DIETARY CARBOHYDRATE INTERACTS WITH DIETARY FAT TO INFLUENCE

LEPTIN RESPONSIVENESS IN RATS

by

SAMANTHA JEAN HARING

(Under the Direction of Ruth Harris)

ABSTRACT

To determine whether leptin resistance caused by a high fructose diet is due to fructose specifically or to increased dietary monosaccharide, we tested leptin responsiveness in rats fed 30% kcal fat diets containing 50% kcal glucose, 40% kcal fructose, or 15% kcal fructose. Intraperitoneal injections of 2.0 mg leptin/kg inhibited 14 hour weight gain and food intake in rats fed 40% fructose or glucose diet for 9 weeks, but not in those fed the 15% fructose diet. Leptin stimulated phosphorylation of signal transducer and activator of transcription 3 (PSTAT-3) in the medial and central nucleus of solitary tract in 40% fructose-fed animals, but not in the hypothalamic arcuate nucleus of any group. In a second study only glucose-fed animals responded to central leptin infusions although all animals remained insulin sensitive. Therefore, dietary monosaccharides may act at sites other than the hypothalamus to reverse high-fat diet induced leptin resistance.

INDEX WORDS: leptin, fructose, STAT-3, immunohistochemistry, arcuate nucleus, nucleus of the solitary tract, leptin resistance

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Again, this page is optional. You do not have to provide an acknowledgements section in your thesis or dissertation. You may use this section to express acknowledgements of those who have helped you with this document and your academic career.

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# CHAPTER 1

## INTRODUCTION

The prevalence of overweight and obesity among Americans is on the rise and currently 66% of Americans are overweight or obese (12, 13). In Westernized societies, impaired food intake regulation contributes to the obesity epidemic. Appetite signaling in the brain plays a key role in regulation of food intake (9, 16). There are several appetite signals that have been associated with the control of food intake including neuropeptide Y, ghrelin, cholecystokinin, and leptin (14). Leptin, an adipocyte-derived cytokine, has received a lot of attention as a factor that determines energy balance. In experimental conditions, leptin causes a decrease in food intake and an increase in energy expenditure. Obese individuals, when compared to normal weight controls, have high circulating levels of leptin, yet they fail to decrease food intake; this is a condition known as "leptin resistance" (5). Although the exact mechanism of leptin resistance is not known, a current theory of leptin resistance involves an inhibition of leptin transport across the blood-brain barrier due to an elevation of circulating triglycerides (1). This results in failure of central leptin receptors to be activated, which can be confirmed by measuring the signal transducers and activators of transcription-3 (STAT-3) phosphorylation, a transcription factor that is phosphorylated following leptin receptor activation (2). Another potential cause of leptin resistance involves increased expression of suppressor of cytokine signaling-3 (SOCS-3), a transcription factor that acts as a negative feedback signal to leptin receptor activation (3).

On the rise with an increased positive energy intake is the consumption of the monosaccharide, fructose (4). Excessive consumption of fructose has been related to changes in appetite signaling and metabolic pathways (6, 10), causing it to be a dietary factor of interest when looking at appetite regulation. One main concern for an increased fructose consumption is

related to its metabolic effects. Unlike glucose metabolism, fructose metabolism stimulates lipogenesis (8). Increased fructose consumption not only results in increased triglycerides and VLDLs, but it also alters certain appetite hormones (11). Fructose metabolism results in a small amount of glucose production, meaning insulin secretion may be stimulated by low intakes of fructose but when fructose intake increases there is little further stimulus for insulin release. Because consumption of high levels of fructose increases circulating concentrations of triglycerides, it is possible that high fructose diets can cause leptin resistance.

Preliminary data from our lab has shown that a diet high in fructose and fat leads to the development of leptin resistance in rats. Leptin resistance was measured as an inhibition of energy intake and body weight gain following a peripheral injection of leptin. The experiments described here determined if leptin resistance was associated with a failure of leptin to activate central leptin receptor by testing STAT-3 activation in the hypothalamus and brainstem of rats fed diets of varying fructose concentration. In order to demonstrate that this effect was dependent on fructose, the monosaccharide glucose was also tested. A second study was conducted that administered leptin centrally into the 3$^{rd}$ ventricle of the brain, to test whether leptin resistance in fructose fed rats was central or peripheral. Glucose tolerance was also measured in this experiment to determine whether chronic fructose consumption caused simultaneous changes in leptin and insulin sensitivity because leptin and insulin receptors activate similar proteins in the post-receptor signaling cascade (7, 15).

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# CHAPTER 2

# LITERATURE REVIEW

## OBESITY

The prevalence of overweight and obesity among Americans is increasing and currently 66% of Americans are overweight or obese and 32% are obese (37, 38). An increased body weight is often associated with other health problems such as diabetes, cancer, cardiovascular disease, and metabolic syndrome (1). An increased body weight is most often due to an excess energy intake. Appetite signaling in the brain has been shown to play a key role in the regulation of food intake (26, 48). This monitoring of adiposity by the brain is of interest in obesity research because subjects have ample fat stores, but still eat more energy than they need. There are several appetite signals that have been associated with the control of food intake including neuropeptide Y, ghrelin, cholecystokinin, and leptin (47).

## LEPTIN

The *ob* gene was first designated when certain mice out of a group became obese, gaining as much as four times the weight as their normal-weight counterparts, these mice were thus designated ob/ob mice (25). Another group of mice were also found to be obese and to develop severe diabetes; these mice are referred to as db/db (11, 14). Parabiotic studies were performed with ob/ob mice and db/db mice that suggested the presence of circulating factors in the shared blood supply; these studies showed a decreased body weight in the ob/ob mice and no change in body weight of the db/db mice (13). It was hypothesized that ob/ob mice were missing a satiety factor, whereas db/db mice could not respond to the signal. This circulating factor was identified as leptin in 1994 (49). Friedman's lab found that ob/ob mice lacked the gene that codes for

leptin production, describing the animals as leptin deficient (18) and the db/db mice then were found to lack the leptin receptor (28).

Since leptin's discovery in 1994, many studies have been performed to understand its functions and its relationship with obesity. Leptin has often been referred to as an adipostat, that is it helps to control the amount of fat in the body (17, 18). Leptin acts in several tissues, but in situ hybridization has shown high levels of leptin receptors in the hypothalamus, specifically in the arcuate nucleus, ventromedial nucleus, paraventricular nucleus, and the ventral premamillary nucleus (34). When leptin receptors in these areas are activated, leptin signaling results in a decreased food intake and increased energy expenditure. Lack of this signaling in both humans and rodents results in effects that are opposite to those of leptin; increased food intake and decreased energy expenditure that lead to an increased body weight (12, 18). When leptin was administered to ob/ob and db/db mice, ob/ob mice lost body weight whereas there was no change in db/db mice, validating the parabiotic study hypothesis that ob/ob mice are deficient in leptin and db/db mice are deficient in the receptor (20). Because obese individuals have an increased food intake, leptin administration has also been tested to determine if it has comparable effects as in the ob/ob mice. In non-obese subjects, leptin administration shows no significant effect on energy expenditure or food intake (30). In one study, obese subjects who were treated with leptin showed a dose-dependent effect in some of the subjects (22), but other studies show conflicting results (31). A deficiency in leptin is not found in most obese individuals, rather, it has been found that obese individuals have increased levels of leptin when compared to individuals of normal weight (15). This has led to the concept of leptin resistance, a condition in which circulating concentrations of leptin are elevated, but they have no effect on energy intake or expenditure. Leptin resistance is discussed in more detail below.

## LEPTIN RECEPTORS

The leptin receptor, Ob-R, was identified in the mouse choroid plexus, as well as other tissues such as the hypothalamus, and was classified as a member of the class I cytokine receptors (36, 43). Leptin receptors may be spliced into different isoforms and are designated as Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re, Ob-Rf (18, 28, 36). These receptors are further classified into long and short forms: short forms include Ob-Ra, Ob-Rc, Ob-Rd, Ob-Rf and the long form is Ob-Rb (28, 43). ObRe is a circulating receptor that sequesters free leptin in the circulation (28, 42). The long isoform is considered to be the most important for mediating the effect of leptin on energy balance and is found at high levels in the hypothalamus (19, 28, 43). Further studies found leptin receptor mRNA to be most concentrated in the arcuate nucleus (ARC), ventromedial and dorsomedial nuclei of the hypothalamus, validating the importance of the hypothalamus as a site of leptin activity (41). A comprehensive study by Sahu found that leptin receptor activation in the hypothalamus may regulate, down-stream, other neurohormones including galanin, melanin-concentrating hormone, neurotensin, proopromelanocortin, and neuropeptide Y (40).

Leptin receptors have 3 domains: a ligand-binding domain that is extracellular, a transmembrane domain, and a signaling domain in the cytoplasm (36, 43). When leptin binds to the extracellular domain of the long form of the receptor it activates Jak2, part of the signaling domain of the receptor (24). When Jak2 is activated it induces phosphorylation of tyrosines. Jak2, recognizes the phosphorylated tyrosines and induces further activation and helps to control the signaling process (27, 36). Two tyrosines associated with leptin signaling are Tyr985 and Tyr1138 (2, 19, 43). While Tyr986 phosphorylation results in extracellular signal-related kinases (ERK) functioning, its function is unclear at this time, Tyr1138 phosphorylation induces the signal transducers and activators of transcription-3 (STAT-3) protein (2, 43, 45). Leptin's

main effects, decreased food intake and increased energy expenditure, are reliant on STAT-3 activation (5), however other factors of leptin signaling, such as ERK have been correlated with leptin's effects on energy homeostasis (39). With increased activation of the leptin receptor, suppressor of cytokine signaling-3 (SOCS-3) is also activated, which binds to Jak2 to inhibit further activation of STAT-3 and inhibits the effects of leptin; this system represents a negative feedback system (2, 7). In situ hybridization has shown that measurement of expression of SOCS-3, predominately in neurons in the ARC, can be used in addition to measurement of STAT-3 activation as an indirect indication of leptin receptor activation in the brain (4, 7).

## LEPTIN RESISTANCE

Circulating leptin levels are highly correlated with the percentage of body fat in an individual and are high in obese individuals (15). Obese individuals treated with leptin do not show any significant leptin response, indicating that they are resistant to leptin (31). Several potential mechanisms have been proposed for leptin resistance; however, two have gained the most attention: inhibition of leptin transport into the brain and increased inhibitor expression. Burguera et al. found that in obese individuals leptin has diminished transport across the blood brain barrier (10). They have also hypothesized that saturation of the leptin receptors at the blood brain barrier may be contributing to this decreased transport (10). Increased triglycerides were found to also inhibit leptin transport across the blood brain barrier, a state that exists in both starved and obese individuals, leading to the hypothesis that starvation-increased triglycerides potentially protect the brain from receiving a leptin signal in order to prevent a further decrease in food intake (3).

With prolonged activation of Ob-Rb, suppressor of cytokine signaling (SOCS-3) is activated and inhibits further activation of leptin signaling proteins. Increased levels of this

transcription factor have been associated with obesity in rodents and could possibly be related to a failure of leptin receptor activation in conditions of leptin resistance (6, 8, 36).

## FRUCTOSE

In parallel with the increased incidence of obesity is an increased consumption of the monosaccharide, fructose. An increase of fructose consumption from 37 g/day in 1977 to 54.7 g/day in 2007 is related to an increase in fructose-containing beverages and snack foods available on the market today (9, 46). One main concern for an increased fructose consumption is related to its metabolic effects; unlike glucose metabolism, fructose metabolism stimulates lipogenesis (21). With glycolysis, an influx of glucose is regulated via the enzyme phosphofructokinase (PFK); when fructose is consumed, it also enters glycolysis, but it does so at a point that is not regulated by PFK. Due to this lack of regulation, an excessive fructose consumption can lead to increased pyruvate, the final product of glycolysis, leading to an increase in acetyl-coA production and ultimately acyl glycerols and very low density lipoproteins (VLDLs) (21, 32).

