of inclusion)- Ground wheat, ground corn, wheat middlings, dehulled soybean meal, corn gluten meal, soybean oil, calcium carbonate, dicalcium phosphate, brewers dried yeast, iodized salt, L- lysine, DL-methionine, choline chloride, kaolin, magnesium oxide, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, ferrous sulfate, zinc oxide, niacin, calcium pantothenate, copper sulfate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, calcium iodate, vitamin B12 supplement, folic acid, biotin, vitamin D3 supplement, cobalt carbonate.		

Table 1 Dietary Composition of Teklad Diet

Table includes breakdown of energy density, caloric contribution of macronutrients to diet and ingredients.

	Initial Body Weight (g)	Final Body Weight (g)	Body Weight Change (g)	Percent Fat Mass
Control (n=26)	313.9 ±2.9	523.7 ±9.5	178.0 ±7.2	19.9%
Sucrose (n=21)	318.4 ±2.7	531.7 ±11.7	182.7 ±9.5	19.9%

Table 2 Body weight Change and Body Composition

Presented in Table 1 are group mean initial body weights (g) (p=0.2629), final mean body weight (p=0.5933) and mean body weight change from day 1 to day 42 (p=0.6891) Percent fat mass are based on carcass data after brain and liver dissection. (Control group n=26 Sucrose group n=21)

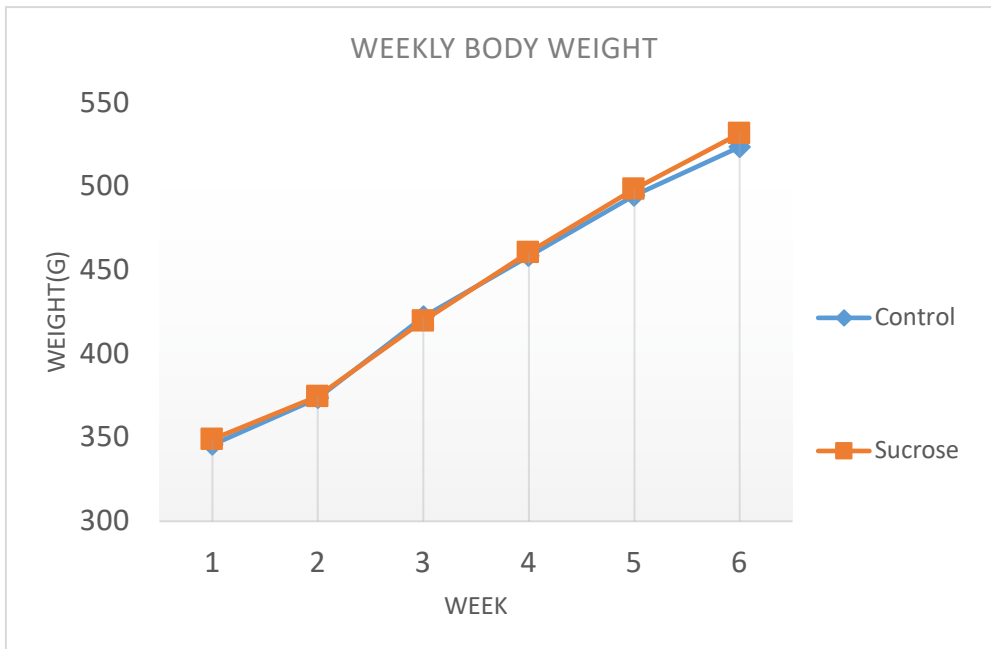


Figure 1 Average Weekly Body Weight

Data are average weekly body weight in grams (g) of control and sucrose groups. Effect of time on all rats $p < 0.0001$.

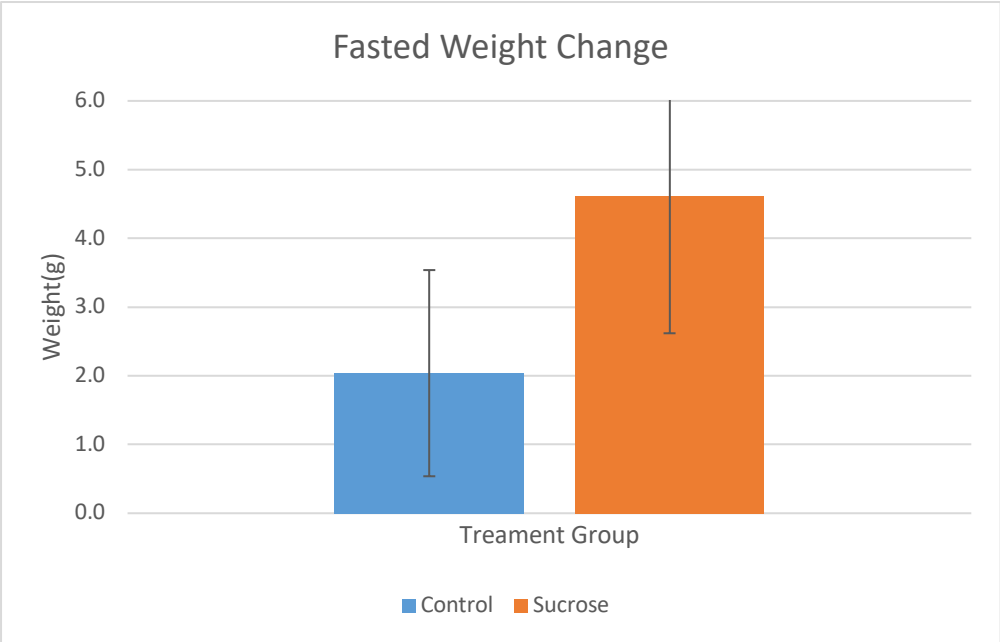


Figure 2 Fasted Weight Change

Average weight change from overnight fast of control and sucrose fed rats \pm SEM ($p= 0.2948$).
(Control $n=26$ Sucrose $n=21$)

