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The Effects of Intracerebroventricular Leptin on Milk Availability in Lactating Rats

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by

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ABSTRACT

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Reports have linked energy balance along with adipocyte derived leptin action to improved fertility. Recent evidence indicates that leptin hormone is present in breast milk and leptin receptors are well expressed in mammary epithelial cells. The hypothesis that insufficiency of leptin restraint in the hypothalamus may underlie infertility in rodents and the failure of lactating breast to express adequate amount of milk was tested. Female Sprague-Dawley rats were injected leptin through intracerebroventricular cannulation (ICVC) of the third ventricle. Female rats were mated with stud males and observed throughout gestation. Compared to the control groups, leptin treatment increased prolactin levels in the dams and increased milk transfer to pup. Hypothalamic mRNA leptin levels and brain size in the offspring from leptin treated dams were significantly higher than the control. These findings support the involvement of leptin in reproduction and could lead to better understanding of leptin transfer from dam to offspring.

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CHAPTER 1

INTRODUCTION

Leptin

Since its discovery in 1994 by Zhang et al., leptin has been the focus of a wide variety of research. Leptin, a 16kD protein product of the obese gene, is a hormone expressed mainly in white adipose tissue (Zhang et al. 1994). The function of leptin was first reported to act as an adiposity signal linked to food intake and obesity, where leptin acts as an important homeostatic regulator and nutritional guide for the body. However, further research has reported the presence of leptin in other areas of the body including the stomach, brain, skeletal muscle, placenta, and mammary glands, to name a few (Bluher and Mantzoros 2004). Since this time, leptin has been described as a pleiotrophic hormone that affects various physiological functions throughout the body (Morrison 2009).

Leptin Functions in Energy Metabolism

Aside from day to day fluctuations in food consumption and physical activity, most mammals maintain a steady body weight over a period of many years. This is possible because cumulative energy intake from the consumption of food is relative to energy expenditure. Over the years it has been proposed that there are a set of homeostatic regulators that work in synchrony to control this energy balance. There are numerous circulating peptides and steroids produced in the body that are known to influence appetite through their actions on certain areas of the brain, most importantly the hypothalamus (Coll et al. 2007). Arguably, one of the most important regulators of food intake and energy expenditure is leptin.

In mammals leptin was first described to function as an adiposity signal. Adipose tissue is a major site of energy storage within the body. With focus on secretion from this adipose tissue, circulating leptin fluctuates in proportion to fat mass and acts on areas of the hypothalamus to regulate appetite and food intake (Denver et al. 2011). The areas around the hypothalamus have a delicate blood brain barrier (BBB), a necessity to the long-term effects of leptin action. This allows leptin to cross the barrier more readily in this location than in other parts of the brain. The hypothalamus is known for many functions, one of which is to regulate appetite and energy metabolism by detection of peripheral signals, such as leptin. Leptin acts on many different areas of the hypothalamus. The leptin receptor expression is highest in neurons within the nuclei of the basomedial hypothalamus that include the arcuate (ARC), dorsomedial hypothalamic, and ventromedial hypothalamic nuclei. Leptin acts on these areas to inhibit food intake, increase thermogenesis, and increase pituitary hormone secretion (Denver et al. 2011).

In the case of energy homeostasis and food intake, leptin effect is most closely associated with the ARC of the hypothalamus. The ARC contains 2 populations of neurons that are sensitive to leptin. The first population contains the orexigenic peptides Neuropeptide Y (NPY) and Agouti-Related Protein (AgRP). Recent studies have found that central injections of the aforementioned peptides into the hypothalamus lead to an increase in food intake and reduction in energy expenditure. Therefore, leptin acts to suppress or inhibit these neurons from proper functioning, leading to a decrease in food intake and an increase in energy expenditure alike (Morrison 2009). In contrast, the second population of peptides are those of anorexigenic origin: proopiomelanocortin (POMC) and cocaine-and amphetamine-regulated transcript (CART). Leptin acts on the ARC to increase the production of these anorexigenic peptides that generate an anorectic signal, causing a decrease in food intake (Denver et al. 2011).

In addition to its inhibitory effects on food intake and regulation of metabolic rate, leptin has also been found to stimulate actual physical activity in order to maintain energy balance and homeostasis. Evidence shows that this is due to an increase in α-melanocyte-stimulating hormone and CART, both of which are homeostatic peptides that can be found in the ARC (Lenard and Berthoud 2008).

Leptin acts on a variety of centers and pathways to regulate energy homeostasis within the body. For example, leptin plays a role in energy metabolism of peripheral tissues. The adenosine monophosphate-activated protein kinase (AMPK) is the downstream component of a protein kinase cascade that acts to maintain the energy homeostasis within a cell. Leptin has been found to manipulate this medium in order to alter metabolic pathways in skeletal muscle tissue. Once activated, AMPK will use ATP-generating pathways such as fatty acid oxidation and avoid ATP-consuming pathways such as fatty acid synthesis; therefore, restoring the energy homeostasis of the cell (Andersson et al. 2003). Evidence also suggests that leptin plays a role in the regulation of the mammalian target of rapamycin (mTOR). mTOR is a conserved energy sensor that is elicited in hypothalamic regulation of energy homeostasis. Leptin activates hypothalamic mTOR that is responsible for the suppression of food intake and direct regulation of NPY (Lenard and Berthoud 2008).

Overall, the effect that leptin has on food intake and energy expenditure is clearly reported. Leptin acts as an anorectic hormone to decrease appetite and food intake by use of various pathways including peripheral and central modes of action previously described (Denver et al. 2011). Leptin also works to increase energy expenditure, thus regulating energy balance and homeostasis throughout the body. However, the action of leptin does not merely include

food intake and energy metabolism. Research has uncovered a plethora of other physiological effects including those involved in reproduction.