## FRUCTOSE AND INSULIN SENSITIVITY

Fructose metabolism results in a small amount of glucose production, meaning insulin secretion may be limited following fructose consumption. Although most studies show a negligible change in insulin secretion after fructose ingestion, a low fructose consumption of 7.5 g/day in adults has been shown to increase glucose response in adult type 2 diabetics (16, 35). Although acute consumption of fructose seems to be neutral in relation to insulin release, chronic consumption may indirectly lead to hyperinsulinemia. Potential explanations of fructose-induced insulin resistance include inhibition of insulin signaling and hepatic glucose production, as well as indirect effects resulting from increased accumulation of lipids in the liver and muscle (21).

## FRUCTOSE AND APPETITE HORMONES

Increased fructose consumption not only results in increased serum triglycerides and VLDL concentrations, but it also alters certain hormones involved in the control of food intake (33). In humans, ghrelin, a hormone that stimulates food intake, has been shown to decrease by 30% after consumption of a diet with 30% kcal of glucose, but not to change after the consumption of a diet with 30% kcal of fructose (21, 44). This same study tested leptin and triglyceride levels after 6 months consumption of the 30% kcal fructose diet and found a 24% increase in leptin levels and a 35% increase in triglyceride levels in comparison to the glucose diet, suggesting that a fructose diet can alter leptin responsiveness and significantly raise triglyceride levels in comparison to a glucose diet (44). Another study compared the effects of rodent consumption of a high fat diet (20% kcal lard) versus a high fructose diet (60% kcal fructose) (23). The results show that the animals on the high fat diet had higher body weights, but the fructose fed animals had higher triglyceride levels and higher plasma leptin concentrations (23). Another study tested various carbohydrate solutions (sucrose, glucose, and fructose) on rodents' appetite and serum levels. The results indicated that all sources caused an increase in body weight and leptin levels (29). It is obvious that fructose leads to an increase in leptin levels; however these leptin levels are not associated with a decrease in body fat or food intake. What remains to be understood is how fructose is inducing leptin resistance at the receptor level.

## SUMMARY

Leptin is an adipocyte cytokine that is of major interest in appetite signaling and satiety. Recent data suggest that rats fed a high fructose diet are leptin resistant. Leptin resistance is a phenomenon that is not yet well understood, but has recently been linked to an inhibition of leptin transport across the blood brain barrier by elevated concentrations of triglycerides in the

blood (3). This idea involves failure of the leptin receptors in the brain to be activated when circulating leptin concentrations are high. Leptin receptor activation can be confirmed by measuring the phosphorylation of STAT-3, a critical protein in the leptin receptor signaling cascade (5). By testing the activation of this transcription factor in a model of fructose-diet induced leptin resistance, we will gain insight into the effects of dietary fructose on leptin responsiveness. Another experiment that will help us to understand this model is administration of leptin into the 3$^{rd}$ ventricle of the brain, to determine whether leptin resistance is occurring in the periphery or in the brain. Lastly, insulin responsiveness of the rats will determine whether chronic fructose consumption results in the simultaneous development of insulin resistance.

**Hypothesis**

Leptin resistance induced by medium fructose, high fat diets is associated with a failure of leptin to activate central leptin receptors and is associated with a decrease in STAT-3 activation in the hypothalamus and brainstem.

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# CHAPTER 3

# DIETARY CARBOHYDRATE INTERACTS WITH DIETARY FAT TO INFLUENCE LEPTIN RESPONSIVENESS IN RATS[1]

[1] Haring, S.J., Harris, R.B.S. To be submitted to the *American Journal of Physiology*

ABSTRACT

Previously we showed that a diet high in fructose induces leptin resistance in rats. To determine whether changes in leptin responsiveness were due to fructose specifically or increased dietary monosaccharide, we performed a study to test leptin responsiveness in rats fed diets containing 30% kcal fat and either 50% kcal glucose, 40% kcal fructose, or 15% kcal fructose . After 9 weeks, i.p. injections of 2.0 mg/kg leptin inhibited 14 hour weight gain and food intake in the 40% fructose fed rats and the glucose rats, but not in the 15% fructose fed rats. Phosphorylation of signal transducer and activator of transcription 3 (PSTAT-3) in the medial and central nucleus of solitary tract was significantly stimulated by leptin only in the 40% fructose fed animals. No animals exhibited significant PSTAT-3 levels in the hypothalamic arcuate nucleus. A second study tested central response to leptin infusions in animals fed the same experimental diets for 9 weeks. Only the glucose-fed animals responded to the leptin infusions. All animals in the second study remained insulin sensitive. These data suggest that dietary fat and carbohydrate have independent effects on leptin responsiveness and signaling and monosaccharides may reverse leptin resistance produced by a high-fat diet by acting at sites other than the hypothalamus.

# INTRODUCTION

Leptin, an adipocyte-derived cytokine, is an essential regulator of food intake. Leptin acts in several tissues, but in situ hybridization has shown high levels of leptin receptors in the hypothalamus, specifically in the arcuate nucleus, ventromedial nucleus, paraventricular nucleus, and the ventral premamillary nucleus (24). When leptin receptors in these areas are activated, leptin signaling results in a decreased food intake and increased energy expenditure. A lack of leptin signaling in both humans and rodents results in effects that are opposite to those of leptin; increased food intake and decreased energy expenditure that results in increased body weight (8, 14). Overweight and obese individuals with ample fat stores, have higher amounts of circulating levels of leptin than normal weight individuals (9). Despite the fact that adipose tissue produces leptin in proportion to the size of body fat stores, obese individuals do not have a diminished food intake to prevent further weight gain. This has led to the concept of leptin resistance, a condition in which circulating concentrations of leptin are elevated, but have no effect on energy intake or expenditure.

One theory of leptin resistance is the idea of increased circulating triglycerides inhibiting leptin transport across the blood brain barrier (1). One source of increased triglycerides is excessive fructose consumption. When high amounts of fructose are consumed, lipogenesis occurs, resulting in increased amounts of circulating triglycerides in the blood. A study by Huang et al. compared the effects of consumption of a high fat diet (20% kcal lard) versus a high fructose diet (60%) in rats for 6 months. The results show that the animals on the high fat diet had higher body weights, but the fructose fed animals had higher triglyceride levels and higher plasma leptin concentrations than the animals fed the high fat diet (19). The effects of fructose have also been compared to those of glucose. In a study by Stanhope et al., overweight and

obese subjects were given either a glucose or fructose beverage that consisted of 25% kcal of daily caloric intake (35). Visceral adiposity was significantly increased in the fructose group when compared to the glucose group. In addition, dyslipidemia increased de novo lipogenesis and decreased insulin sensitivity was seen in the fructose group (35). Analyzing biochemical markers such as leptin, triglyceride, and insulin levels are critical for understanding these experiments, but measuring signal transducers and activators of transcription-3 (STAT-3) levels in the brain may also provide an indication of the activity of leptin receptors (5) . Measuring PSTAT-3 levels in both the nucleus of the solitary tract in the brainstem and in the arcuate nucleus in the hypothalamus, we can compare the leptin receptor activation of rats consuming either a fructose or glucose diet. After analyzing the differences of the leptin activation in these areas of the brain, we can gain insight into the effects of dietary carbohydrate on central leptin receptor activation. Additionally, by testing leptin responsiveness using central infusions of rats consuming high fructose and high glucose diets, we can compare this data with the PSTAT-3 data and the current leptin literature in order to determine the effects of dietary monosaccharides on central and peripheral leptin signaling.

## METHODS

All animal procedures were approved by the University of Georgia Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### *Experiment 1: Testing leptin responsiveness, via intraperitoneal injections, after 9 weeks of monosaccharide experimental diets*

Preliminary data from our lab showed that high-fat, high fructose diets lead to leptin resistance, but leptin responsiveness in rats fed glucose-containing diets has not been tested. The objective of Experiment 1 was to test whether a high-fat, high-glucose diet also led to leptin resistance. This would demonstrate whether leptin resistance in rats fed a fructose diet was due to the fructose specifically or was simply an effect of monosaccharide.

Experimental Protocol: Thirty-two male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) with baseline weights of approximately 250 g were housed in individual hanging wire cages in a climate controlled room at 23ºC with lights on 12 hr/day from 07:00 hours. After one week on a standard chow diet the rats were weight-matched into 3 groups. Each group was randomly assigned to one of three experimental diets, all of which contained 30% kcal fat. Eleven rats were assigned to the Medium Fructose diet (MFruc HF), 10 rats were assigned to the Low Fructose diet (LFruc HF), and 11 rats were assigned to the Glucose diet (Gluc HF) (**Table 1)**. The MFruc HF diet consisted of 40% kcal fructose, 10% kcal glucose; the LFruc diet consisted of 15% kcal fructose, 10% kcal glucose; and the Gluc HF diet consisted of 50% kcal glucose and no fructose (Research Diets, Inc., New Brunswick, NJ) (**Table 1**). Body weights were recorded two times a week. Preliminary data had previously shown that rats fed the Medium Fructose diet for 5 weeks were leptin resistant, therefore on days 36 and 39, rats were tested for leptin responsiveness. On the first test day half of the rats received leptin and half received PBS and on the second test day rats received their counterpart, i.e. rats receiving leptin on test day one received PBS on test day two. Food was taken away at 08:00 hours on the morning of the test; rats were injected i.p. later the same day at 18:00 hours with either 2.0 mg/kg leptin (rat recombinant leptin, R+D Systems, Minneapolis, MN) or an equivalent volume

of PBS. At 19:00 hours, one hour after the injections were administered, food was replaced. Food intakes were then measured 14, 24, and 36 hours post injection. After food intakes and body weights were analyzed, it was determined that all treatment groups were leptin responsive, therefore the experiment was continued and the rats were again tested for leptin responsiveness on days 64 and 67.

*Experiment 2: Testing STAT-3 activation in the hypothalamus and brainstem, after 10 weeks of consumption of experimental diet*

Experiment 2 tested whether the rats fed diets that caused leptin resistance in Experiment 1 show a decreased STAT-3 activation, which is often used as an indication of leptin receptor activation. The objective of Experiment 2 was to determine differences in dietary monosaccharide effects on leptin-induced STAT-3 activation.

Experimental Protocol: On days 70 and 71, 3 or 4 days following the final leptin responsiveness test in Experiment 1, rats were food deprived at 08:00 hours and injected with either 2.0 mg/kg leptin or PBS at 12:00 hours. Twenty minutes after the injections of either leptin or PBS, rats were anesthetized with 100 mg/kg Ketamine. An incision in the skin and muscle was made transversely under the rib cage, followed by another incision made perpendicular to the first incision along the rib cage, opening the thoracic cavity for access to the heart. A needle was inserted into the heart and the following ice-cold fluids were infused into the rats' circulation: 75 ml heparinized saline, 200 ml 4% paraformaldehyde, and 25 ml heparinized saline. All solutions were pumped at a rate of 50 ml/min from a peristaltic pump. After perfusions, the rats were decapitated and brains were collected and stored in 4% paraformaldehyde at 4ºC.