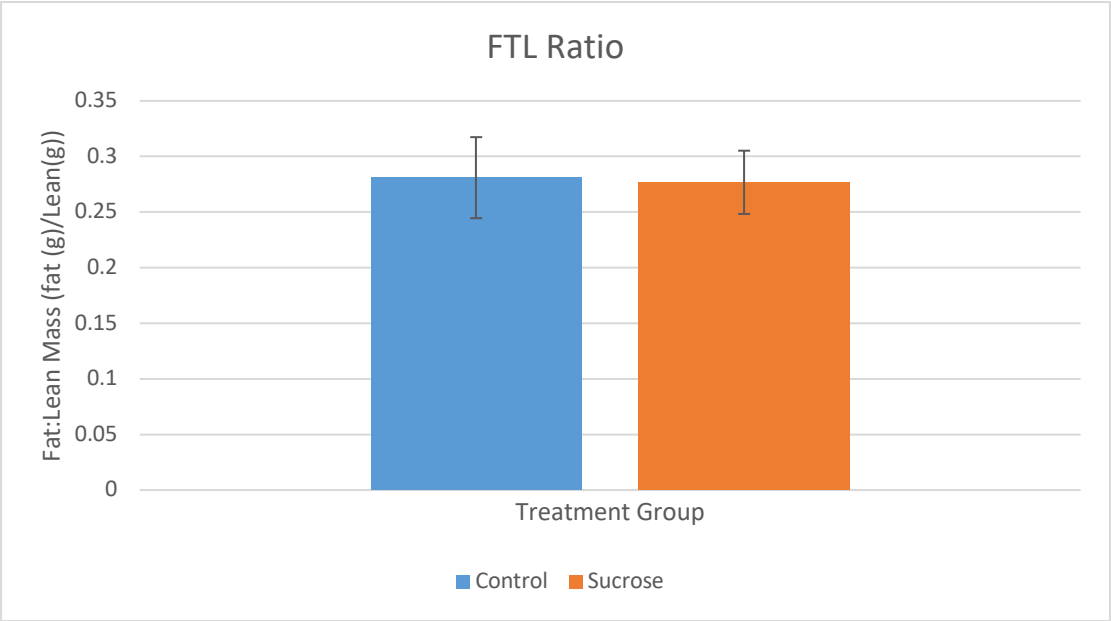


Figure 3 Fat to Lean (FTL) Mass Comparison

Average fat mass (g):lean mass (g) (FTL) ratio derived from echo MRI data \pm SEM ($p=0.6411$) (Control $n=26$ Sucrose $n=21$)

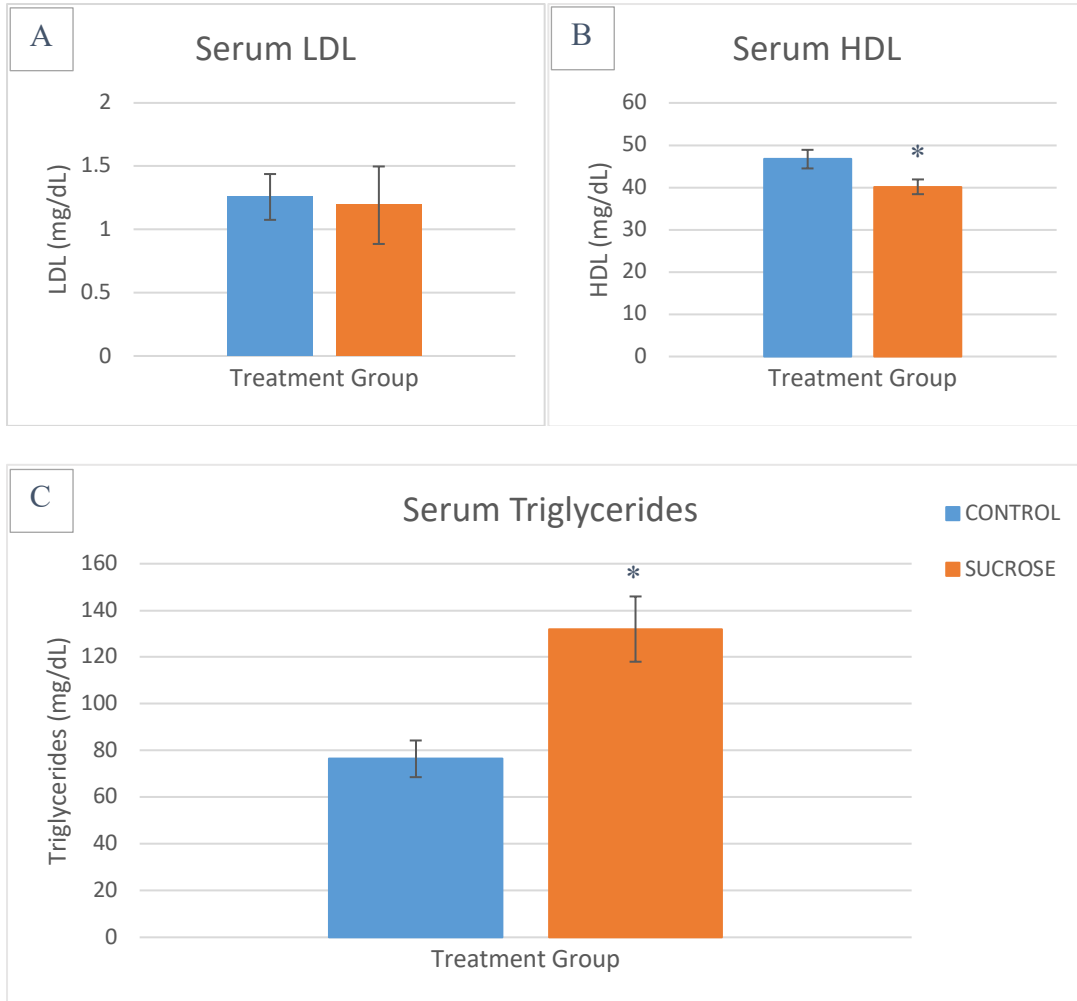


Figure 4 Lipid Profile

Serum circulating factors from truncal blood (n=16) ± SEM (* denotes significant data p<0.05) A. Serum LDL (p=0.8550) B. Serum HDL (p=0.0278) C. Serum triglycerides (p=0.002)



Figure 5 Glucose and Hormonal Profile

Serum circulating factors from truncal blood (n=16) ± SEM (* denotes significant data p<0.05) A. Serum leptin (p=0.0362) B. Serum insulin (p=0.9451) C. Serum glucose (p=0.0210)