Leptin Links to Reproduction

It has long been postulated that fertility in mammals requires adequate nutrition and a sufficient reserve of metabolic fuel. Individuals experiencing severe caloric restriction, weight loss, and critically increased energy expenditure have been shown to have impaired reproductive systems. The effect of nutritional status on reproduction could reflect the action of unknown metabolic signals which are recognized by the brain and serve as indicators of metabolic state (Hoggard et al. 1998). Therefore, there must be relationships between energy storage, adipose tissue, and reproductive function. It is well established that leptin plays a pivotal role in food intake and energy metabolism (Caprio et al. 2001). Hence, leptin could act as a fitness signal of reproduction for the organism. There has been much research on this topic and leptin has since been found to regulate many reproductive actions including areas of fertility, onset of puberty, pregnancy, and the regulation of other sex hormones (Hoggard et al. 1998).

Fertility

A recognizable feature of the homozygous *ob/ob* mutation, which causes leptin unaffectedness in mice, is sterility of both male and female subjects (Barash et al. 1996). In earlier work by Hoggard et al. (1998), they found that sterility of the male can sometimes be reversed when the animal is maintained on a restricted diet; however, females of this mutation are sterile no matter the diet. Serum levels of reproductive hormones of the *ob/ob* females are also uniformly reduced, suggesting a defect in functional development of what is called the hypothalamic-pituitary axis (Hoggard et al. 1998). The hypothalamic-pituitary axis is the main

site of reproductive action in the brain. More current research shows that sterility cannot be reversed in either sex of the *ob/ob* mice by caloric restriction alone. Thus, the sterility is not caused by the obesity or amount of food intake but rather genetically implied leptin deficiency (Caprio et al. 2001).

Hoggard et al. (1998) concluded that the repeated administration of leptin to female *ob/ob* mice results in increased ovarian weight and corrected their sterility. Thus, reproductive functions like ovulation, pregnancy, parturition, and lactation were restored (Barash et al. 1996; Hoggard et al. 1998). However, subsequent generations of the *ob/ob* female pups were unable to reproduce without leptin treatment. This demonstrates that correction of sterility requires continuous leptin treatment (Hoggard et al. 1998). Therefore, leptin is directly related to the modification of reproductive capacity and fertility.

Puberty

Another link between leptin and reproduction lies within the onset of puberty. Due to the ability of leptin to restore fertility to mice, this suggests that the hormone may also be an important signaling trigger in the onset of reproductive function, puberty (Goumenou et al. 2003). Puberty is the culmination of a prolonged series of prepubertal events. In order to determine the effects of leptin, many other aspects must also be monitored, including the secretion of other sex hormones involved in reproduction (Hileman et al. 2000). In an experiment, normal, prepubertal mice that were injected with leptin reproduced earlier (Hileman et al. 2000; Goumenou et al. 2003). They also showed earlier maturation of the reproductive tract that was determined by timing of the vaginal opening, progress toward the first oestrus cycle, and the weights of the uteri, ovaries, and oviducts. There was also a marked decrease in

luteinizing hormone (LH) and oestradiol when compared to the control, saline treated mice (Hoggard et al. 1998).

Pregnancy

The functions of leptin during pregnancy are of great importance. During pregnancy, maternal serum leptin levels are found to be greater than those shown in nonpregnant women (Holness et al. 1999; Henson and Castracane 2000). Leptin levels are shown to increase through the second trimester and remain elevated until parturition (Henson and Castracane 2000). However, these elevated leptin levels cannot be distinguished solely by the increase in weight gain during pregnancy (Caprio et al. 2001). Therefore, leptin levels must play another role in this stage of reproduction.

The role of leptin in pregnancy is not only of importance to the mother, but to the fetus as well. High levels of expression of both leptin and its receptors in the fetus suggest that it plays an important role in fetal development. Fetal leptin could provide a signal to the mother about fetal growth and development (Holness et al. 1999). Leptin concentrations in cord blood are correlated with placental weights, infant length, and head circumference (Henson and Castracane 2000), thus suggesting that leptin may act to modulate the growth hormone (GH) secretion at the level of the hypothalamus (Hoggard et al. 1998; Henson and Castracane 2000).

With respect to the circulating leptin that occurs during gestation, significant cross-talk may occur between the placenta, fetus, and the maternal adipose stores. Mechanisms mediating leptin receptor synthesis must therefore be sensitive to the changing endocrine signals that occur during gestation and alter leptin's role throughout pregnancy (Henson and Castracane 2000).

Lactation

The role of leptin in lactation can be confirmed by the failure of milk production in the ob/ob female mouse after normal delivery (Moschos et al.2002). During pregnancy leptin levels are increased in the dam when mammary epithelial cell proliferation is initiated (Moschos et al.2002). This evidence supports that leptin could be linked to mammary gland growth, which is a necessity for lactation to occur. Leptin has also been found to have a positive relationship with prolactin, a hormone that stimulates milk synthesis (Feuermann et al. 2009). During pregnancy and lactation, exogenous leptin levels increase with the secretion of prolactin (Roy 2007). This supports leptin as an important hormonal regulator of lactation. During suckling, nitric oxide synthase (NOS) has been shown to regulate milk transfer from dam to pup (Otukonyong, Okere, et al.2000). It has been demonstrated that leptin plays an indirect role in the regulation of NOS (Otukonyong, Okutani, et al. 2000). Therefore, leptin could regulate not only the production of milk, but also the transfer of milk through various pathways.