One day prior to slicing, brains were transferred to a 25% sucrose, 0.01% azide solution. Using a Vibratome UltraPro 5000 (Vibratome®, St. Louis, MO), two areas of the brain were sliced and collected: the nucleus solitary tract (NTS) in the brainstem and the arcuate nucleus (ARC) in the hypothalamus. Using the coordinates given in the Paxinos Rat Brain Atlas (28), the following two areas were sliced (all coordinates are given in relation to bregma): ARC (Anteroposterior: -2.12 mm to -4.13 mm) and NTS (Anteroposterior: -13.68 mm to -14.30 mm) (28). Using the vibratome, 30 µm serial coronal slices were collected in a 24-well plate with sucrose azide and stored at 4°C until immunohistochemistry (IHC) procedures were performed. The protocol used for phosphorylated STAT-3 immuhistochemistry was as described by Ellacott et al (13). Slices from the well plate were transferred into glass scintillation vials using a paint brush. Four wells were used for collection of each brain, but only slices from one well from each of the four wells containing slices of each rat were used for the STAT-3 protocol. Therefore PSTAT-3 was detected on every fourth slice (120 µm interval) through the arcuate nucleus and brainstem. On the first day of the IHC protocol, tissues were incubated as follows: 10 minutes in PBS, 20 minutes in 1% sodium hydroxide/hydrogen peroxide, 10 minutes in 0.3% glycine in PBS, 10 minutes in SDS in PBS, 1 hour in 3 mL PBS triton with 150 µL donkey serum, and finally a 1:3000 dilution of rabbit anti-PSTAT-3 (Catalogue # ab16030, Abcam, Cambridge, MA) was added for overnight incubation. On day 2 of the protocol, tissues were washed and incubated as follows: 5 minutes in PBS triton, 5 minutes in PBS triton, 5 minutes in PBS, 1 hour of biotinylated antibody (Vectastain ABC kit, Vector Labs, Burlingame, CA), 5 minutes in PBS triton, 5 minutes in PBS triton, 5 minutes in PBS, 5 minutes in the second antibody or ABC reagent, 5 minutes in PBS triton, 5 minutes in PBS triton, 5 minutes in PBS, 5 minutes in DAB-nickel substrate, 5 minutes in PBS triton, 5 minutes in PBS triton, and 5 minutes in PBS.

Following IHC, slices were mounted onto subbed slides using a paint brush and allowed to dry overnight. Slices were then counterstained with cresyl violet and coverslipped using Permount. Slices were allowed to dry overnight and PSTAT-3 was visualized using a Nikon microscope.

Images on the slides were captured on the computer using ImagePro Plus Software (Media Cybernetics, Inc., Bethesda, MD) and counted using Adobe Photoshop software (Adobe Systems, Inc., San Jose, CA). A 27 mm reticule 1 mm square 10x10 grid (Nikon Instruments, Melville, NY) was inserted into the lens of the microscope to enhance the ability to count the slices. This grid was then aligned to a 10x10 grid image in Photoshop that was superimposed onto the slice. Using the Paxinos Rat Brain Atlas as a guide, the areas to be counted were outlined on the image of the slice. The image on the computer was then compared to the image on the microscope to allow only PSTAT-3 activation sites to be counted. Data was then collected for each rat, and the sum of the counted sites was analyzed.

*Experiment 3: Testing leptin responsiveness, via third ventricle infusions, after 9 weeks of monosaccharide experimental diets*

In Experiment 1, leptin was administered as i.p. injections. Because leptin can act in both peripheral tissue and the brain, an i.p injection did not differentiate between central and peripheral leptin responsiveness. The objective of this experiment was to determine whether the medium-fructose diet caused central leptin resistance.

Experimental Protocol: Thirty male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) with baseline weights of approximately 300 g were housed in individual hanging wire cages in a climate controlled room at 23ºC with lights on 12 hr/day from 07:00 hours. The rats were

given a standard chow diet prior to surgery. Rats underwent stereotaxic surgery to place guide cannulas in the third ventricle of the brain using coordinates from the Paxinos Rat Brain Atlas (anteroposterior -2.8mm, lateral 0.0mm, ventral -8.3mm relative to bregma). One week after surgery, cannula placement was tested. Rats were infused with 20 ng angiotensin II in 2 µL sterile saline. Rats that drank water within 2 minutes of the infusion were included in the experiment; all thirty rats responded positively to the angiontensin test. After surgeries and a 7 day recovery period, rats were weight-matched and assigned to one of the three experimental diets. The diets were the same as described for Experiment 1 (**Table 1**). Rats were tested for leptin response at 9 weeks (day 57 and day 60). Food was taken away at 08:00 hours on the morning of the test; rats were injected later the same day at 18:00 hours. To test for leptin responsiveness, rats were infused with 1.5 µg leptin/2 µL or 2 µL PBS into the third ventricle. On day 57, half of the rats received PBS and the others received leptin, and then on day 60 rats received the other treatment. At 19:00 hours, one hour after the infusions were administered, food was replaced. Food intakes and body weights were then measured 14, 24, and 36 hours post injection.

*Experiment 4: Testing insulin sensitivity after 10 weeks of monosaccharide experimental diets*

Because high-fat and medium fructose diets are reported to cause insulin resistance and because leptin and insulin use common post-receptor signaling proteins, we used the rats from Experiment 4 to determine whether the three diets described above had differential effects on insulin sensitivity of the rats.

Experimental Protocol: Insulin sensitivity was tested on day 66. Food was removed from the cages at 08:00 hours. Starting at 14:00 hours each rat was injected i.p. with 0.75 U/kg insulin (Eli Lilly Corp.). Blood samples were collected from the rat immediately before injection (Time 0) and at 10, 20, 30, 45, 60 and 75 minutes after the insulin injection. At time 0 and 10 min, approximately 0.5 ml of blood was collected to allow measurement of insulin (Rat Insulin RIA kit; Millipore Corp.) at all other time points only small volumes of blood were collected to allow measurement of blood glucose concentration (Glucometer Elite: Bayer Corp.). Food was returned to the cages after collection of the last blood sample.

Statistics: Body weights and food intakes were analyzed using t-tests, two-sample assuming unequal variances (Excel 2003, part of Microsoft Office Professional Edition 2003.) PSTAT-3 activation was measured using one-way ANOVA and Duncan's Multiple Range test for post hoc comparisons between groups (Statistica, Stat Soft, Tulsa OK).

## RESULTS

*Experiment 1: Testing leptin responsiveness, via intraperitoneal injections, after 9 weeks of monosaccharide experimental diets.*

The average starting weights for the rats were 250 g and the weights at the end of the study averaged 440 g. Rats were given leptin to test for responsiveness at 5 weeks, however, all animals were responsive (data not shown). After 64 days on experimental diets, animals consuming the glucose and the medium fructose diets showed a significant inhibition of weight gain 12 hours after receiving an injection of 2.0 mg/kg leptin (**Figure 1**; Glucose: $P<0.002$, Medium Fructose: $P<0.04$). Two-way ANOVA revealed that a leptin effect was seen during the

leptin and PBS administration (P<0.0001). There was no effect of leptin at later time points. There was no significant difference in weight change in the rats consuming the low fructose diet after leptin administration (**Figure 1**; P<0.1). Leptin significantly inhibited food intake (P<0.007), but the difference was not significant for any of the three dietary groups. There was no effect of diet on suppression of food intake after leptin injections occurred (**Figure 2**; Glucose: P<0.06, Medium fructose: P<0.16, Low fructose: P<0.18).

*Experiment 2: Testing STAT-3 activation in the hypothalamus and brainstem, after 10 weeks of consumption of experimental diet*

Three areas of the brain were analyzed for PSTAT-3 as a measure of leptin receptor activation using immunohistochemical methods. PSTAT-3 in the nucleus of the solitary tract was measured at both the medial and central nucleus of the solitary tract. Using the coordinates given in the Paxinos Rat Brain Atlas (28), the following two areas were sliced (all coordinates are given in relation to bregma): ARC (Anteroposterior: -2.12 mm to -4.13 mm) and NTS (Anteroposterior: -13.68 mm to -14.30 mm) (28) (**Figure 3 & 7**). In the medial nucleus of the solitary tract, PSTAT-3 was increased in leptin treated animals (P<0.01), but this was significant in only the rats consuming the medium fructose diet when compared to PBS controls (**Figure 5**; Medium fructose: P<0.0001). In the central nucleus of the solitary tract, leptin stimulation of PSTAT-3 by leptin (P<0.007) was significant only in rats fed the medium fructose diet; rats fed glucose and low fructose diets did not show an increase in activation with leptin administration (**Figure 6**; Medium fructose: P<0.0001, Glucose: P<0.09, Low fructose: P<0.8). Although there was no effect of leptin, the level of PSTAT-3 activation present in tissue of PBS-injected rats fed the low fructose diet was the same as the level found in leptin-injected rats fed the medium

fructose diet. In the arcuate nucleus of the hypothalamus, there was no significant effect of leptin or diet treatment on PSTAT-3 (**Figure 9**; Glucose: $P<0.06$, Medium fructose: $P<0.4$, Low fructose: $P<0.4$).

*Experiment 3: Testing leptin responsiveness, via third ventricle infusions, after 9 weeks of monosaccharide experimental diets*

After 9 weeks of consumption of experimental diets, central leptin responsiveness was tested by recording food intake and changes in body weight after infusion of leptin into the third ventricle. Weight change of rats recorded at 14, 24, and 36 hours post 1.5 µg leptin/2 µL or 2 µL PBS infusions showed no differences in weight change for animals consuming the fructose diets (**Figures 10-12**) but weight gain was inhibited by leptin in animals consuming the glucose diets at both 24 and 36 hours post infusions **(Figures 11-12)**. Diet composition had a significant effect on food intake at all three time points measured: 14, 24, and 36 hours (**Figures 13-15**; $P<0.006$, $P<0.0004$, $P<0.0007$). Rats consuming the medium fructose diet ate significantly less than the rats consuming the low fructose diet at 24 and 36 hours **(Figures 14-15)**. There was no effect of leptin on cumulative food intake in any of the diet groups (**Figures 13-15**).

*Experiment 4: Testing insulin sensitivity after 10 weeks of monosaccharide experimental diets*

Insulin tolerance test results show that after 10 weeks of consumption of the experimental diets, insulin sensitivity was the same in all of the animals (**Figures 16-17**). Basal insulin levels were significantly different for each diet group at time 0 versus time 15.

# DISCUSSION

Leptin is an adipocyte cytokine that contributes to energy homeostasis. Normal signaling of leptin results in a decrease in food intake and an increase in energy expenditure. It is well known that increased amounts of body fat are associated with elevated levels of circulating leptin (9). Adequate functioning of leptin is not observed in overweight individuals with increased amounts of leptin, as exemplified in their static or even increased food intake patterns and reduced energy expenditure. The mechanistic basis of this resistance to leptin is yet to be understood. Previous data from our lab suggests that leptin resistance can also develop when a high amount of fructose is present in the diet. We had not tested whether leptin resistance was due to fructose specifically or if it was caused by having high levels of monosaccharide, rather than polysaccharide, in the diet. The objective of the experiments described here was to determine if changing the dietary monosaccharide from fructose to glucose influenced the development of leptin resistance. Our data indicated that while glucose fed animals remain both centrally and peripherally responsive to leptin after 9 weeks of diet consumption, medium and low fructose fed animals show differential responses to leptin. Medium fructose rats were responsive to leptin only in the periphery, while low fructose rats responded to neither modes of leptin administration. Measured PSTAT-3 in the brain indicated leptin responsiveness that was inconsistent with the changes in food intake and body weight, suggesting that other aspects of leptin signaling, such as ERK, should be utilized to indicate leptin's effects.

Experiment 1 tested the effects of chronic consumption of three high fat diets with varying levels of monosaccharide content on leptin response in rats. In Experiment 1, rats fed the medium fructose (40% kcal) diet and rats fed the glucose (50% kcal) diet were leptin responsive, in that an injection of leptin decreased food intake, resulting in an inhibition of

weight gain. Surprisingly, the leptin-treated low fructose (15% kcal) diet fed rats did not experience the significant inhibition of weight gain like the other animals; these rats were leptin resistant. Although we had previously found that a high fructose, low fat diet caused leptin resistance, these results suggested that increasing the monosaccharide content of a high fat diet prevented the development of leptin resistance in high-fat fed rats.