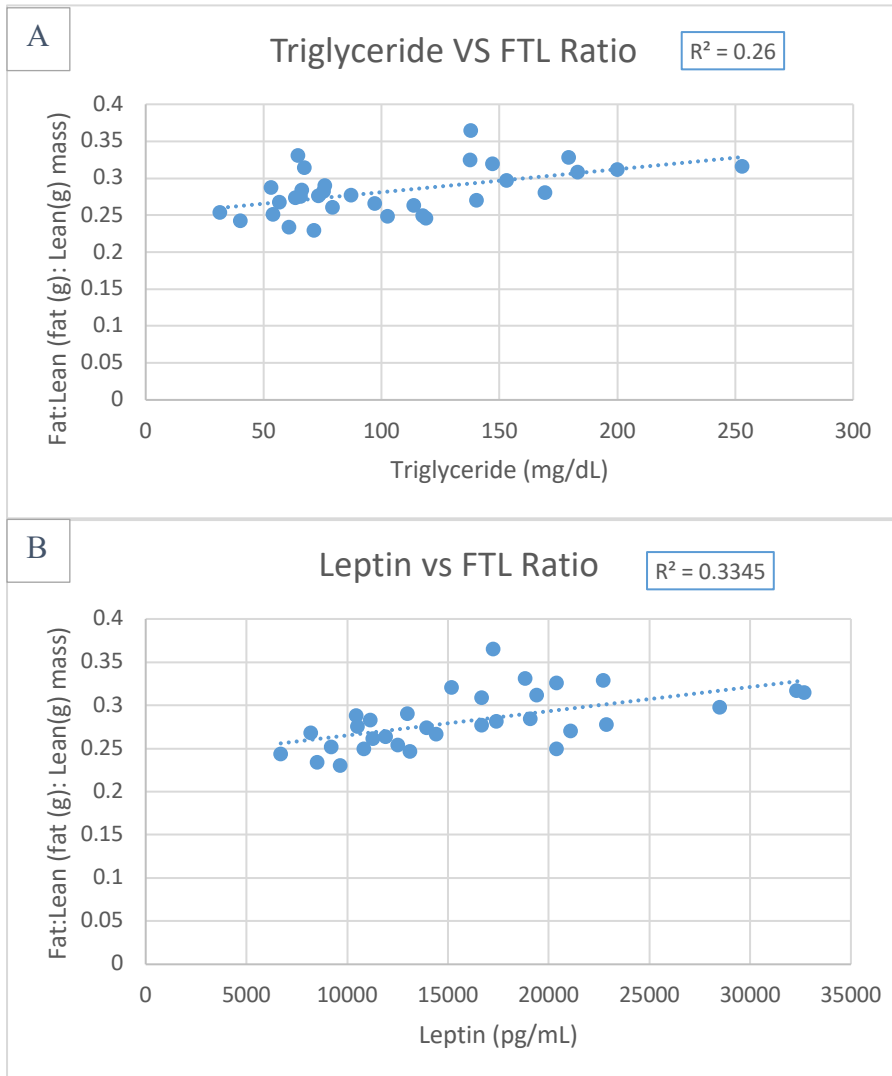


Figure 6 Circulating Lipid Factors Correlate to Body Composition

Correlations between Fat:Lean Mass and circulating factors (n=32) were examined A. Serum triglycerides (p=0.0029)
 B. Serum leptin (p=0.0005)

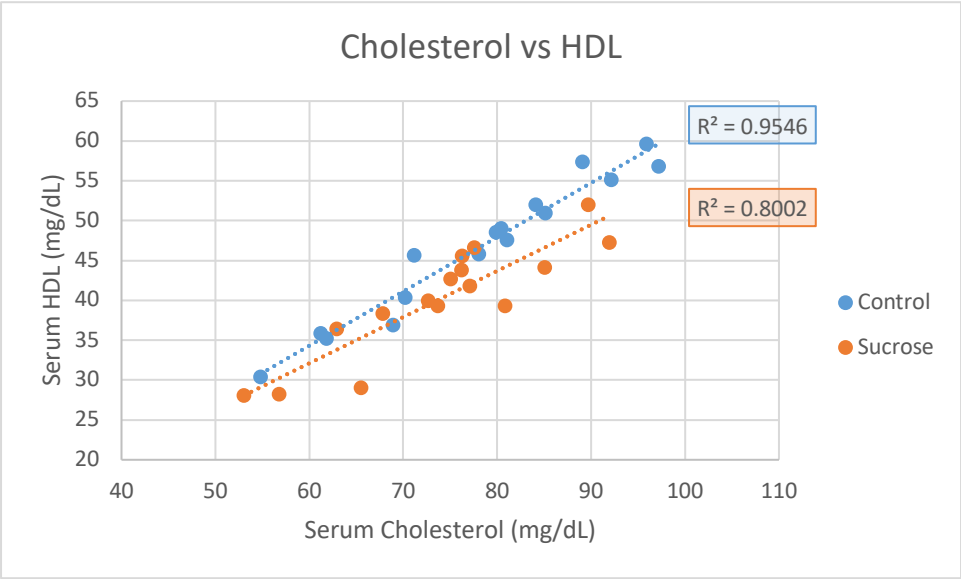


Figure 7 Sucrose Treatment Reduces HDL Correlation to Cholesterol

The correlation between serum cholesterol and serum HDL in both treatment groups (n=16) (p=0.045925) revealed that sucrose access suppressed the correlation of the two factors.

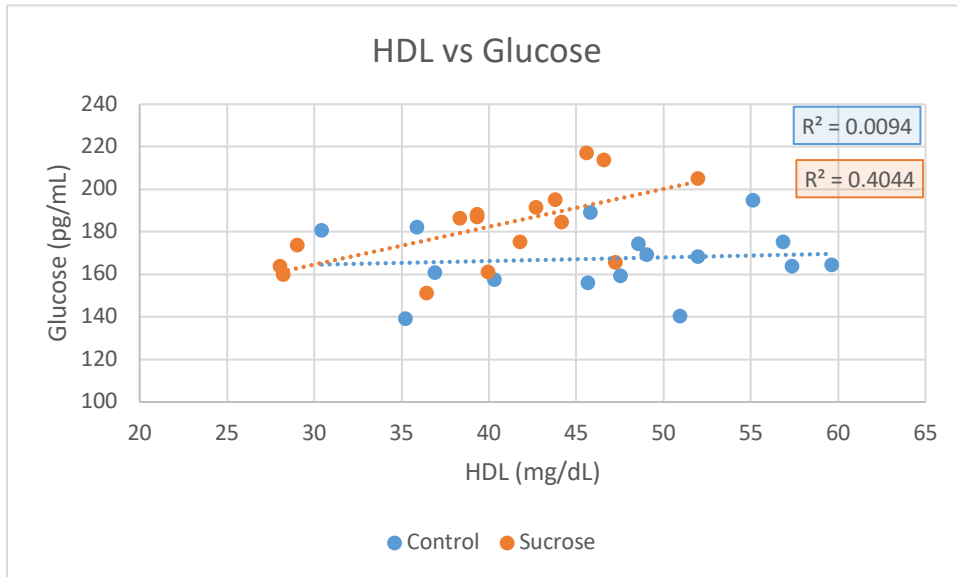


Figure 8 Sucrose Treatment Increases Correlation of HDL to Glucose

No difference was found between treatment groups (n=16) in the correlation between serum HDL and serum glucose (p=0.095338)

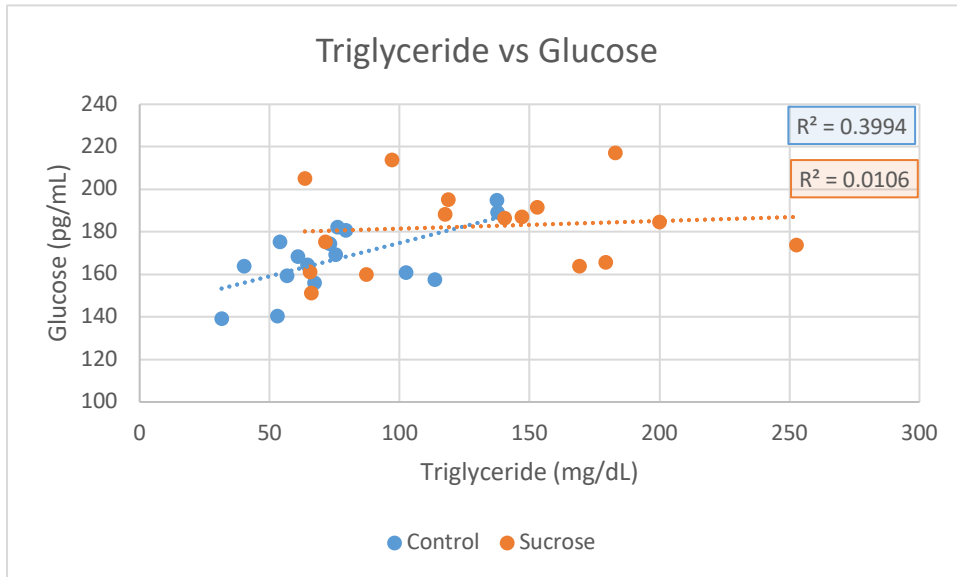


Figure 9 Treatment Effect of Correlation Between Triglyceride and Glucose

No difference was found between treatment groups (n=16) in the correlation of serum triglyceride and serum glucose (p=0.1028)