Leptin Actions on Other Reproductive Hormones

Research has demonstrated that there is a correlation between the levels of leptin and other hormones that effect reproductive ability at levels of fertility, pregnancy, and even lactation (Hoggard et al. 1998). At the level of the hypothalamus, leptin acts indirectly to stimulate the amount of gonodotropin-releasing hormone (GnRH) that is released, which leads to an earlier rise of pubertal behaviors (Moschos et al. 2002). Previous work has demonstrated that low levels of leptin have also been associated with low levels of luteinizing hormone (LH); leptin administration has been shown to restore LH levels and thus, reproductive function at the level of pubertal onset (Holness et al. 1999; Donato et al. 2009).

Feuermann et al (2009) illustrated the relationship between estrogen, leptin, and prolactin. Leptin has been found to regulate the amount of estrogen receptor alpha (ERα) in mammary epithelial cells; an increased amount of leptin led to an increased expression of ERα in these cells (Feuermann et al. 2009). During pregnancy increased prolactin levels are paired with an increase in leptin secretion. In turn, a decrease in prolactin leads to a decrease in leptin (Feuermann et al. 2009). These findings support the notion of crosstalk between the 2 reproductive hormones; they are dependent on each other. Leptin's mechanism of action in reproduction can be viewed in Figure 1.

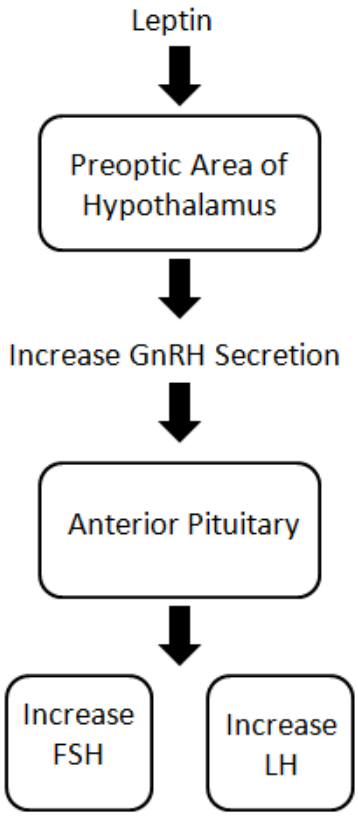

Figure 1: Leptin mechanism of action in reproduction. Leptin acts on the preoptic area of the hypothalamus to increase GnRH secretion. GnRH acts on the anterior pituitary to increase the release of FSH that stimulates follicular growth and LH that initiates ovulation.

The focus of this study involves the reproductive function of leptin on lactation. Leptin and energy balance have been linked to improved fertility in rodents and humans (Caprio et al. 2001). Recent evidence indicates that leptin is present in breast milk and leptin receptors are well expressed in mammary epithelial cells (Feuermann et al. 2009). Therefore, leptin could be involved in stimulating the growth of mammary epithelial cells and thus improving milk yield in lactating breast. The hypothesis was tested that insufficient levels of leptin in the brain feeding center, the hypothalamus, may underlie infertility and the failure of lactating breast to express an adequate amount of milk. Therefore, if enough leptin can be administered to the hypothalamus, fertility and milk yield could improve.

CHAPTER 2

MATERIALS AND METHODS

Animals

Fifteen female Sprague-Dawley rats (Harlan Laboratories, VA) of the same age, each weighing 200-250g, were acquired through the Division of Laboratory Animal Resources (DLAR) of East Tennessee State University (ETSU) and used in this study. The animals were housed individually and kept in a controlled, specific pathogen-free room located in the Brown Hall DLAR facility at ETSU. Regular chow (Harlan Laboratories, VA) and water were made available ad libitum. Light cycles consisted of 12h light at 7a.m. and 12h dark at 7p.m. The following experimental protocols were approved by the University Committee for Animal Care (UCAC) at ETSU.

Determination of the Oestrus Cycle

Oestrus cycle was determined daily for each female rat. Only rats with a regular 4-day cycle were used in this study. A regular 4-day cycle consists of the following 4 stages: proestrus, estrus, metestrus, and diestrus (Westwood 2008).

Vaginal Cytology

To determine the individual stages of the oestrus cycle, a wet mount process was conducted to visualize the cells lining the vagina. Ten µl of normal saline was introduced into the vagina with a glass pipette and the saline was then aspirated and placed onto a clean slide. The cells were examined under a compound microscope at 10X and 40X for analysis as previously described (Marcondes et al. 2002). Pictures were then taken with an Axioskop 40

microscope (Zeiss, NY) with a Powershot A640 camera (Canon, NY) at 10X magnification. A sample of these pictures can be viewed in Figure 2.