Metabolic studies in both rats and humans show that increased consumption of fructose results in increased amount of circulating triglycerides due to higher rates of lipogenesis, and that increased triglycerides may inhibit leptin transport across the blood brain barrier (1, 17). Thus it is ambiguous as to why the diet with higher amounts of fructose resulted in leptin response and the diet with lower amounts of fructose induced leptin resistance. A potential explanation for this effect involves changes in levels of saturation of leptin transporters with chronic consumption of fructose. Banks et al. suggests that following consumption of a high fat diet, increased leptin levels cause diminished regulation of the leptin transporter and further accumulation of leptin results in increased saturation of the transporters (2). All of the diets used in this study were high in fat, so the idea of the fat component of these specialized diets causing increased saturation is invalid and is considered a control variable. Banks et al. determined that circulating triglycerides, more specifically, may be contributing to reduced transport (1). Levels of triglycerides were not tested in this study, but it has been suggested that excessive intakes of fructose results in increased lipogenesis (17). There have been conflicting studies in the literature however, that show inconsistent effects of fructose inducing hyperlipidemia (3, 16, 27, 37). A previous study from our lab measured triglycerides after consumption of the medium fructose diet and found no difference in triglyceride levels. If the fructose content of these diets does not increase serum triglycerides, this factor can also be excluded as being involved with leptin resistance.

The objective of Experiment 2 was to determine whether different dietary monosaccharides effected leptin-induced STAT-3 activation. When the leptin receptor is activated, STAT-3 is phosphorylated and results in the expression of leptin's main effects, a decrease in food intake and an increase in energy expenditure. Phosphorylation of this transcription factor has been found to be necessary in mediating leptin's effects (5). Several brain sites are associated with leptin action, but the hypothalamic arcuate nucleus (ARC) has been shown to be a direct target of leptin signaling (10). Additionally, the brainstem has more recently been found to mediate leptin activity in the nucleus of the solitary tract (NTS) (15, 18). By measuring and comparing leptin activity in both of these areas, differences in leptin transport, saturation mechanisms, and receptor activity may be identifiable.

When testing both the central and medial nuclei of the solitary tract in the brainstem, it was observed that only the medium fructose diet showed a significant elevation in PSTAT-3 levels in groups treated with leptin compared with those treated with receiving PBS. There was no increase in the level of PSTAT-3 in glucose or low fructose fed animals in any of the brain areas tested. This is expected for the low fructose rats, since their food intakes and changes in body weight were not indicative of leptin responsiveness in Experiment 1. Glucose rats however, were responsive to leptin in Experiment 1, but showed no increase in PSTAT-3 in the NTS. Concerning the ARC, we found that leptin did not increase the phosphorylation of STAT-3 in any of the treatment groups.

Although the ARC is generally accepted as a direct target of leptin signaling, PSTAT-3 is not necessary or sufficient in determining adequate leptin response (21, 26). More specifically, Myers et al. found that STAT-3 signaling is not necessary for regulation of the orexigenic neurons in the ARC (26). Leptin regulates two LRb-expressing neurons,

proopiomelanocortin/cocaine- and amphetamine-regulated transcript (POMC/CART) neurons and Agouti-related peptide/neuropeptide-Y (AgRP/NPY) neurons. Leptin inhibits AgRP/NPY-producing neurons thus inhibiting increased food intake and leptin activates POMC/CART neurons which ultimately cause a decrease in food intake (4, 12). If the activity of the orexigenic neurons is essential to leptin signaling, as has been suggested by van de Wall et al. (38), measuring STAT-3 may be unnecessary for determining whether leptin is functioning in this area. This could explain why we saw a leptin response in glucose and medium fructose rats in Experiment 1, but not when looking at their corresponding PSTAT-3 levels in the ARC. In addition to STAT-3 not being an adequate indicator of leptin sensitivity, some groups, such as Rahomouni et al., suggest that extracellular signal-related kinases (ERK) in the leptin signaling pathway is responsible for leptin's actions on food intake and body weight (30). If we measure activation of ERK for the glucose diet fed animals, we may find that these rats have increased levels of phosphorylated ERK. Using ERK in this way may further validate the idea of using phosphorylated ERK as an additional marker of leptin receptor activation.

Glucose and medium fructose fed rats were leptin responsive in Experiment 1, but showed dissimilar activation in the medial NTS, i.e. medium fructose rats showed significant levels of PSTAT-3 whereas glucose rats did not. One possible explanation for the discrepancy between PSTAT-3 activation in the ARC and NTS involves POMC/CART neuronal expression. These anorexigenic neurons are not vital to signaling only in the ARC; Grill and Bjorbaek's groups have shown that POMC neurons in the NTS do not show PSTAT-3 activation by leptin, and they hypothesized that these neurons are regulated entirely differently to the leptin-responsive neurons in the ARC (21). Furthermore, Cone et al. demonstrated that POMC neurons in the NTS do not express CART, unlike those in the ARC (13). As mentioned above, STAT-3

signaling was found to be inessential for acute or chronic regulation of POMC neurons (1, 26). This may explain why we saw a discrepancy between changes in food intake and activation of PSTAT-3 in the brain of glucose fed rats. Measuring leptin-induced changes in CART and POMC expression in both the ARC and NTS may provide insight into melanocortin activity and its association with leptin signaling and communication between the forebrain and hindbrain.

Few studies have tested the effects of dietary fructose on leptin responsiveness. One recent study, by Shapiro et al., tested hypothalamic PSTAT-3 levels in rats consuming either a 60% fructose or a standard chow diet (no fructose) for six months (33). Western blots of the hypothalamus were performed to measure PSTAT-3 levels. Shapiro et al.'s results show that fructose fed animals were leptin resistant and control animals were responsive, and leptin resistance corresponded with a 25.7% decrease in hypothalamic PSTAT-3 in basal conditions. While we did find a decrease of PSTAT-3 in rats fed the medium fructose diet, we found this decrease only in the brainstem and not the hypothalamus. Differences in results between the two studies may arise from the amount of fructose in experimental diets, length of time on diets, and method of analyzing PSTAT-3.

It is worth noting that the differences in STAT-3 activation of PBS- and leptin-treated medium fructose rats were relatively small and also were very similar to the levels of phosphorylation found in low fructose fed rats. This may have been due to the time of the day that the rats were tested. Rats were perfused in the early afternoon, so the rats were in a post-absorptive state prior to being killed, and were thus not as hungry as they would have been at the start of their dark cycle. This may mean that all of the rats already had some level of leptin signaling suppressing their appetite. Similar studies have food-deprived animals for 24 hours

before perfusions, preventing animals from eating during the dark cycle before the perfusions, resulting in lower leptin levels before injections and perfusions (20).

The purpose of Experiment 3 was to test central leptin responsiveness in rats fed the same diets from Experiments 1 and 2 and for the same length of time. This was to determine whether the resistance to peripheral leptin injections was due to a failure of leptin to cross the blood brain barrier (BBB) or due to a failure of central leptin receptors to respond to leptin. The only rats that showed any response to the central leptin were the glucose-fed animals in which weight gain was significantly inhibited during the 36 hours after injection. Because the rats were not tested before this time point, no indication of exactly when central leptin resistance developed can be determined in the fructose fed animals. In Experiment 1 medium fructose fed rats responded to a peripheral injection of leptin which suggests that this leptin response was mediated by leptin receptors in the periphery or in an area of the brain that was distant from the third ventricle.

Glucose fed rats were responsive to both central and peripheral leptin administration, whereas low fructose fed rats did not respond to either modes of administration. This was expected because glucose fed rats were leptin responsive and low fructose fed rats were leptin resistant in Experiment 1. Medium fructose rats, however, responded only to peripheral leptin. This can be interpreted as failure of central leptin receptors to respond to leptin. There are at least two plausible explanations for why this is occurring. Firstly, leptin may not be adequately crossing the BBB to get to central receptors (7). Secondly, there may be an increase in suppressor of cytokine signaling-3 (SOCS-3). This negative feedback regulator of leptin signaling is suggested to cause leptin resistance (6). It is thought that when circulating leptin levels are increased, such as in an obese subject, an increase in SOCS-3 expression is exhibited, and in turn diminishes most of the increase in leptin receptor signaling (25). Although the

medium fructose fed rats responded to peripheral leptin, as demonstrated in Experiment 1, it appears that leptin fails to activate central receptors. From Experiment 2, we know that medium fructose fed rats showed a response to leptin in the NTS, but not the ARC. Consistent with our hypothesis, if leptin fails to activate PSTAT-3 in the ARC, the issue could be failure of leptin to cross the BBB. Testing SOCS-3 levels in this experiment would indicate whether leptin is crossing the BBB or if there is an over expression in SOCS-3.

In Experiment 4, the animals from Experiment 3 were given insulin tolerance tests. All animals were insulin responsive. This is surprising because an increase in fructose intake has been linked with a decrease in insulin sensitivity (23). Additionally, leptin resistance is often associated with obesity and insulin resistance (22). The glucose diet did not contain fructose and the rats were leptin responsive, thus it was expected that this group would respond to insulin. Despite the amount of fructose that the medium fructose diet contained, the rats remained responsive to leptin after chronic consumption of the diet, thus insulin responsiveness is not completely unexpected. Havel points out that there have been discrepancies in the literature concerning fructose consumption and insulin response (17). Explanations for the variability include the amount of sugars in the diet, length of diet consumption, and total diet content (11, 36, 37). He also hypothesizes that subjects of normal weight and insulin sensitivity may be resistant to the effects of chronic fructose consumption on insulin resistance, while those subjects that are initially insulin resistant are more likely to further exacerbate insulin resistance with fructose consumption (17). This may explain why medium fructose-fed animals were responsive to insulin. The fat content of each diet also may have played a role in insulin sensitivity. Discrepancies have been shown in the literature when analyzing fat intake and insulin sensitivity (31). Reasons for these differences are due to length of study, type of fat, and total fat content.

All rats consumed the same amount of fat in this study, which can be assumed to be a control variable in terms of any of its effects that it may have on the rats' metabolism.

Another important function of leptin is glucose homeostasis. Leptin has an ability to regulate glucose metabolism independent of leptin signaling (10, 29, 32, 34). Leptin signaling in the ARC successfully regulates the effect of leptin on glucose metabolism (10). A more recent study by Chua suggests, however that there may exist another cell type in the hypothalamic ARC that also helps with glucose homeostasis or that there are additional ARC neurons that respond to leptin and regulate glucose metabolism when other ARC neurons have no leptin receptor (38). Based on these studies and hypotheses, perhaps we can suggest that glucose homeostasis is still being regulated sufficiently, despite the lack of leptin response in the ARC. This may explain why animals are still maintaining normal blood glucose levels and are remaining insulin sensitive.

In conclusion, rats that consumed the high fat, high glucose diet responded to peripheral and central leptin administration although the activation of STAT-3 in the ARC and NTS did not increase with leptin treatment. Therefore, the responses observed in the glucose-fed animals may have been the result of activation of a different aspect of the leptin-receptor signaling pathway, such as through activation of ERK. Rats consuming the medium fructose diet are responding to leptin via the NTS and possibly, additionally through ERK. Rats consuming the high fat, low fructose diet did not respond to leptin centrally or peripherally. Why the low fructose diet caused leptin resistance while medium fructose diet did not is unclear, but it is possible that the metabolism of fructose prevented development of leptin resistance in high-fat fed animals based on some aspect of its metabolism that is different from that of starch or glucose. More studies should be conducted with these diets that look at energy expenditure, fat pad analysis,

triglyceride levels, SOCS-3 activation, and ERK signaling. Future studies may provide insight into metabolic differences produced by diets of different fructose content that provide a greater understanding of how dietary carbohydrate modifies leptin receptor activation and leptin responsiveness.