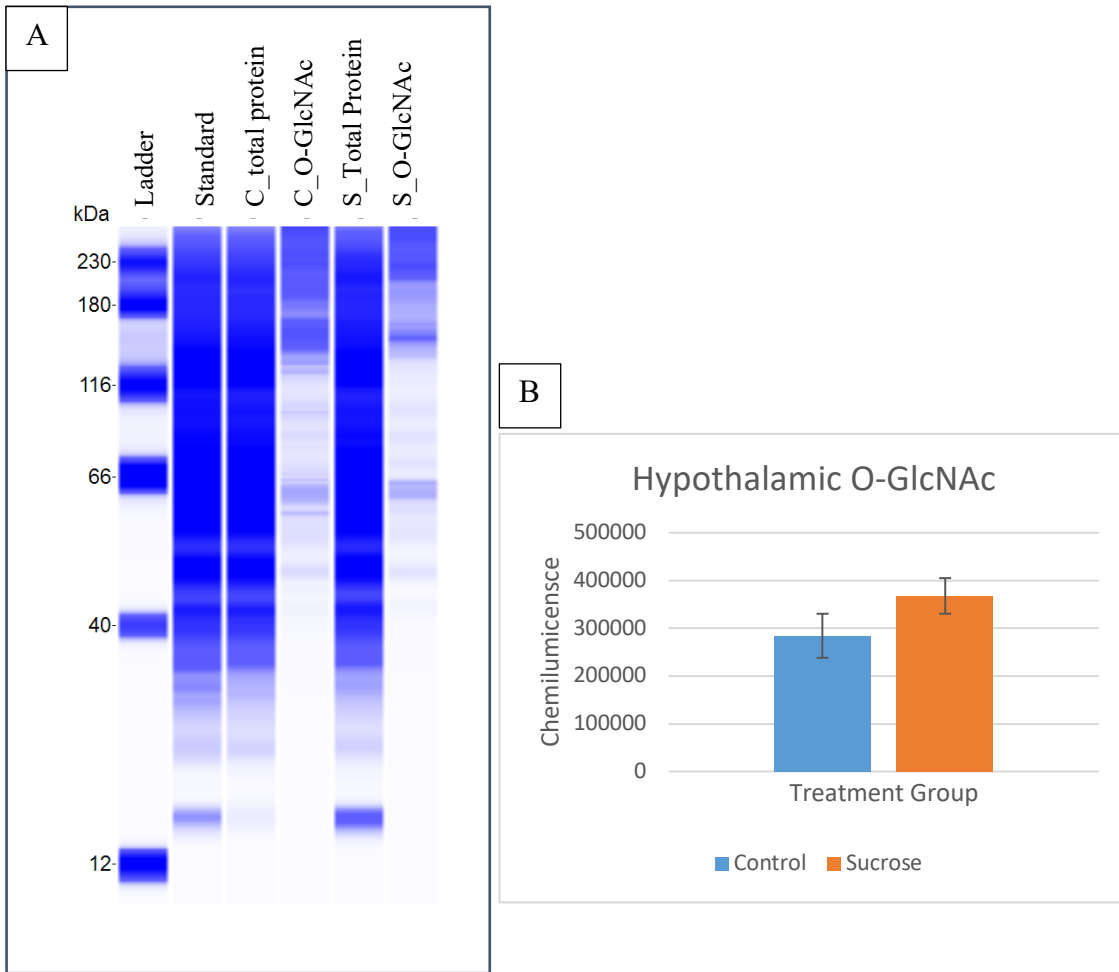


Figure 10 Hypothalamic O-GlcNAc Levels

A. Representative blot of O-GlcNAc in hypothalamus of both groups of rats. Standard and Total Protein levels were used to normalize data. B. Data are average O-GlcNAc levels from hypothalamic tissue \pm SEM ($p=0.1698$)

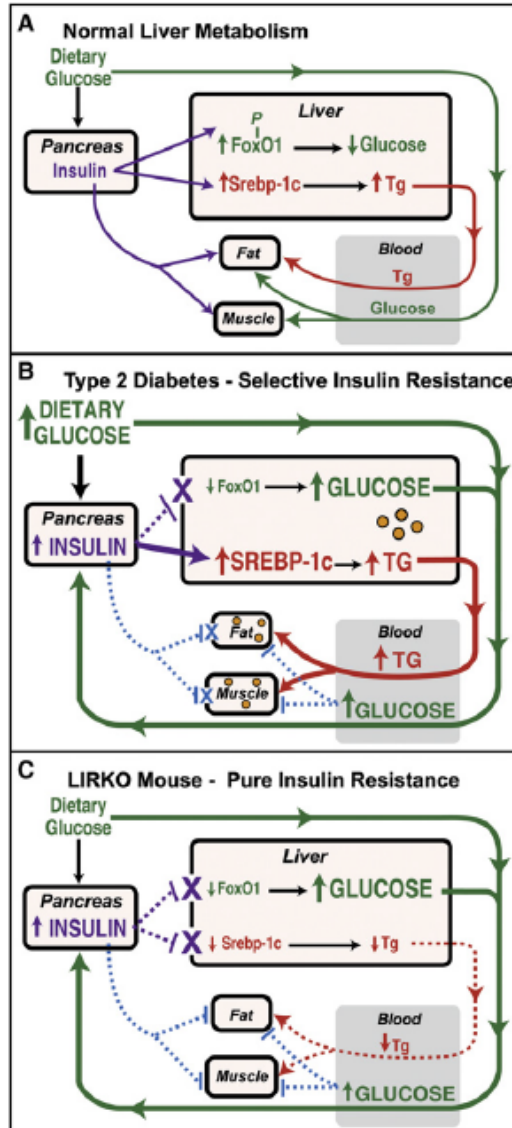


Illustration 8 Selective Insulin Resistance of the Liver

Illustrated above are three models of insulin sensitivity in the liver. A. Normal insulin sensitive level with normal glucose and triglyceride(TG) levels B. Diabetic selective insulin resistant liver with elevated glucose and TG levels (Brown & Goldstein, 2008)

Appendix

Cannulation Verification and Leptin Injection

Ventricular cannulation using stereotaxic equipment is very precise; however, the cannula placement must be verified for accuracy by some sort of measure. A common method is to employ an angiotensin II challenge followed by measuring water intake immediately post injection. Polydipsia following the injection indicates an appropriate placement. Typically, 1 in 10 animals fail this challenge. However, in this experiment fewer than half of the rats passed the angiotensin II challenge. Further, among those who met the criterion, by consuming more than 3 mL in an hour, several measurements were omitted due to water bottle leakage (see Figure 11-A). For this reason, a test at the molecular level was used as a backup verification procedure. STAT3 phosphorylation at the tyrosine 705 residue is a known response to leptin signaling and was previously shown to be unaffected by a high sucrose diet.(R. B. Harris & Apolzan, 2015) However, analyses of pSTAT3 (Y705) data measured in the Sucrose and Control groups show no significant increase in pSTAT3 (Y705) in leptin injected rats (see Figure 12-A). The mixed and in some case conflicting, results of cannula placement make hypothalamic protein analysis difficult to analyze and interpret. For these reasons, the data are interpreted by treatment group without including the ICV injection as a variable. This Appendix summarizes the results of the analyses that were performed on all of the rats of both the Sucrose and Control groups, all the while fully acknowledging that only dietary treatments could be used for inferences about the effects of sucrose access on hypothalamic function.