Oestrus Cycle

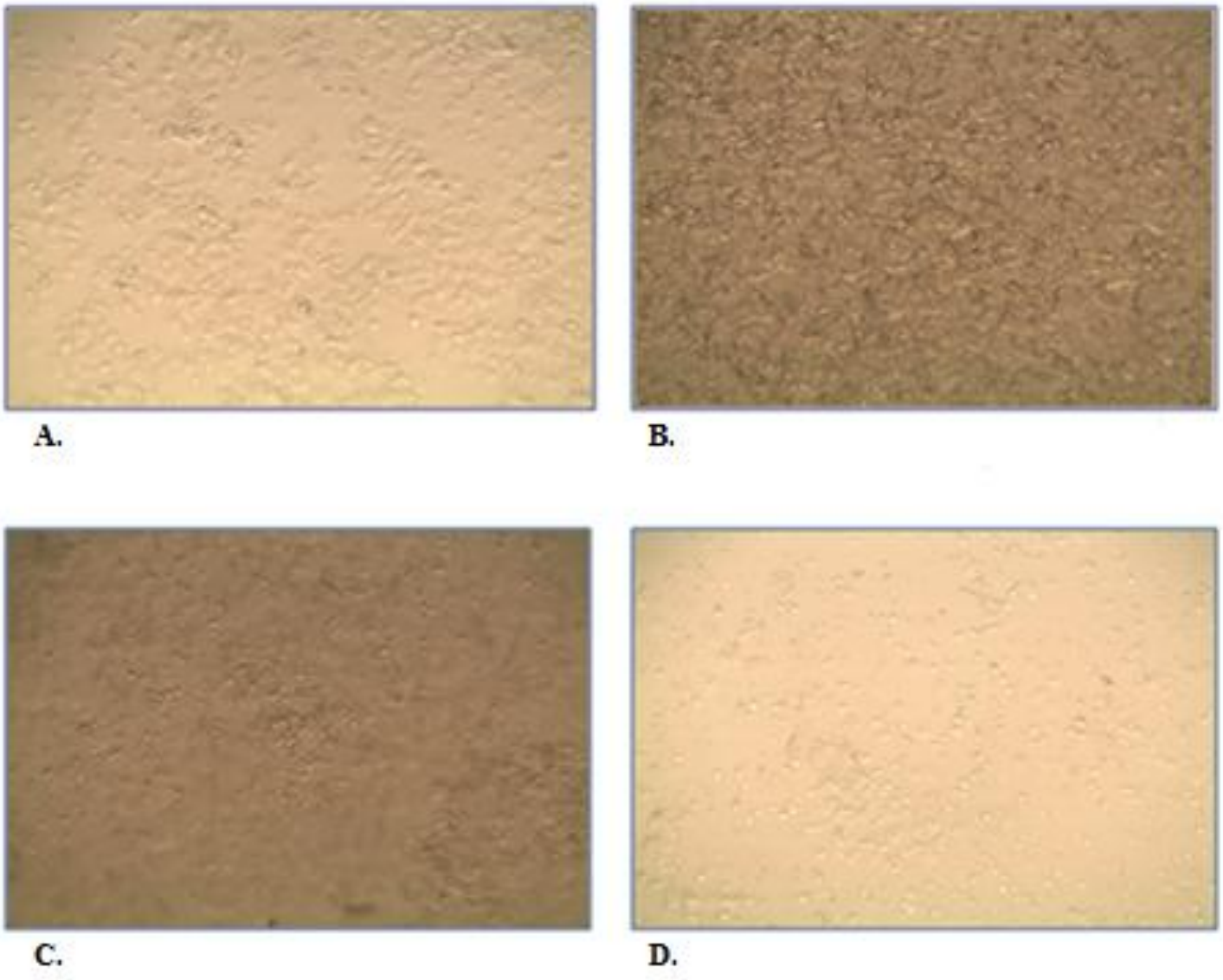

Figure 2: Pictures of vaginal smears were taken at 10X using a digital camera microscope. Each figure represents 1 of the 4 cycles of oestrus. (A) Proestrus: predominance of nucleated epithelial cells. (B) Estrus: predominance of anucleated cornified cells. (C) Metestrus: includes nucleated epithelial cells, anucleated cornified cells, and leukocytes. (D) Diestrus: predominance of leukocytes.

Stereotaxic Surgery

Overview

Several reports have linked energy balance as well as leptin action to improved fertility (Barash et al. 1996; Hoggard et al. 1998; Caprio et al. 2001). In order to study the direct effects of leptin on the the hypothalamus, a cannula was inserted into the third ventricle of the brain, an area adjacent to the hypothalamus. Implantation of the cannula was achieved through the process of stereotaxic surgery. This surgery technique uses a 3-dimensional coordinate system to locate specific brain sites. Coordinates for the location of the third ventricle in a rat brain were established with the use of The Rat Brain in Stereotaxic Coordinates, A Brain Atlas by Paxinos and Watson (1998). Once the cannula was inserted, leptin could be administered directly to the third ventricle. The efflux of cerebrosprinal fluid (CSF) indicated correct placement of the cannula.

Anesthesia and Medication Administration

Anesthesia and analgesic were given prior to and during the stereotaxic surgery. Isoflurane was used with an inhalational anesthesia system powered by a table top anesthesia machine (VetEquip Incorporated, CA). A picture of this anesthesia machine can be seen in Figure 3. Delivery of the isoflurane anesthesia was achieved before the surgery by inhalation in an enclosed chamber and during the surgery via a nose cone around the animal's nostrils. Meloxicam was given for pain associated with the surgery. This analgesic was administered through subcutaneous injection before surgery at 2 mg/kg. If pain was noted after surgery, another half dose of Meloxicam at 1 mg/kg was given until a full recovery was reached.