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Table 3-1. Composition of experimental diets.

| Diet | Glucose + High Fat | | Medium Fructose + High Fat | | Low Fructose + High Fat | |
|---|---|---|---|---|---|---|
| | gm % | kcal% | gm % | kcal% | gm % | kcal% |
| Protein | 21.5 | 20 | 21.5 | 20 | 21.5 | 20 |
| CHO | 54.2 | 50 | 54.2 | 50 | 54.2 | 50 |
| o Glucose | 52.1 | 50 | 10.5 | 10 | 10.5 | 10 |
| o Fructose | 0 | 0 | 42.5 | 40 | 15 | 15 |
| o Corn Starch | 0 | 0 | 0 | 0 | 25.5 | 25 |
| Fat | 14.1 | 30 | 14.1 | 30 | 14.1 | 30 |
| *TOTAL* | | *100* | | *100* | | *100* |
| kcal/gm | 4.3 | | 4.3 | | 4.3 | |

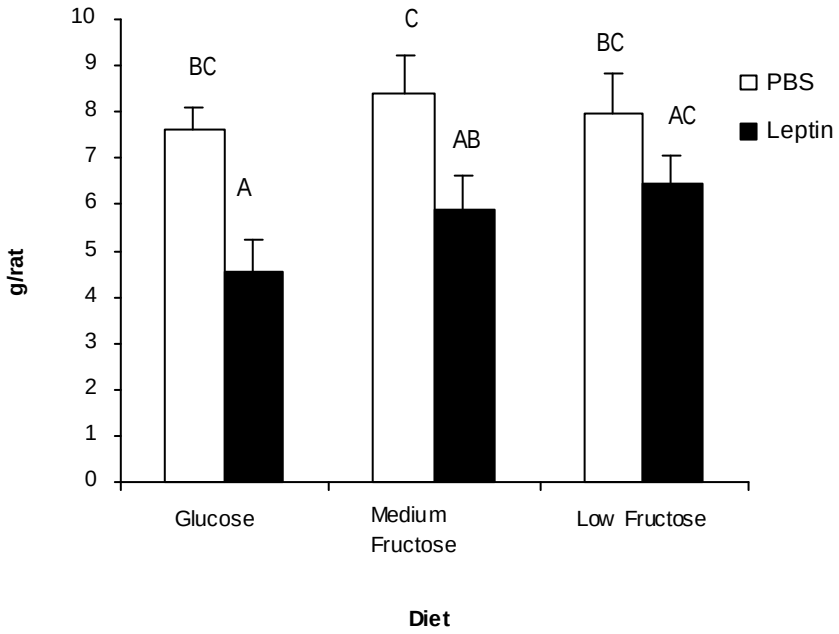


Figure 3-1.Change in body weight during the 14 hours following i.p. injection of leptin or PBS in rats fed experimental diets for 64 days in Experiment 1. Data are means + SEM for groups of 9 or 10 rats. Values that do not share a common superscript are significantly different at $P<0.05$.

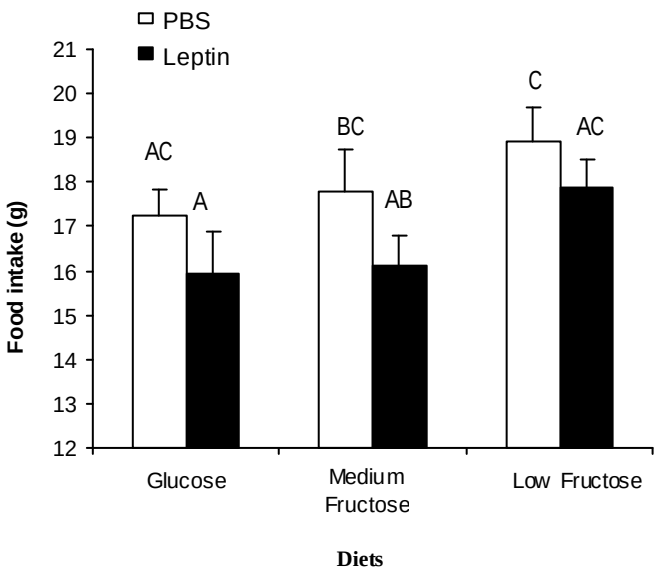


Figure 3-2. Fourteen hour food intake of rats injected with leptin or PBS after 64 days on experimental diets. Data are means + SEM for groups of 9 or 10 rats. Values that do not share a common superscript are significantly different at $P<0.05$.

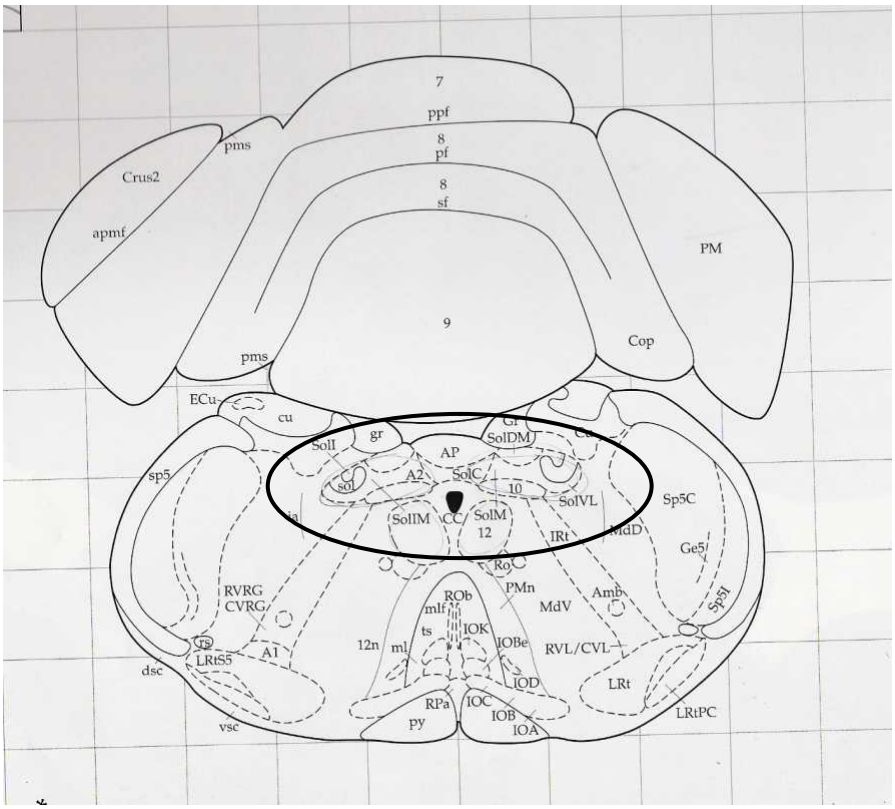


Figure 3-3. Two areas of the brainstem, the central nucleus of the solitary tract (SolC) and the medial nucleus of the solitary tract (SolM), were analyzed in Experiment 2. Coordinates from the Paxinos Rat Brain Atlas denotes the segment of the brainstem that was sliced: nucleus of solitary tract Anteroposterior: -13.68 mm to -14.30 mm.

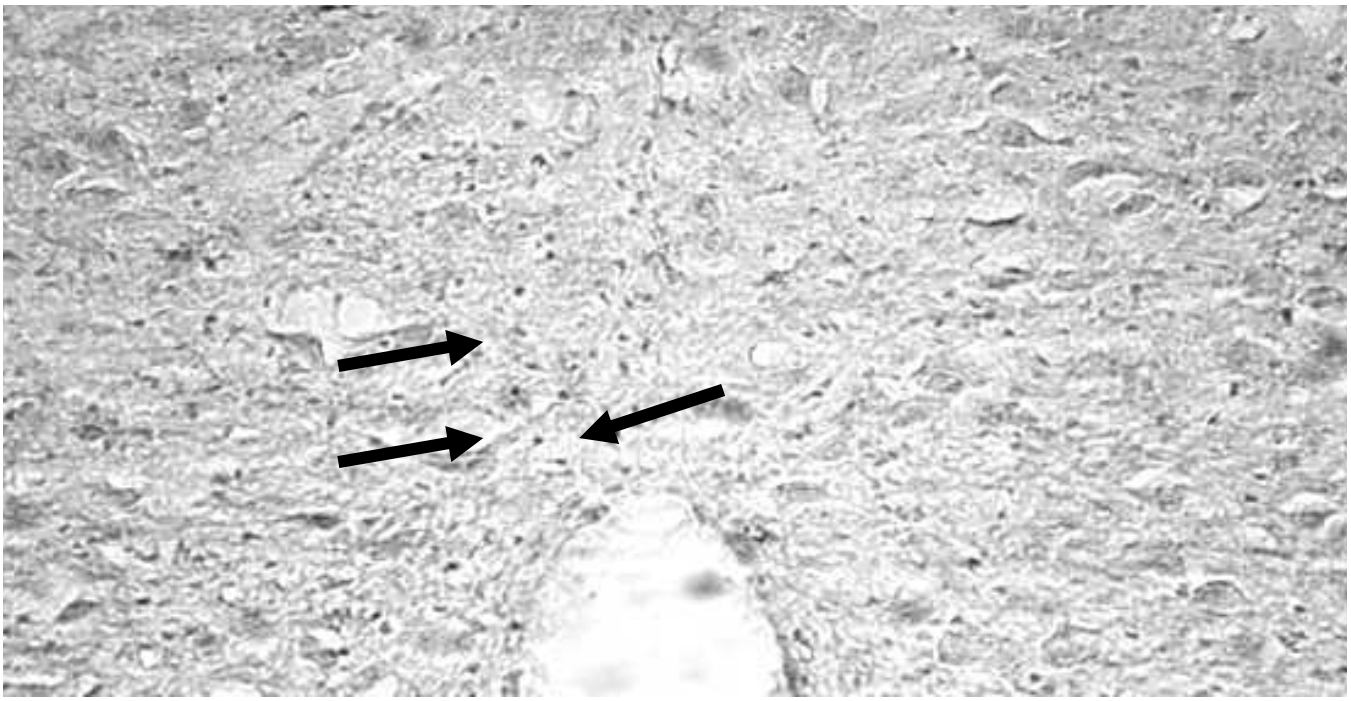

Figure 3-4. Image of PSTAT-3 immunohistochemical staining of the NTS. Area highlighted is central NTS, superior to central canal. Arrows denote positive PSTAT-3 staining of activation sites.

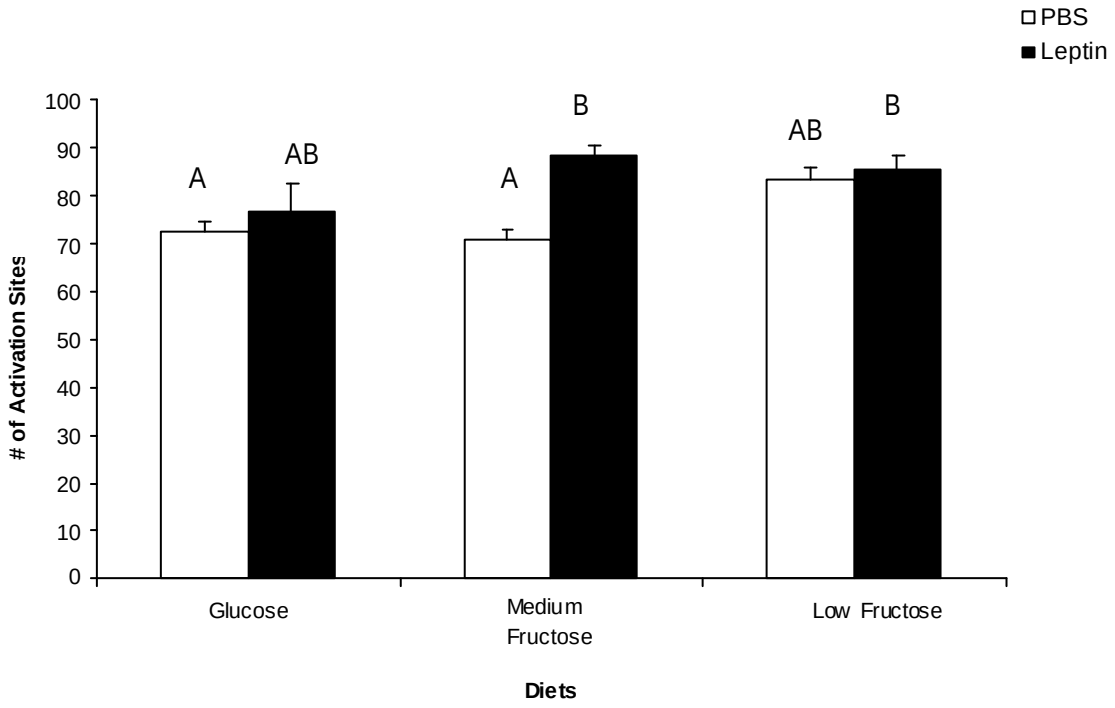


Figure 3-5. P-STAT3 immunohistochemistry activation sites in the medial nucleus of solitary tract. Data are means + SEM for groups of 9 or10 rats. Values that do not share a common superscript are significantly different at $P<0.05$.