Hypothalamic Protein Quantitation

Methods

Right hypothalamic tissue was used to extract protein using the complete tris lysis buffer (R60TX) by Meso Scale Discovery (Rockville, MD) at ratio of 100 ul buffer to 10 mg tissue. Tissue was homogenized by passing it through an 18 gauge needle, followed by sonication. Homogenates were incubated on ice followed by centrifugation. Supernatant was collected and frozen at -80°C. Immunoassay analysis for STAT3 and AKT/phosphorylated AKT (pAKT) were completed following Meso Scale Discovery's protocol for pSTAT3 (Y705) (cat K150SVD) and AKT/pAKT (cat K15100D), respectively. To analyze FoxO1, lysates were diluted to within range of the Signalway Antibody LLC (College Park, MD) Rat FoxO1 ELISA kit (cat EK2219). Of these data only 12 samples were sufficiently replicated or within range (Control n=6 Sucrose n=6).

Immunoassay data

No significant difference between control fed and sucrose fed PI3K pathway proteins in hypothalamic tissue were observed. Hypothalamic AKT levels were virtually identical between Control at 220680.8 signal units and Sucrose group at 221569.1 signal units (see Figure 13-A i). Similarly, no differences between group pAKT and FoxO1 measures were observed, see Figure 13-A ii and 14-A respectively. Interestingly, there was no significant correlation between pSTAT3 and pAKT, as increased pSTAT3 would indicate activation of the PI3K pathway thereby increasing pAKT. The lack of correlative data may be due to high variability at basal levels as leptin is not the only ligand that regulates PI3K signaling. However, we predict a bolus of leptin would yield a correlation as the two pathways would be activated under the same stimulus.

O-GlcNAc Enrichment

In order to analyze O-GlcNAcylation of proteins, enrichment prior to mass spectrometry analysis was required. While the components required to enrich O-GlcNAcylation are commercially available, the process of enrichment is extremely complicated and failure rates are very high. The levels of O-GlcNAc in complex protein samples are usually so low in comparison to other components that the glycosylate is lost during analysis. In conjunction with previous research, our analysis of two unenriched hypothalamic samples, one control fed, and one sucrose fed, displayed only a few sites of O-GlcNAcylation under higher energy collisional dissociation (HCD). However, one modified site stood out among the rest. Pro-low-density lipoprotein receptor-related protein 1 isoform X1 (LRP-1) was shown to be O-GlcNAcyated at the Threonine 1808 residue (AtALAIImGDK; Tryptic peptide range 1807-1816) in both samples at different sample sizes. Further, no reference of O-GlcNAcylation at the threonine residue of LRP-1 has been found.

LRP-1 is a cell surface receptor, expressed ubiquitously but highly expressed in central tissue. Without further analysis the implication of O-GlcNAcylation of LRP-1 cannot be determined. But these results suggest LRP-1 may be regulated by O-GlcNAcylation. As a major part of lipid metabolism, LRP-1 is an important component of energy homeostasis. Deletion of central LRP-1 has been shown to increase intake and reduce energy expenditure, resulting in obesity. Effects on energy homeostasis may stem from LRP-1 co-expression in AGRP neurons which stimulate hunger.(Q. Liu et al., 2011) Further, LRP-1 is not expressed in POMC neurons which stimulate satiety. Previous reports demonstrated O-GlcNAcylation of proteins in AGRP neurons is highest during fasting states rather than fed states.(Y. Liu et al., 2012) As AGRP neurons are highly expressed in the hypothalamus and animals were sacrificed after fasting, the

discovery of O-GlcNAcylated LRP-1 in both sample lysates is not surprising. Further, LRP-1 has been shown to bind directly to the leptin receptor complex and is required for phosphorylation of the receptor and there by STAT3 phosphorylation.(Q. Liu et al., 2011)

LRP-1 was not the protein of interest during this study so an enrichment protocol needs to be developed. Future directions for LRP-1 O-GlcNAcylation include more replicates, localizing the modification to AGRP neurons and effects of feeding state on the modification. An enrichment protocol was adopted by combining *Hart and Ma* enrichment protocol with the simplified click reaction developed by *Hsieh-Wilson* (see Trypsin digestion and Enrichment sections below). Initial trials used lysate with the intention to monitor the reactions via various assays. However, few assays were available and were not definitive. These reactions need to be monitored via mass spectrometry. Complex samples are a poor choice for monitoring reactions. As a result, a protein control, alpha-crystallin, was used. Initial tagging via enzymatic addition of GalNAz was monitored via mass spectrometry but the protocol failed to successfully complete the reaction.

Future development of the O-GlcNAc enrichment should begin with a control peptide. Control O-GlcNAcylated peptides can be difficult to find as they are available as a custom order and are not commonly produced by peptide synthesizing companies. However, the peptide is essential as the purified peptide reduces possible errors and variability. For example, the peptide does not undergo trypsin digestion and can have known O-GlcNAcylation sites as well as controlled levels of O-GlcNAcylation by mixing the positive control peptide with its negative control counterpart. For example, alpha-crystallin has known O-GlcNAcylation sites and is known to be O-GlcNAcylated about 20% at all times. However, the variability in O-GlcNAcylated sites and its local concentration make this procedure less than ideal.

Trypsin Digestion

Lysate undergoing enrichment was trypsin digested using Trypsin Gold by Promega (Madison, WI). Briefly, lysate was thawed and precipitated with -20°C chilled acetone and incubated for 1 hour at -80°C. The precipitate was pelleted, washed with chilled acetone and dried. The pellet was then dissolved in resuspension buffer (8 M urea, 50 mM NH₄HCO₃, 10 mM Dithiothreitol) at 37°C for 30 minutes. Samples were then reduced by adding 500 mM iodoacetamide to a final concentration of 30 mM and incubated at room temperature for 30 minutes in the dark. The reduction was quenched with the addition of quenching buffer (50 mM NH₄HCO₃, 10 mM Dithiothreitol) and incubation at 37°C for 30 minutes. Samples were then diluted with 50 mM NH₄HCO₃ to reduce urea concentration to ~1M urea. Trypsin was added at 1 ug trypsin/50 ug protein and incubated overnight at 37°C. Samples were then desalted on SMMV18 column (Nest Group, Southborough, MA) and dried before enrichment.