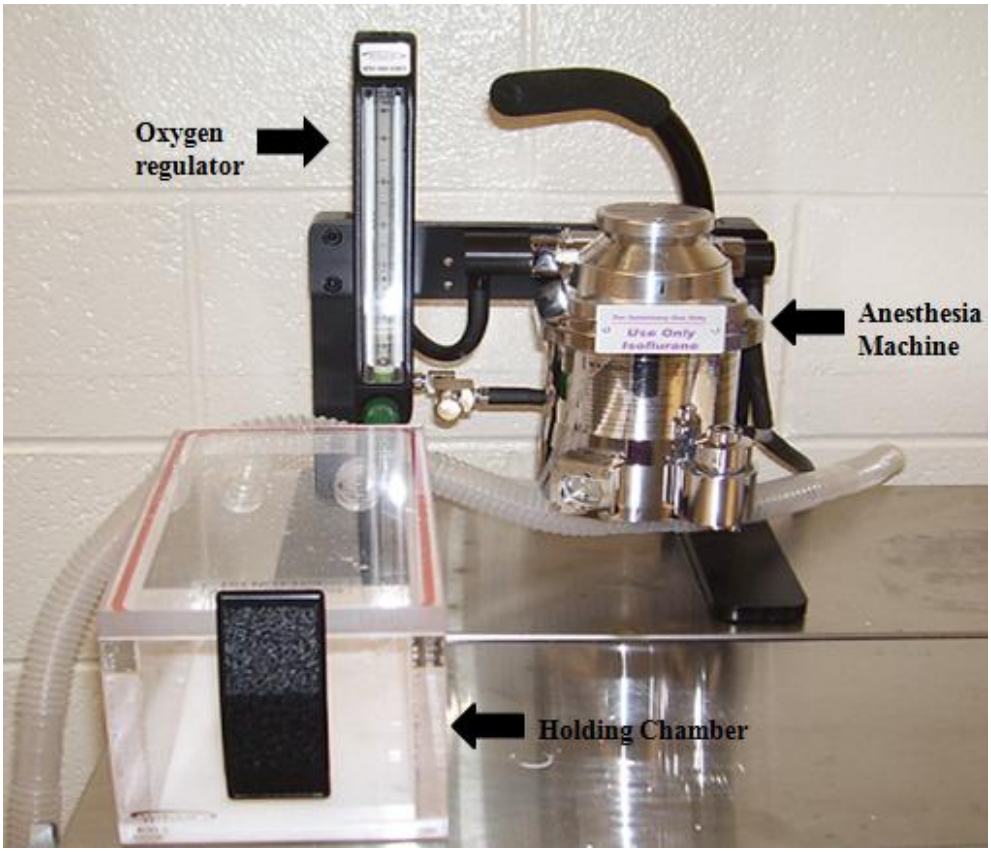

Figure 3: VetEquip anesthesia machine and holding chamber. Oxygen was regulated at 14.7 PSIA (1.0 bar) and 21°C throughout the surgery.

Surgery Procedure

A lab standard stereotaxic apparatus (Stoelting, IL) was acquired for use in the implantation of the stainless steel cannula (BASi, IN). The rat was removed from the anesthesia chamber and placed upon the stereotaxic apparatus. Tapered ear bars were inserted into each ear canal and locked into place, which ceased horizontal movement of the head. A bite bar was then adjusted into the animal's mouth and secured into position, which rendered the head immobile. The nose cone was fixed around the animals nostrils and isoflurane anesthesia was administered throughout the surgery at 2.5 PSIA with 14.7 PSIA of oxygen.

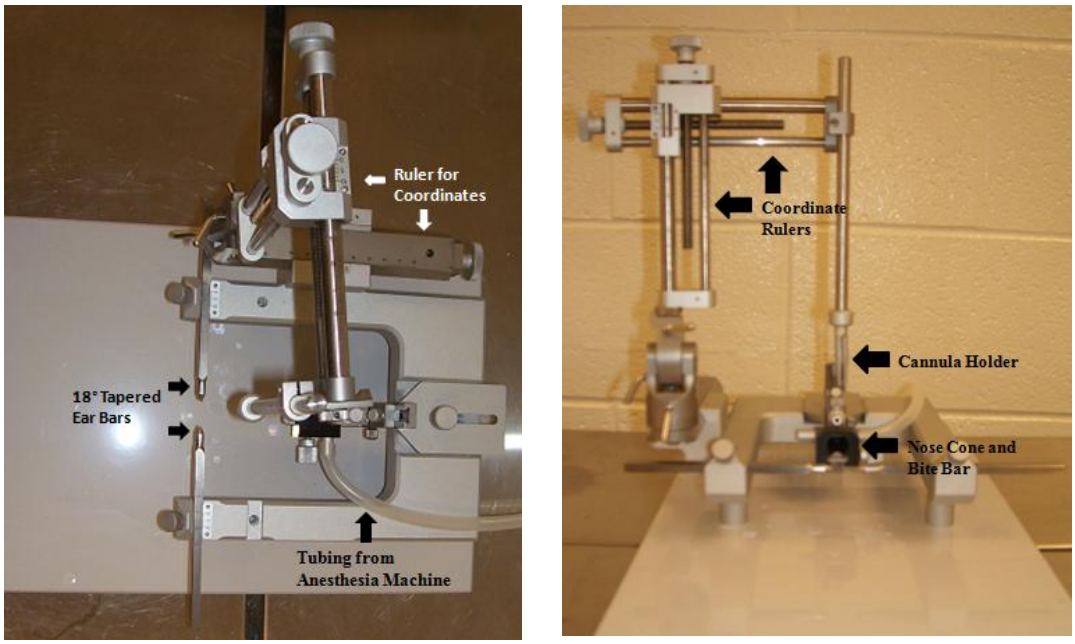

Figure 4: Two views of the stereotaxic apparatus depicting coordinate rulers, ear bars, tubing from anesthesia machine, nose cone, bite bar, and cannula holder.

Once the animal was secured in the stereotaxic apparatus (Figure 4), a small midsagittal incision was made on the scalp and held back using forceps. This allowed for visualization of bregma, an important landmark used to determine the coordinates. The coordinates used in this study for the implantation of the cannula into the third ventricle of the brain are as follows: Anterior to Posterior (AP) = -0.8mm, Medial to Lateral (ML) = 0.0mm and Dorsal to Ventral (DV) = -8.0mm. Using a pencil, a circular area was marked on the skull to represent these AP and ML coordinates from bregma. In the area surrounding these coordinates, 4 brain screws (BASi, IN) were anchored into the skull for stabilization. An OmniDrill35 (World Precision Instruments, FL) with a 0.5mm bit was then used to drill the skull bone until the brain surface was exposed.

When the brain surface was reached, the stainless steel brain cannula (BASi, IN) was then lowered into the third ventricle of the brain at the exact coordinates listed above. The

cannula was affixed to the skull using dental cement (Lang Dental Manufacturing Company Incorporated, IL) around the cannula and brain screws. After the cement had set, the skin was sutured together around the cannula. The rat was removed from the apparatus and monitored until recovery was reached. Images were taken during and after surgery (Figure 5).