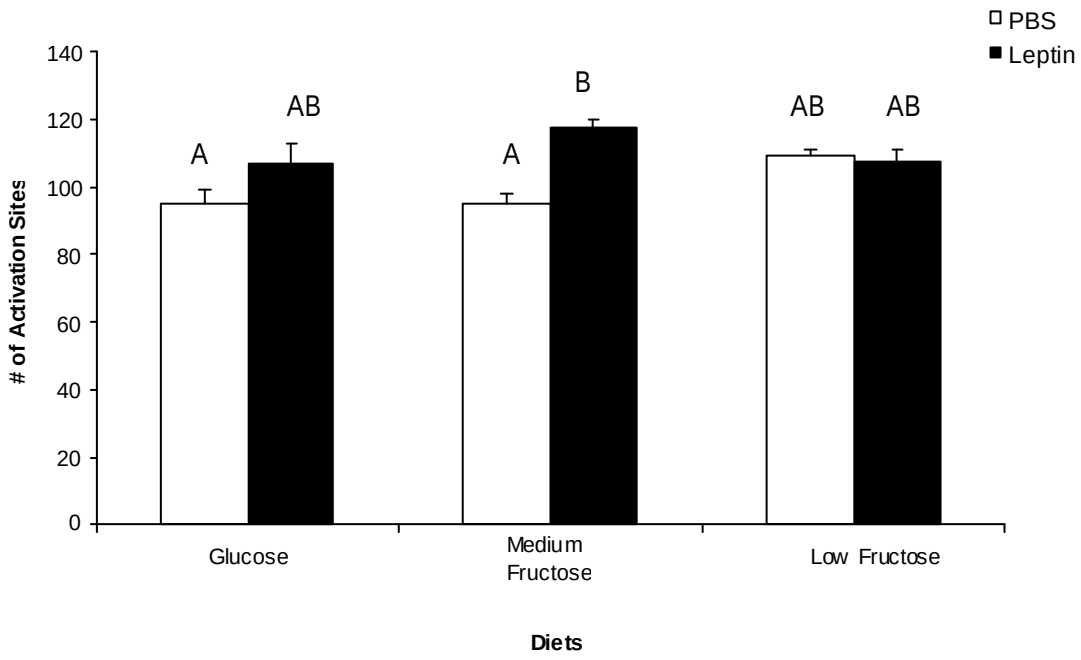


Figure 3-6. P-STAT3 immunohistochemistry activation sites in the central nucleus of solitary tract, superior of central canal. Data are means + SEM for groups of 9 or 10 rats. Values that do not share a common superscript are significantly different at $P<0.05$.

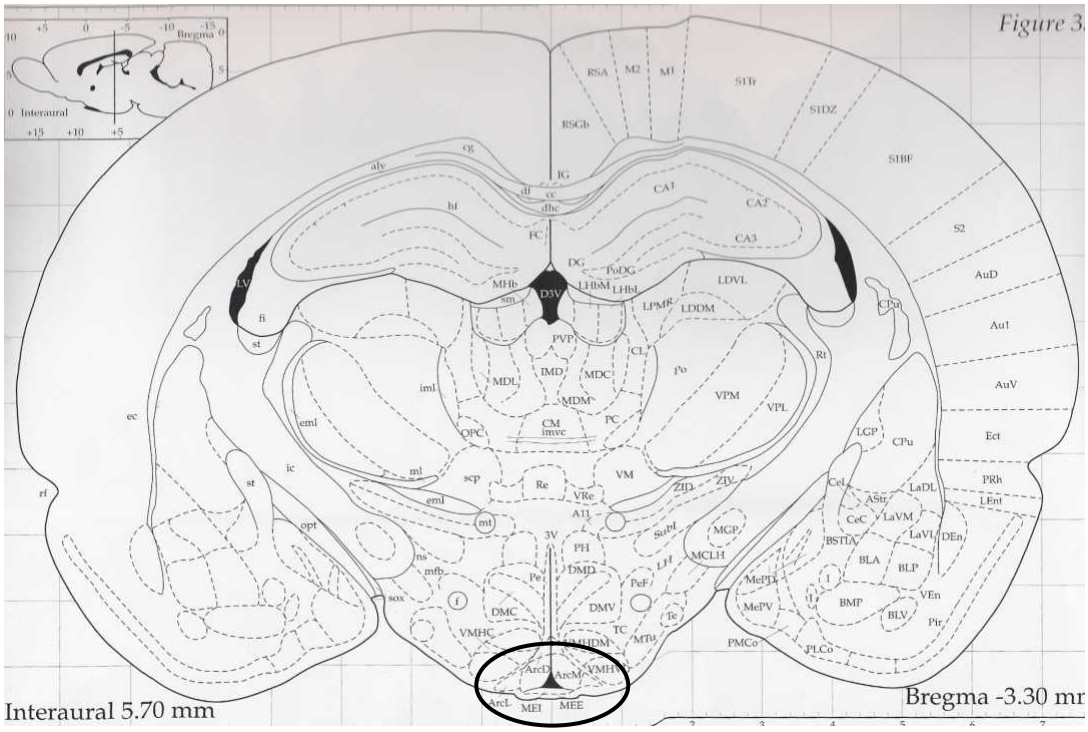


Figure 3-7. One area of the hypothalamus, the arcuate nucleus (Arc) was analyzed in Experiment 2. Coordinates from the Paxinos Rat Brain Atlas denotes the segment of the hypothalamus that was sliced: arcuate nucleus Anteroposterior: -2.12 mm to -4.13 mm.

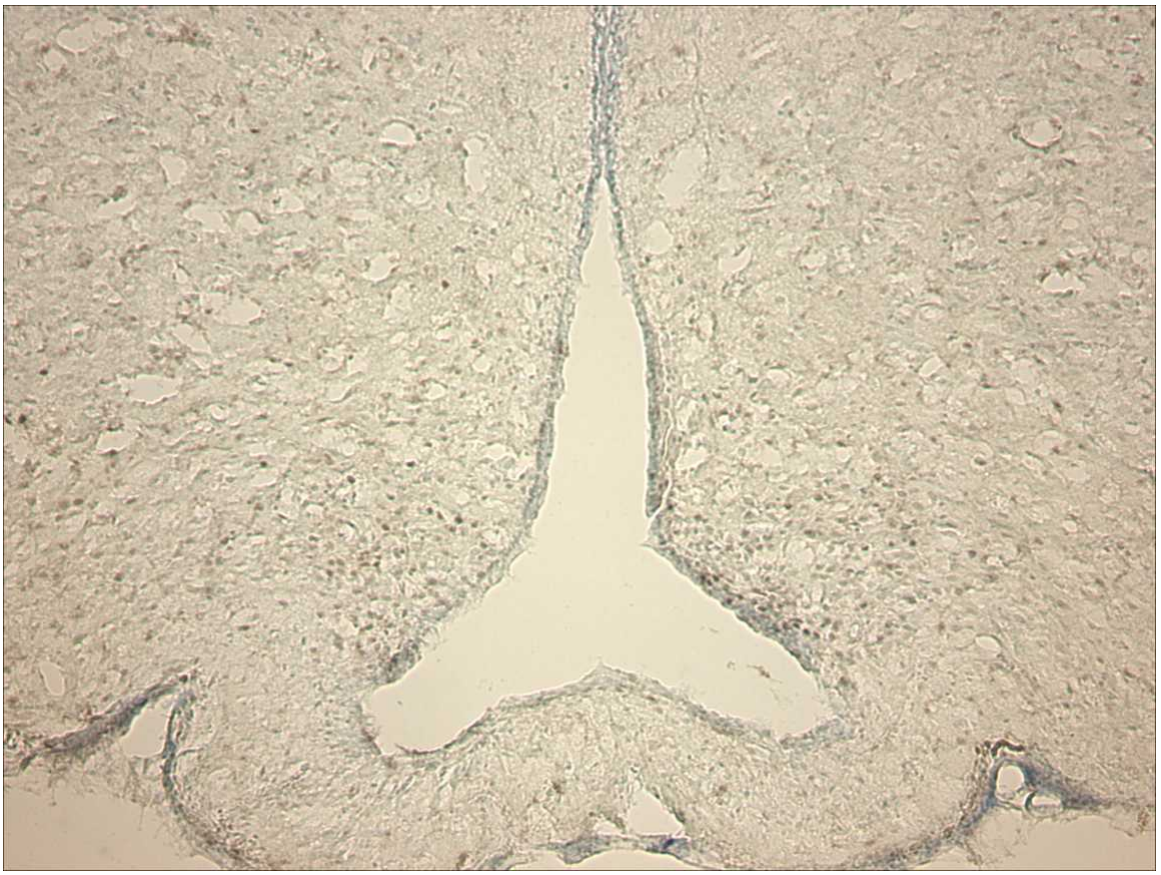

Figure 3-8. Image of PSTAT-3 immunohistochemical staining of the ARC. Area highlighted is ARC of the hypothalamus. Arrows denote positive PSTAT-3 staining of activation sites.

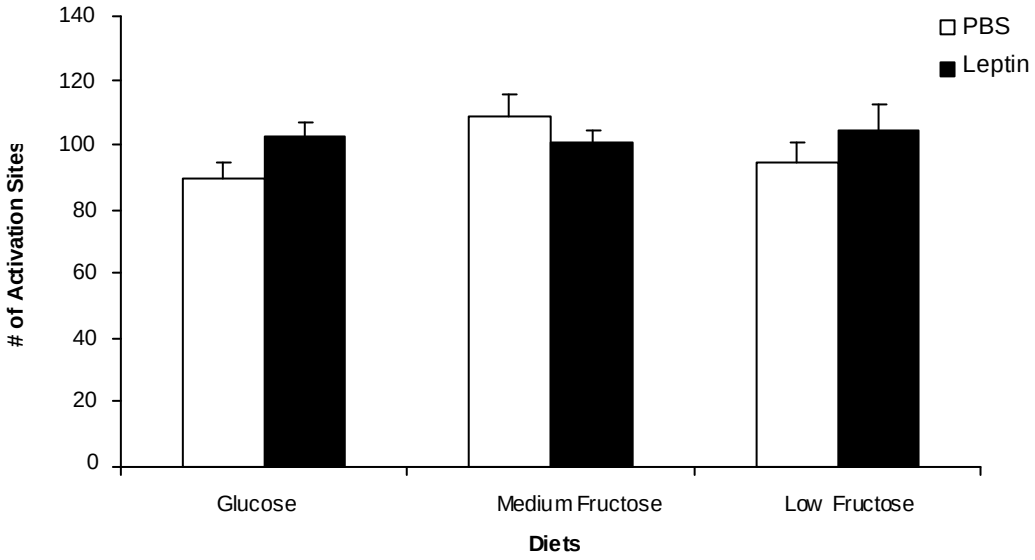


Figure 3-9. P-STAT3 immunohistochemistry activation sites in the arcuate nucleus. Data are means + SEM for groups of 9 or 10 rats. Values that do not share a common superscript are significantly different at $P<0.05$.