Enrichment

Digested samples underwent enzymatic tagging using Thermo-Fisher's Click-It system, which conjugates an azide analogue, O-linked N-azidoacetylgalactosamine tetraacylated (O-GalNAz), to O-GlcNAc. Briefly, dried peptides were solubilized in 10 mM HEPES pH 7.9. The enzymatic reaction was set up by adding MnCl₂ and substrate UDP-GalNAz followed by mixing. The enzyme Gal-T1 was added to samples in addition to PNGase and CIP subsequently mixed and incubated overnight at 4°C. Finally, samples were desalted using SMMV18 column (Nest Group, Southborough, MA) and dried down. Enzymatically labeled samples were biotinylated

via click chemistry reaction with alkyne-dde-biotin. Dried peptides were resuspended in 1x PBS before adding copper-click chemistry reagents. Samples were prepared with 2.5 mM alkyne-dde-biotin and 100 mM THPTA. The reaction was initiated by the addition of 20 mM CuSO₄ and 300 mM sodium ascorbate. The reaction was incubated for 1 hour at room temperature protected from light. Excess alkyne-dde-biotin was removed using SCX column (Nest Group, Southborough, MA). Biotinylated peptides are collected using Neutravidin Agarose (Thermo Fisher, Waltham, MA). Briefly, Neutravidin agarose was prepared in microspin tubes and washed with PBS. Samples were added to column and incubated for 1 hour at room temperature. Columns were washed with PBS to remove unbound protein. Peptides were cleaved from the column by incubating column with 2% hydrazine monohydrate at room temperature for 1 hour. Samples were spun to elute peptides and washed with PBS to remove remaining peptides. Samples were frozen at -80°C until mass spectrometric analysis.

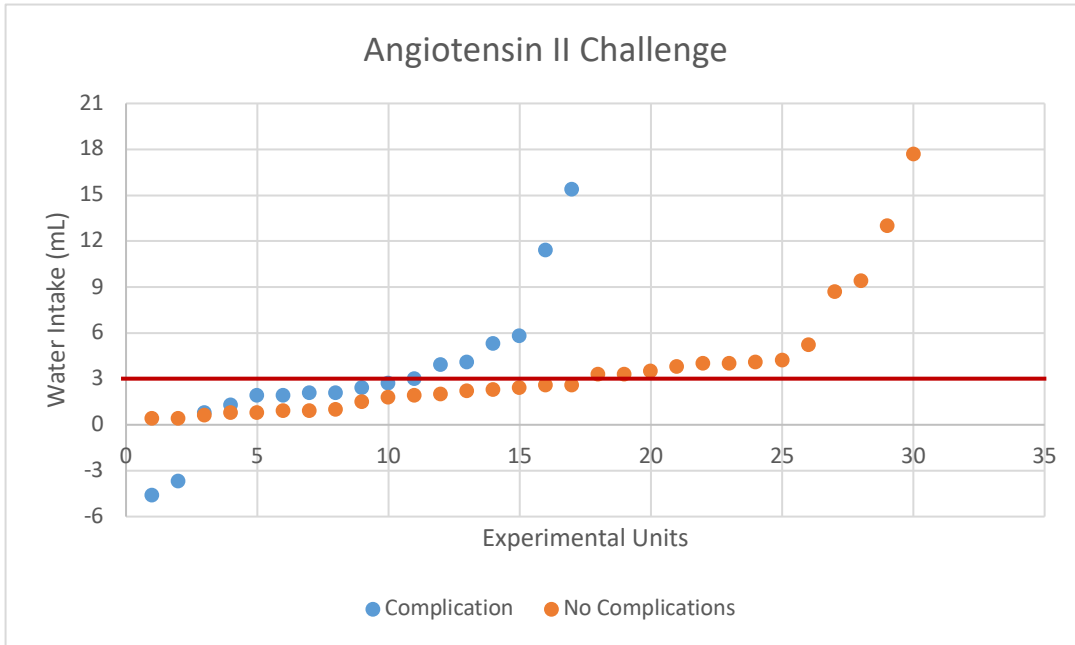


Figure 11-A Angiotensin II Challenge

Water intake in 1 hour after ICV angiotensin II administration grouped by experimental units who completed the protocol without complications and by experimental units who completed the protocol with complications. Values above the red line are considered passing intake volumes

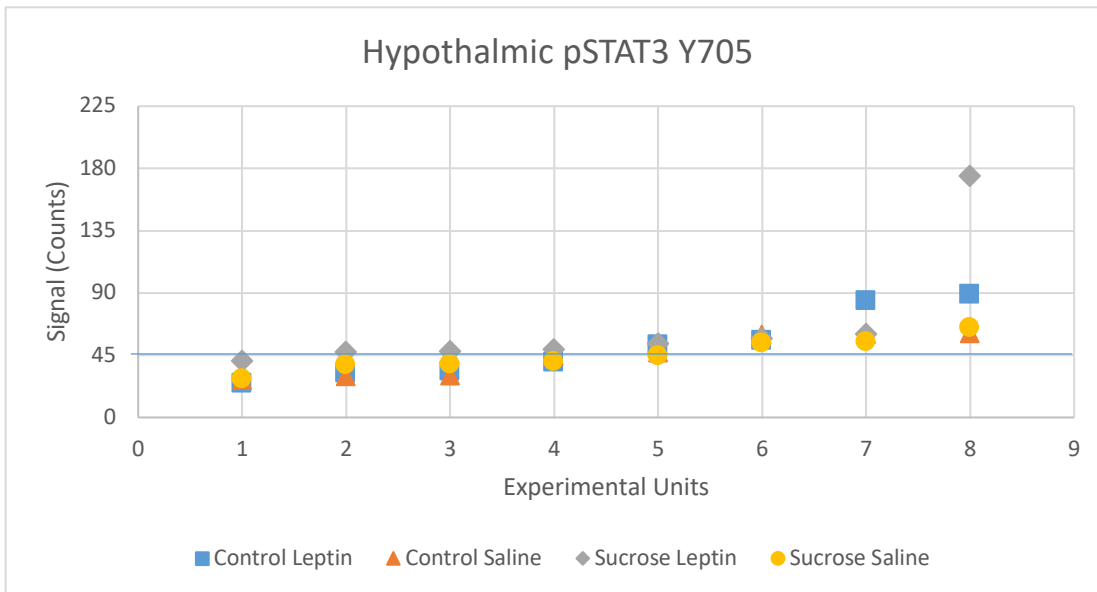


Figure 12-A Hypothalamic STAT3 Phosphorylation

Right hypothalamic pSTAT3 Y705 scatter plot grouped by treatment group. Blue line demonstrates average pSTAT3 (Y705) of Control Saline group (n=8)