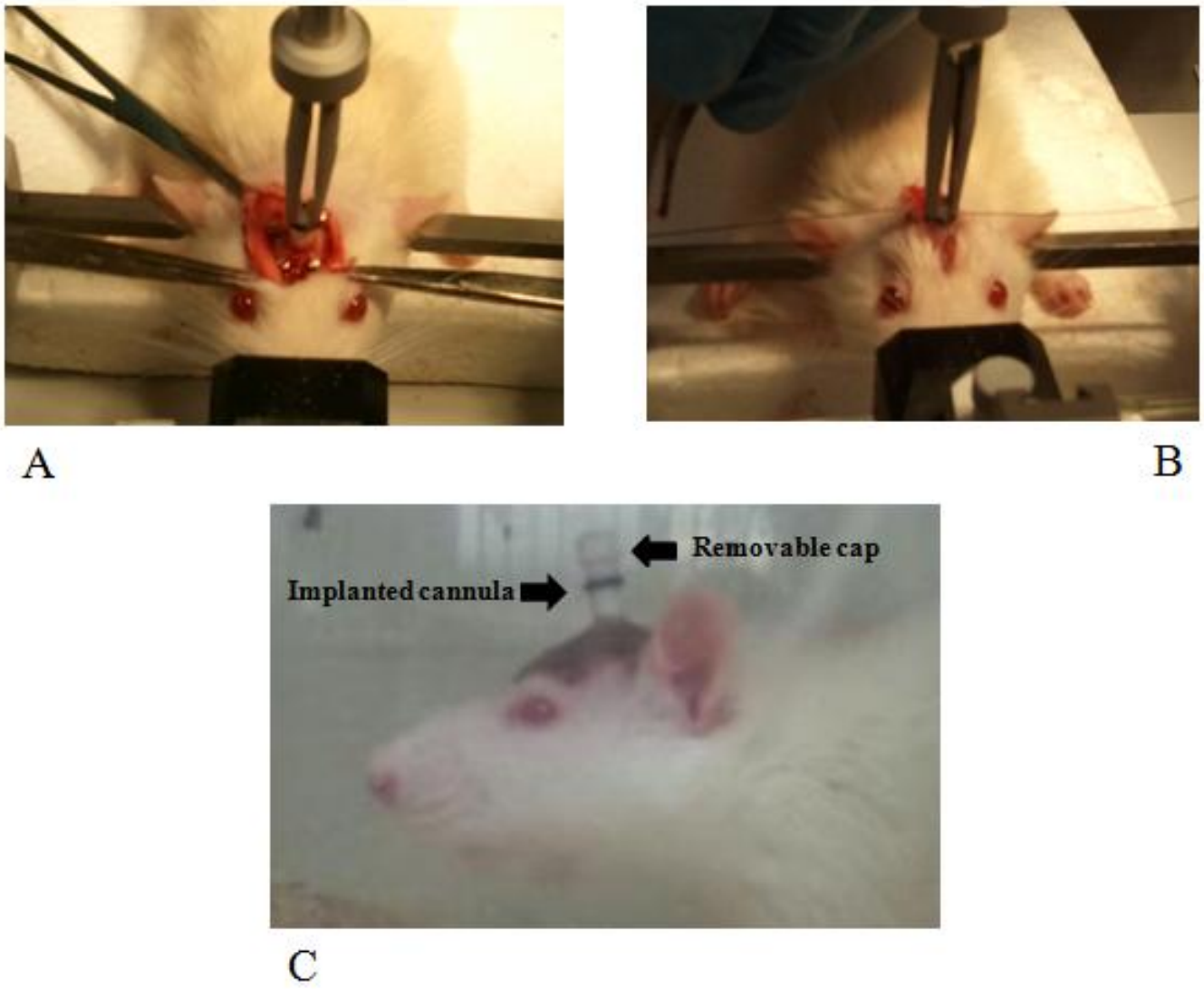

Figure 5: (A) An image was taken of a rat in the stereotaxic apparatus at time of cannula insertion. The anchored brain screws can be seen here. (B) The skin was sutured around the cannula after dental cement was set around the cannula and brain screws. (C) An intracerebroventricular implanted cannula on a recovered rat. The cap was removed from the cannula and injections could then be administered.

Recovery

After surgery, each rat was placed on a heat pad until full recovery from anesthesia. If any signs of pain were noted, a 1 mg/kg dose of Meloxicam was then given subcutaneously. After recovering to normal behavior, each rat was placed back into its individual housing and kept in the specific, pathogen-free room at the Brown Hall DLAR facility. Rats were given a full week of recuperation after the surgery in which they were not handled except for subcutaneous analgesic administration if needed.

Leptin Administration

After full recovery, the female rats were separated into a Leptin Treatment Group (n=5) and a Control Group (n=10). One week post surgery, 5µl leptin or 5µl normal saline was injected through the cannula into the third ventricle daily for 4 days according to their respective groupings.

Mating

Upon completion of leptin or saline injections, the females were then mated with a stud male Sprague-Dawley rat at the proestrus stage of oestrus. The following morning after mating, pregnancy was verified by visualization of a sperm plug. Once pregnancy was established, the females were separated back into individual cages and monitored daily until delivery. Body weight and food intake were measured daily using a digital scale balance (Mettler Toledo,OH) and the period of gestation was also recorded.

Quantification of Milk Transfer

After delivery, the body weight of both the dam and the litter were recorded. The size of the litter was also noted for comparison. The amount of milk transferred between the dams and litter was determined by examination of the feeding habits of each litter. Before the quantification process began, each litter was standardized to 9 pups per litter. Additional pups were sacrificed or designated to a similar litter to give each litter a total of 9 pups. Beginning 4 days after delivery, the pups were separated overnight, beginning at the 7pm dark cycle, in a detached room from the dams. They were then reunited in the morning at the 7am light cycle to suckle for a 1-hour time period. Thereafter, pups remained with the dam until separated again in the evening at 7pm. The body weights of both the dam and litter were measured before and after each period of suckling for a total of 7 days. An image of the pups suckling can be viewed in Figure 6.