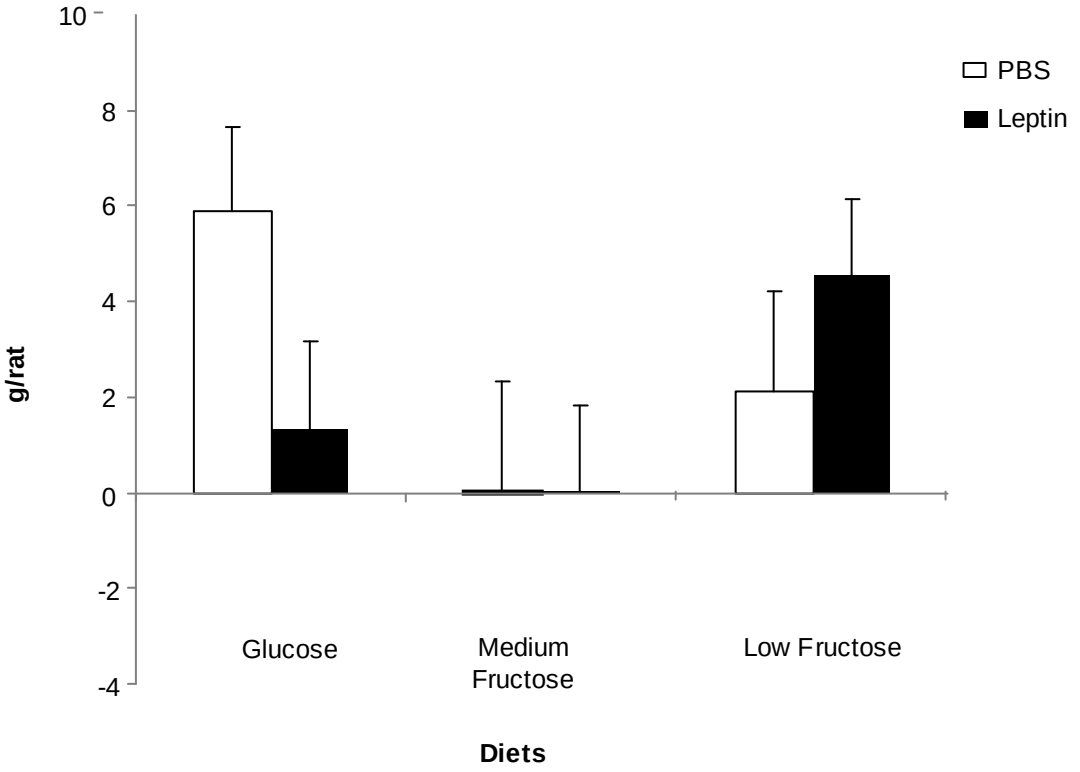


Figure 3-10. Change in body weight 14 hours after third ventricle leptin or PBS administration in rats fed experimental diets for 60 days. Data are means + SEM for groups of 9 rats. Values that do not share a common superscript are significantly different at $P<0.05$.

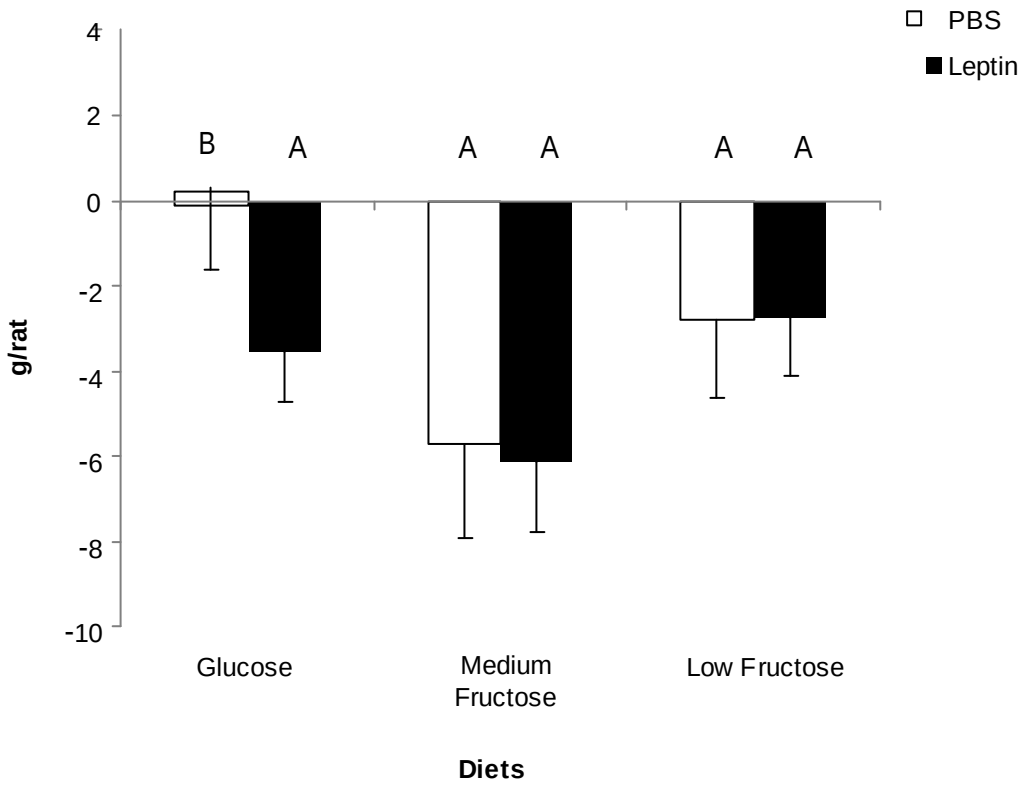


Figure 3-11. Change in body weight 24 hours post leptin or PBS icv administration after 60 days on experimental diets. Data are means + SEM for groups of 9 rats. Values that do not share a common superscript are significantly different at $P<0.05$.

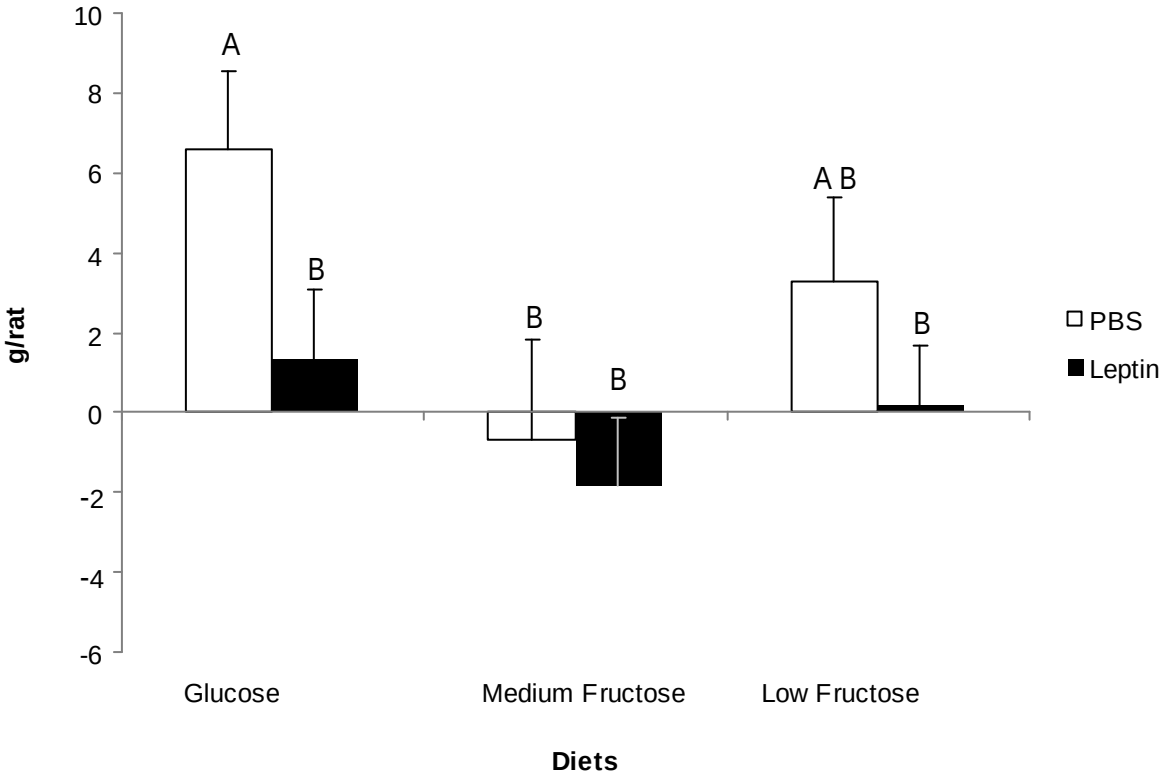


Figure 3-12. Change in body weight 36 hours post leptin or PBS icv administration after 60 days on experimental diets. Data are means + SEM for groups of 9 rats. Values that do not share a common superscript are significantly different at $P<0.05$.

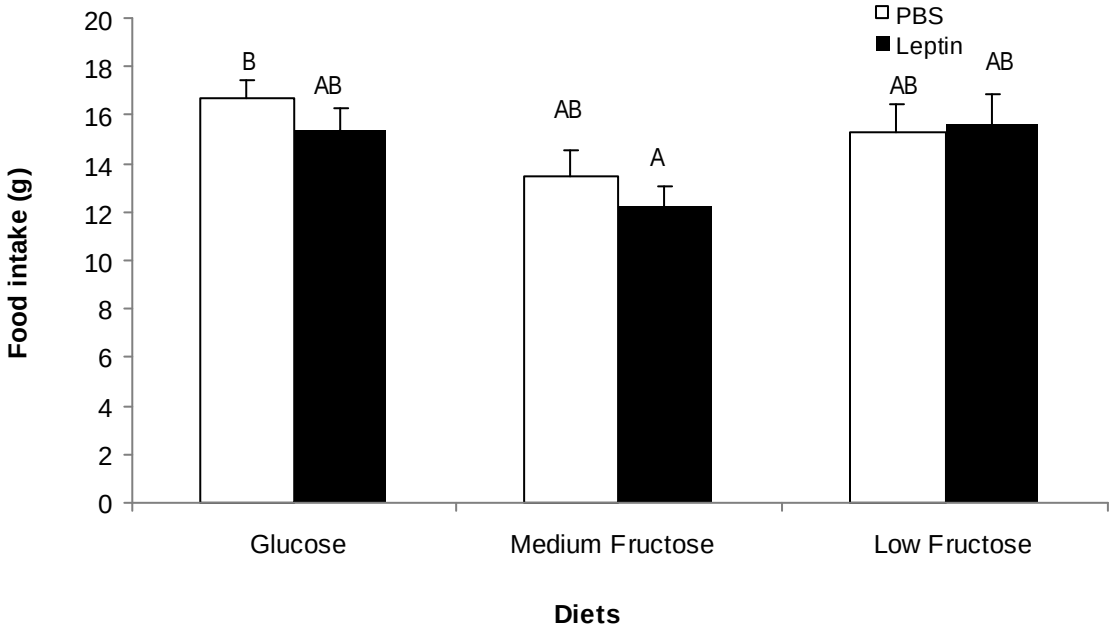


Figure 3-13. Food intake 14 hours post leptin or PBS icv administration after 60 days on experimental diets. Data are means + SEM for groups of 9 rats. Values that do not share a common superscript are significantly different at $P<0.05$.