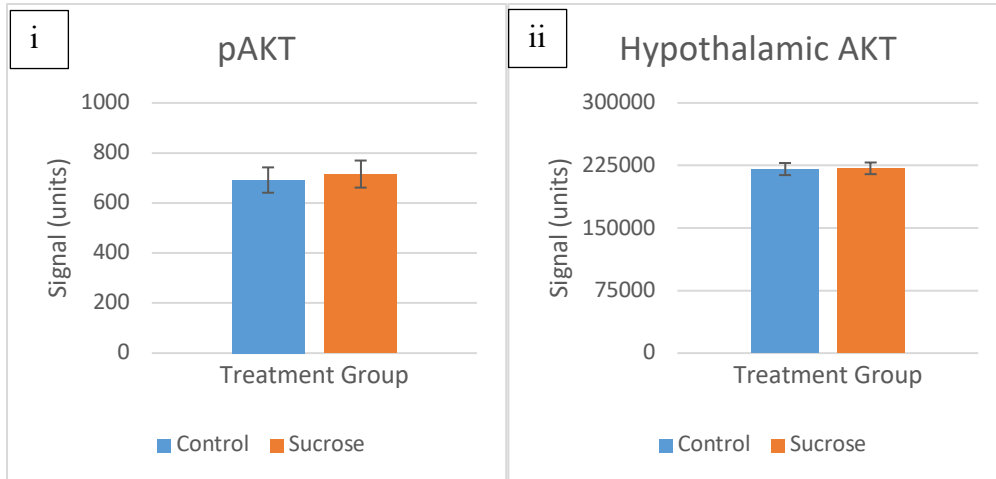


Figure 13-A Hypothalamic AKT and Phosphorylated AKT

i. Right Hypothalamic pAKT Serine 473 by treatment group (n=16) (p=0.7493) ii. Right Hypothalamic AKT by treatment group (n=16) (p=0.9303)

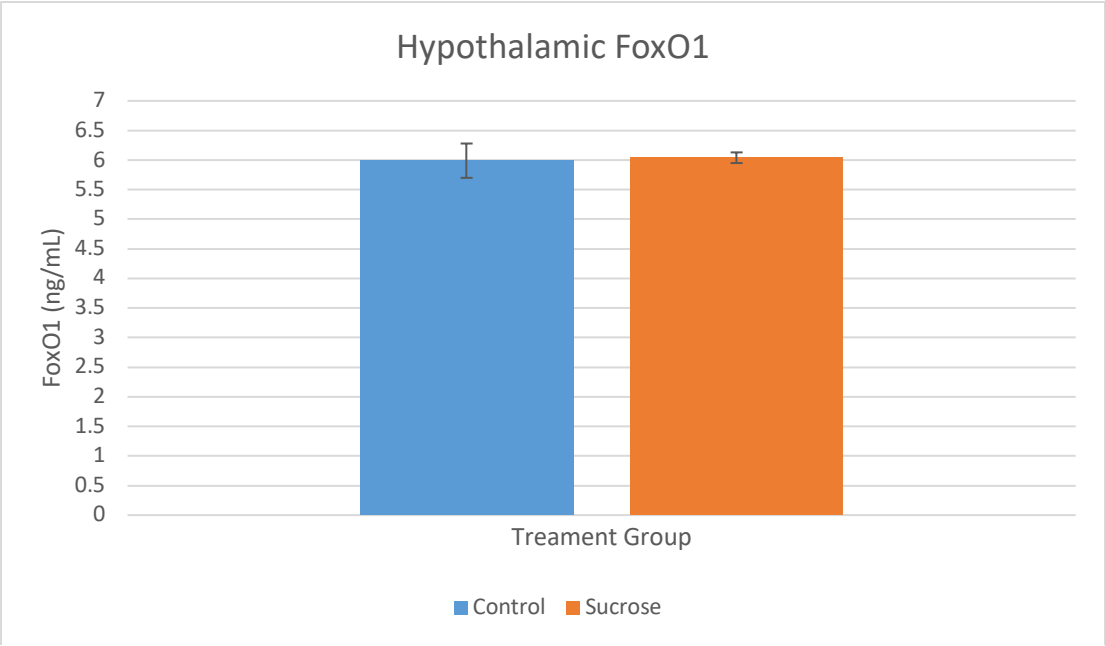


Figure 14-A Hypothalamic FoxO1

Right Hypothalamic FoxO1 by treatment group (n=6) (p=0.8731)

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phosphorylation. *Am J Physiol Regul Integr Comp Physiol*, 308(6), R543-555.
doi:10.1152/ajpregu.00347.2014

Curriculum Vitae

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Work Address:

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University of Maryland
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Educational Background

BA in Dietetics: University of Maryland, 2012

MS in Nutritional Science: University of Maryland, 2014

Thesis Title: Appetite Signals in the Brain: High Fructose Corn Syrup Sweetened Cola Effects
Hypothalamic Activity as Measured by Functional Magnetic Resonance

Ph.D. in Nutritional Science: University of Maryland, Expected Graduation 2018

Dissertation Proposal Title: O-GlcNAcylation of Leptin Signaling Molecules Results in Impaired
Promoter Binding of STAT3 and FoxO1 in the Arcuate Nucleus of Diet Induced Leptin Resistant
Rats

Candidacy Exam: Passed May 2016

Dissertation Defense: Estimated by October 2018

Employment

Graduate Research Assistant (Summer 2015- Present)

Description: Plan and execute research projects related to current project "Aberrant O-GlcNAcylation of leptin signaling proteins by sucrose consumption".

Current research project requires rodent handling and surgery. Laboratory work includes: Protein isolation, enzymatic tagging, Copper "Click" Chemistry tagging, MS sample prep techniques, Streptavidin affinity column enrichments, Simple Western blotting and ELISAs.

Supervisor: Thomas W. Castonguay..... (443) 745-6310

USDA Summer Intern (Summer 2013 and Summer 2014)

Description: Assist in sample processing and analysis, project management and assist during experimental protocols of highly regulated human feeding and room calorimetry studies.
Analyses included analysis of food as well as human urine and fecal samples to determine

moisture content, fat content, protein content, ash weight, fiber content and caloric density. Blood samples were analyzed for markers via ELISA and profiled for lipids and glucose using clinical analyzer. Project management duties included creating data and informational sheets as well as instructing subjects in person and via the telephone as needed.
Supervisor: Theresa Henderson (301) 504-7312

Teaching Assistant

NFSC 100 Introduction to Nutrition (Fall 2013, Fall 2014)

Description: Hold 3 sections of discussion weekly where the current lecture topic was discussed and applied to a worksheet as well as grading homework, exams, worksheets and quizzes.

Supervisor: Thomas W. Castonguay..... (443) 745-6310

NFSC 450 Food and Nutrient Analysis (Spring 2014, Spring 2015)

Description: Prepare for 2-3 sections of laboratory weekly. Labs included moisture content, fat extraction, calorimetry, salt content, Vitamin C quantitation via spectrometry and HPLC, BCA protein assay and aflatoxin assay. Create, administer and grade two practical exams each semester.

Supervisor: Thomas W. Castonguay..... (443) 745-6310

Graduate Administrative and Research Assistant (Summer 2012-Summer 2013)

Description: Administrative duties involved acting as a liaison between UMD NFSC employees and USDA Employees in relation to a cooperative agreement with UMD NFSC and USDA. Responsibilities included posting job descriptions, rating candidates, organizing interviews with selected candidates, preparing questions for interview process, submitting minutes reviewing interviews, processing final candidate selection. Research duties involved assisting during the planning, execution and data collection of laboratory research, primarily rodent feeding studies.

Supervisor: Thomas W. Castonguay..... (443) 745-6310

Publications

2013 Hudgins S, Schlappal A and Castonguay TW. Appetite and reward signals in the brain: sugar intake effects on brain activity as measured by functional magnetic resonance imaging. In: Nutrition in and prevention and treatment of abdominal obesity. R. Watson, (ed.), Elsevier Press, New York. Pp 307-314.