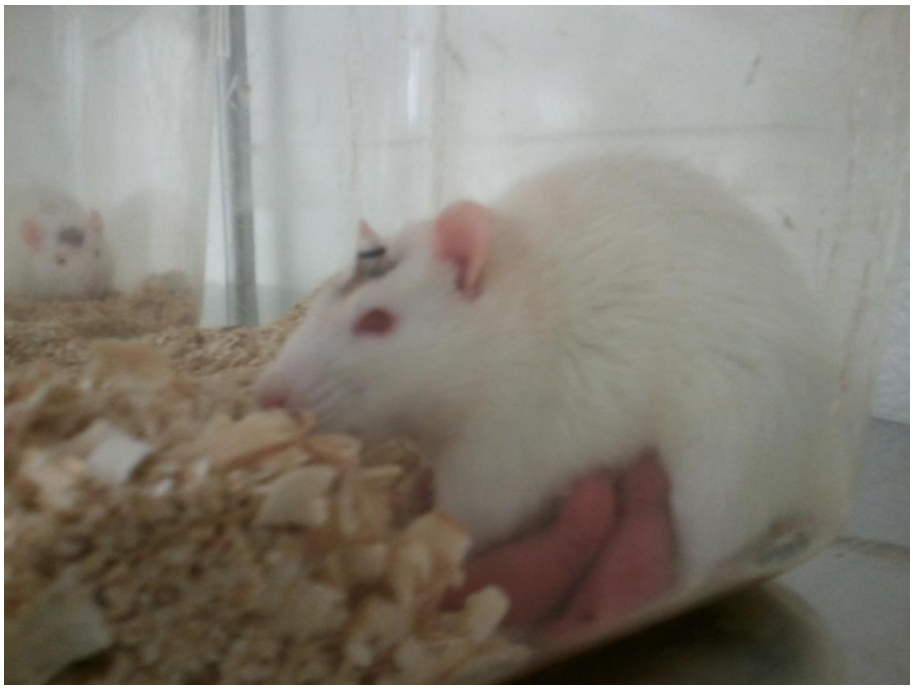

Figure 6: During the 1-hour union of dam and pups, a dam would allow the pups to suckle at the same time. This also depicts acceptance of the litter by the dam.

Maternal Behavior

Maternal behavior towards the pups was also recorded during suckling times. Behaviors included the acceptance or rejection of an individual pup or entire litter. Acceptance in this study referred to the dam caring for the pup(s) by allowing them to suckle. Rejection included negligence by the dam to care for her offspring from delivery to 4 days of development. In some instances this led to untimely death of the litter. In other observations, the litter was sacrificed if the mother was still negligent to care for them by day 4 of development. Cannibalistic actions also counted at rejection from the dam. These actions included eating their offspring at the time of delivery and up to 4 days of development. These actions were usually followed by negligence if any pups survived.

Tissue and Serum Harvesting

At the end of this 7-day observation period, the dams and pups (day 12 of development) were sacrificed by decapitation with the use of a guillotine. From the trunk blood collected, serum was harvested and stored at -80°C for further analysis of hormones. Whole brains from the dams and pups were removed and stored in 10 volumes of RNAlater (Invitrogen, CA) at -20°C for future determination of leptin mRNA expression in the hypothalamus.

RNA Isolation

The hypothalamus was dissected from the whole brain tissue. From 30mg of tissue, RNA was isolated using an RNeasy Mini Kit purchased from Qiagen (Cat. No. 74104). RNA purification was performed as outlined by the manufacturer's protocol. Briefly, the tissue was homogenized using a mortar and pestle technique. The homogenate was applied to a spin filtration column for subsequent purification. The RNA integrity was confirmed using an

Agilent 2100 Bioanalyzer (Agilent Technologies, CA). Samples whose RNA integrity number (RIN) was greater than 7.0 continued in the study.

Gene Expression Analysis

A series of polymerase chain reactions (PCRs) were employed to create a cDNA library from our leptin treated and control samples of isolated RNA. An amplicon was also generated using PCR techniques for our gene of interest (GOI), leptin, which would act as a standard. With the use of specific primers, leptin mRNA was expressed and quantified in each rat hypothalamus.

Two-Step Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Reverse transcription to cDNA was performed by using the Superscript II RT Kit from Invitrogen (Cat. No. 186064-022). The RT-PCR was performed as outlined by the manufacturer's protocol. Reaction method involved the use of oligo dT primers and a uniform input RNA concentration of 200 ng/ml. Transcribed cDNA was verified using standard PCR amplification with target gene specific primers from which the leptin amplicon was generated.

Quantitative-PCR (q-PCR)

Sequence analysis of leptin mRNA was performed prior to q-PCR analysis to determine the feasibility of optimal amplification (NCBI accession# NM_013076). Cyclophilin B was used as an endogenous control based on previous work (Torto et al. 2006). Expression of leptin mRNA levels was determined using the QuantiFast SYBR Green PCR system by Qiagen (Cat. No. 204052). Real time PCR was performed on the Bio-Rad CFX96 optical reaction module (Bio-Rad, CA). Quantitect gene specific primers for leptin and cyclophilin B (Ppib) expression analysis as well as leptin amplicon generation were obtained from Qiagen (Cat. No. Leptin: QT00190960 and Ppib: QT00178703).