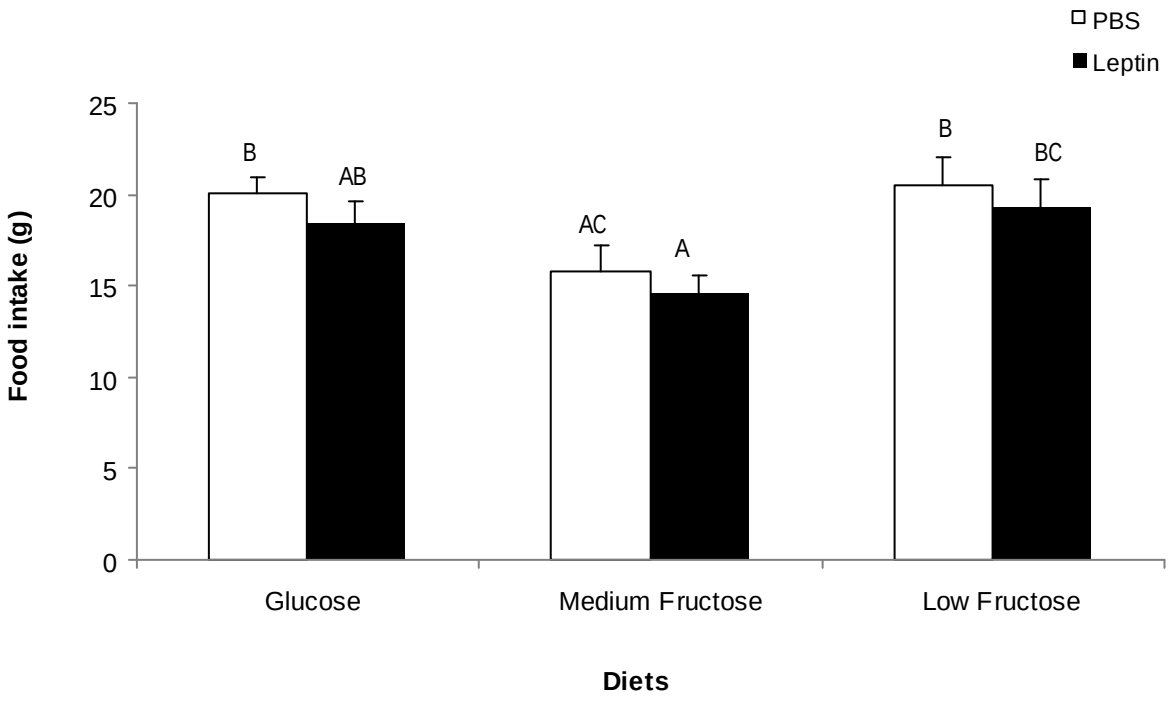


Figure 3-14. Food intake 24 hours post leptin or PBS icv administration after 60 days on experimental diets. Data are means + SEM for groups of 9 rats. Values that do not share a common superscript are significantly different at $P<0.05$.

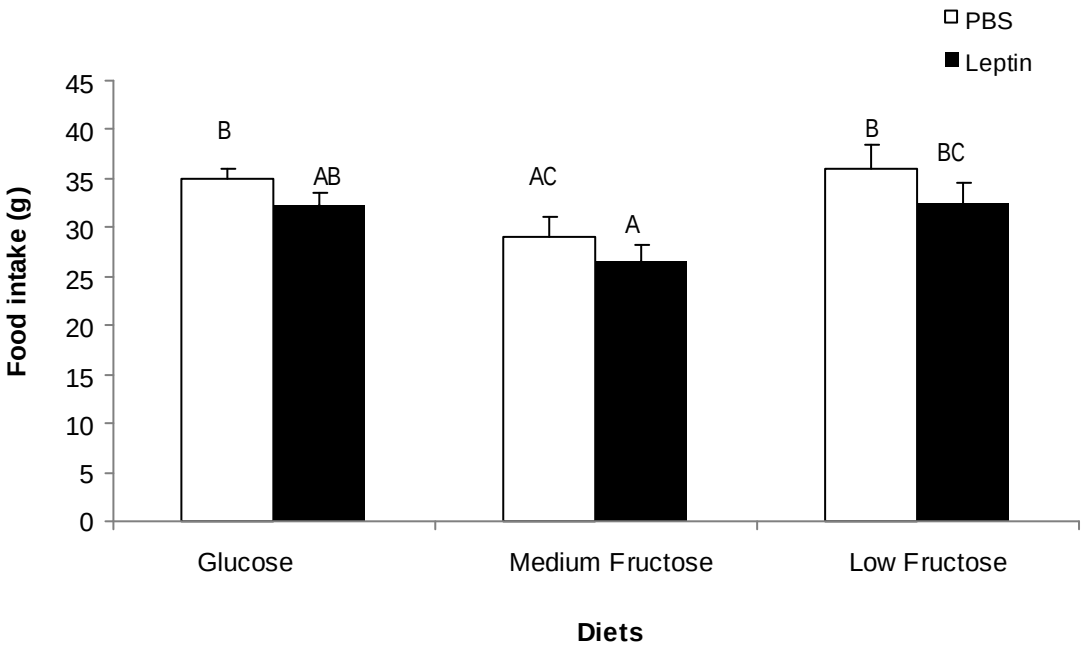


Figure 3-15. Food intake 36 hours post leptin or PBS icv administration after 60 days on experimental diets. Data are means + SEM for groups of 9 rats. Values that do not share a common superscript are significantly different at $P<0.05$.

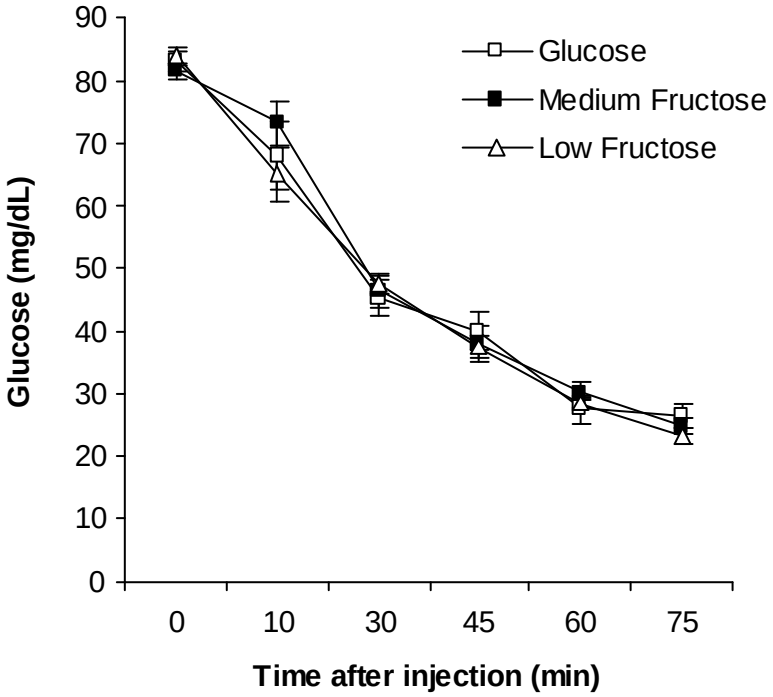


Figure 3-16. Glucose clearance during the ITT performed in Experiment 4 when rats had been on experimental diets for 60 days. Data are means + SEM for groups of 9 rats. Values that do not share a common superscript are significantly different at $P<0.05$.

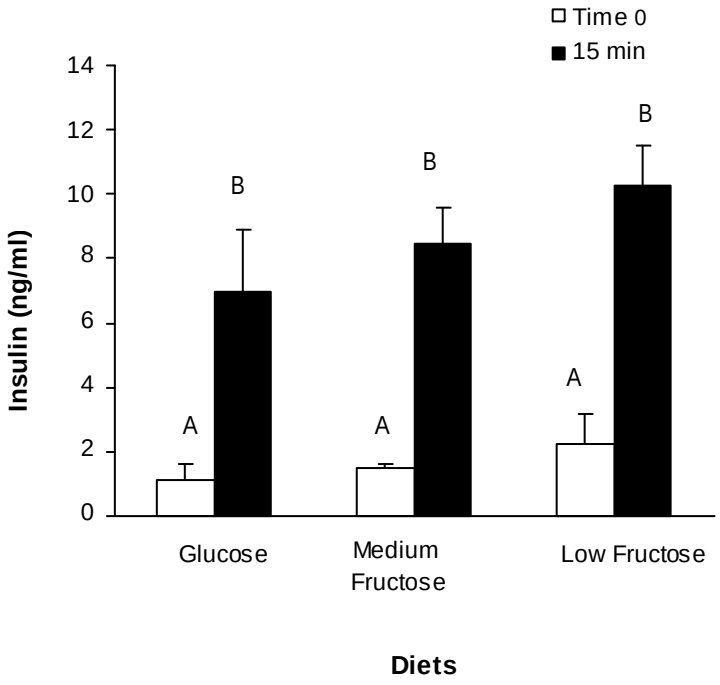


Figure 3-17. Serum insulin concentration measured before and 15 minutes after insulin injection in rats in Experiment 4. Data are means + SEM for groups of 9 rats. Values that do not share a common superscript are significantly different at $P<0.05$.

# CHAPTER 4

## SUMMARY AND CONCLUSIONS

The purpose of these studies was to test whether changes in leptin responsiveness were due to fructose specifically or an increase in monosaccharide content. The first objective of these studies was to test leptin response via intraperitoneal injections of leptin to animals consuming a high fat diet (30% kcal) that contained either 50% kcal glucose, 40% kcal fructose (medium fructose), or 15% kcal fructose (low fructose). After 10 weeks of diet consumption, changes in body weight and food intakes revealed that the glucose and medium fructose fed animals responded to leptin, whereas the low fructose fed animals were leptin resistant. These results indicate that animals consuming an increased amount of glucose remain responsive to leptin, whereas animals consuming fructose vary in their responsiveness, based on their monosaccharide content. Future studies should utilize experimental diets containing additional varied amounts of fructose, to observe any potential dose-related relationship with leptin response and fructose content.

The second objective of these studies was to determine how signals and transducer and activator of transcription 3 phosphorylation (PSTAT-3) in the brainstem and hypothalamus may reflect the variations of monosaccharide type and quantity in the diets. In the hypothalamic arcuate nucleus there was no significant effect of leptin on STAT-3 activation. In the medial and central nucleus of the solitary tract (NTS), medium fructose fed rats treated with leptin showed significant PSTAT-3 activation. From these results, we concluded that other leptin-associated signals should be evaluated to determine how glucose rats are responding to peripheral leptin but not exhibiting increased PSTAT-3 activation (2, 3). These alternate indicators include extracellular signal-related kinase (ERK) and proopiomelanocortin (POMC) neuronal expression

(2, 4). Future studies should evaluate these leptin-associated factors to determine what is occurring with leptin signaling.

Lastly, the final objective of these studies was to determine how these three experimental diets influenced central leptin response and insulin sensitivity. Animals were administered the same three diets for the same duration of time as the previous objective. We found that only the glucose-fed animals responded to central leptin infusions, and all animals remained insulin sensitive by the end of the study. Our results indicate that glucose animals responded to central and peripheral leptin administration, without the associated STAT-3 activation. This further validates the idea of using ERK or other aspects of leptin signaling, to test for leptin signaling (4). Because all of the animals are insulin responsive, we can suggest that if leptin contributes to glucose homeostasis through activation of leptin receptors in the ARC, then glucose and insulin are being regulated independently from food intake and body weight (5). Additionally over-expression of suppressor of cytokine signaling 3 (SOCS-3), a negative feedback inhibitor of leptin signaling, may be causing resistance to leptin in the brain (1). This can be validated in future studies by testing SOCS-3 levels in the brain and comparing them to leptin responsiveness.

Based on these studies, we find it necessary to further research the area of monosaccharide diets and leptin signaling. Aspects of leptin signaling that are in need of additional research include SOCS-3, ERK, and POMC neuronal expression. By completing these additional studies with high monosaccharide diets, we could gain a greater understanding of how dietary carbohydrate modifies leptin receptor activation and leptin responsiveness.

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