2017 Castonguay TW and Hudgins S. Sugars, glucocorticoids and the hypothalamic control of appetite. In: Nutrition and Functional Food for Healthy Aging. R. Watson, (ed.), Elsevier Press, New York. Pp 23-34.

Awards

Nutrition Day Poster Competition-First Place Nutrition May 2014

Nutrition Day Poster Competition- First Place Nutrition May 2016

Nutrition Day Poster Competition- First Place Nutrition May 2018

Honors

Deans Fellowship NFSC 2012-2013

This honor is recognition of academic performance and good standing with University of Maryland and Department of Nutrition and Food Science

Presented Posters

“High Fructose Corn-Syrup Sweetened Cola and the Hypothalamus: A Dose-Response fMRI Study”; Experimental Biology Annual Meeting April 2013, Society for Neuroscience Annual Meeting November 2013, Mid-Atlantic Diabetes Research Symposium January 2014

Related Courses

BIOM601 Biostatistics I
NFSC498A Nutritional Biochemistry
NFSC630 Energy Balance
BSCI447 Endocrinology
NFSC611 Molecular Nutrition
NFSC605 Behavior and Food Intake
ANSC627 Molecular and Quantitative Genetics
PhD
VMSC610 Viral Vectors
ANSC688Y Lipid Metabolism
NFSC678F Nutrition and Chronic Disease
BIOM602 Biostatistics II
BCHM676 Biological Mass Spectrometry

Chris Hakenkamp, PhD
Thomas W. Castonguay, PhD
Thomas W. Castonguay, PhD
Michael Ma, PhD
Wen-Hsing Cheng, PhD
Thomas W. Castonguay, PhD
Iqbal Hamza, PhD Jiuzhou Song,
Georgiy Belov, PhD
Liqing Yu, PhD
Shaik O. Rahaman, PhD
Bahram Momen, PhD
Catherine Fenselau, PhD

Laboratory Techniques

- Chromatography Columns
 - Affinity- Streptavidin
 - Solid Phase Extraction- desalting and detergents
- Protein Tagging
 - Enzymatic
 - Copper-catalyzed azide-alkyne cycloaddition
- Trypsin digestion (Promega)
- Immunoassays
 - Simple Western by Protein Simple
 - Dot Blot
 - ELISA
- Dietary Fiber Extraction and Quantification (AOAC Method 991.43)
- Bomb Calorimetry
- Lyophilization
- Protein Quantification
 - Dumas Method
 - BCA
- Ash Content
- Aflatoxin Testing
- Glucose Quantitation
 - Vitros Clinical Analyzer
 - Blood Glucose Meters
- Triglyceride analysis
 - Vitros Clinical Analyzer-Biological Samples
 - Soxhlet Fat Extraction- Biological and Food Samples
- RT-qPCR
- DNA and RNA Isolations
- SDS-PAGE Gel Protein Separation

Equipment Experience

- Parr Bomb Calorimeters
- Nanodrop 2000 by Thermo
- Simple Western by Protein Simple
- ChemiDoc MP by Bio-RAD
- Room and Benchtop Lyophilizers
- Clinical analyzer
- Automated pipetting systems
- Siemens MAGNETOM Trio, A TIM System 3T
- Muffler Furnace
- Automated Soxhlet

- Leco Nitrogen Analyzer

Laboratory Rodent Experience

- Rodent Handling
 - Sprague Dawley Rats
- Rodent Surgery
 - Intracerebroventricular Cannulation via stereotaxic placement
- Anesthetics
 - Ketamine/Xylazine cocktail
 - Isoflurane
- Rodent Injections
 - Intraperitoneal
 - Subcutaneous
 - Intramuscular
 - Sublingual
- Blood Collection
 - Tail blood draw
 - Truncal blood
- Tissue harvest
 - Brain
 - Liver

Computer/Software Experience

Proficient-SAS/SAS Enterprise, Microsoft Office (Excel, Powerpoint, Word), Mac Software, Microsoft Software
Sufficient-AFNI, Website development, Basic Linux Commands, Jump by SAS, SPSS, R



UNIVERSITY OF
MARYLAND

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

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DATE: February 16, 2016

TO: Thomas Castonguay

FROM: University of Maryland College Park (UMCP) IACUC

PROJECT TITLE: [843690-1] STAT 3 and Foxo1 O-GlcNAcylation results in impaired promoter binding that results in leptin resistance

IACUC REFERENCE #: R-16-01

SUBMISSION TYPE: New Project

ACTION: APPROVED

APPROVAL DATE: February 16, 2016

EXPIRATION DATE: February 16, 2019

Thank you for your submission of the Animal Study Protocol [R-16-01] STAT 3 and Foxo1 O-GlcNAcylation results in impaired promoter binding that results in leptin resistance. The University of Maryland College Park (UMCP) IACUC has APPROVED your submission. This approval is based on the committee's review of the appropriate use and care of animals within your research goals.

Research must be conducted in accordance with this approved submission. All changes must be submitted to the University of Maryland College Park (UMCP) IACUC as a revision. Conducting research outside the scope of your approved submission is reportable to federal entities and will be investigated by the University of Maryland College Park (UMCP) IACUC and may require interruptions to the research project.

Any revision to previously approved materials must be approved by this committee prior to initiation. Please use the appropriate revision forms for this procedure which are found on the IRBNet Forms and Templates Page.

All UNANTICIPATED PROBLEMS involving UNEXPECTED MORBIDITY or MORTALITY to animals or study personnel must be reported promptly to this office.

This protocol requires continuing review by this committee on an annual basis. Please use the appropriate forms for this procedure. Your documentation for continuing review must be received with sufficient time for review and continued approval before the expiration date of February 16, 2019.

Please note that all research records must be retained for a minimum of three years after the completion of the project.

If you have any questions, please contact Renee Kahn at (301) 405-5037 or rkahn002@umd.edu. Please include your project title and reference number in all correspondence with this committee.

Catherine Carr, IACUC Chair



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INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

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DATE: July 28, 2016

TO: Thomas Castonguay
FROM: University of Maryland College Park (UMCP) IACUC

PROJECT TITLE: [843690-2] STAT 3 and Foxo1 O-GlcNAcylation results in impaired promoter binding that results in leptin resistance

IACUC REFERENCE #: R-16-01
SUBMISSION TYPE: Amendment/Modification

ACTION: APPROVED- VVC
APPROVAL DATE: July 28, 2016
EXPIRATION DATE: February 16, 2019

Thank you for your submission of the Animal Study Protocol [R-16-01] STAT 3 and Foxo1 O-GlcNAcylation results in impaired promoter binding that results in leptin resistance. The University of Maryland College Park (UMCP) IACUC has APPROVED proposed changes to the protocol. This approval is based on the committee's review of the appropriate use and care of animals within your research goals.

Research must be conducted in accordance with this approved submission.

Please note that all research records must be retained for a minimum of three years after the completion of the project.

If you have any questions, please contact Renee Kahn at (301) 405-5037 or rkahn002@umd.edu. Please include your project title and reference number in all correspondence with this committee.

Catherine Carr, IACUC Chair

This letter has been electronically signed in accordance with all applicable regulations, and a copy is retained within University of Maryland College Park (UMCP) IACUC's records.