The copy number of leptin was calculated using the total number of base pairs in the sequence. The stock concentration of the leptin amplicon was 1.38×10^{10} copies DNA/µl. Serial dilutions of the leptin amplicon were made to act as standards for the experiment. Standards were run concurrent with the unknown samples to determine quantification of the leptin target.

q-PCR cycling conditions for leptin were as follows: 1) 95.0°C for 5:00 min, 2) 95.0°C for 0:10 sec, 60.0°C for 0:30 sec (annealing/extension) at 39 cycles, 3) 65.0°C to 95.0°C (melt curve acquisition), 4) 10.0°C for 5:00. Cyclophilin B cycling conditions: 1) 95.0°C for 5:00 min, 2) 95.0°C for 0:10 sec, 55.0°C for 0:30 sec (annealing/extension) at 39 cycles, 3) 65.0°C to 95.0°C (melt curve acquisition), 4) 10.0°C for 5:00.

Statistical Analysis

GraphPad statistical software (GraphPad, CA), one-way ANOVA with Tukey's Post Hoc was used to determine the levels of statistical significance ($p<0.05$) of the q-PCR findings.

Hormonal Assays

Prolactin

To determine the amount of circulating prolactin levels, a multiplex assay kit was purchased from Millipore, Inc. (Cat. No. RPT86K) with analytes specific for prolactin. The leptin treated and control dam serum samples were used for prolactin concentration measurement. The prolactin serum levels in the samples were measured according to the manufacturer's instructions on a Luminex 100 System (Luminex, TX).

Oxytocin

The serum levels of oxytocin were measured using an Oxytocin ELISA kit acquired from Enzo Life Sciences, Inc. (Cat. No. ADI-900-153). Dam serum from both leptin treated and control samples were used for oxytocin concentration measurement. Extraction of the samples included the use of a 1g C18 Sep-Pak column (Fisher Scientific, CA). The hormone levels were measured by following the manufacturer's protocols.

Statistical Analysis

Using GraphPad statistical software (GraphPad, CA), a 2 tailed t-test determined statistical significance ($p<0.05$) of the hormone concentration findings of prolactin and oxytocin.

CHAPTER 3

RESULTS

Leptin Actions on Pregnancy

Monitoring of the Oestrus Cycle

During this study, the oestrus cycle of all 15 of the Sprague-Dawley females were monitored until pregnancy was established. All leptin treated females successfully mated and pregnancy was achieved. An observation was made regarding control females. After implantation of a cannula, 2 of the 10 control rat's oestrus cycle became irregular. Their cycles were paused in the diestrus stage, deeming them sterile. Their cycle was monitored throughout the study with no relapse into a normal 4-day cycle. These rats were removed from the study. This observation may suggest that leptin worked to improve reproductive ability of the leptin treated females when compared to the control rats.

Dam Weight Gain

Dam BW was monitored daily throughout the study with the use of a digital scale. This parameter was measured to ensure that leptin did not have a negative effect on the fitness of the dam throughout pregnancy. There was no significant difference between the BW of the leptin treated and control dams (Table 1 and Figure 7). Leptin did not decrease gestational weight of the treated dams.

Table 1: This raw data shows the effects of intracerebroventricular leptin on dam BW throughout pregnancy among leptin treated (n=5) and control (n=5) groups.

Dam Weight Gain Throughout Pregnancy (g)	
Leptin Treated	**Control**
131.6	102.2
83.7	98.3
121.5	131
125.9	71.9
143.4	93.8

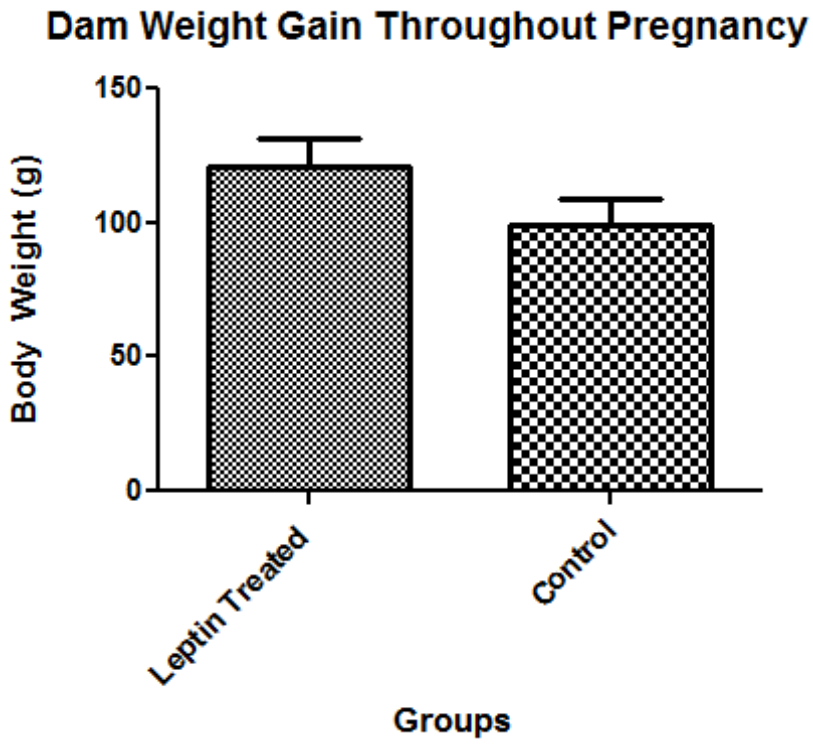

Figure 7: Effects of intracerebroventricular leptin on dam BW throughout pregnancy. There was no significant difference in the BW of the leptin treated rats (n=5) when compared to the control (n=5).

Upon Delivery

When delivery of the pups was completed, dams were given time to recuperate and clean their litter before handled. Once the dam recovered from delivery, there were certain parameters that were measured to determine leptin's effect on reproduction. The number of pups delivered was first recorded along with the BWs of both the dam and the litter. Maternal behavior was also noted during the first 4 days of pup development before separation began.

Leptin Increases the Number of Pups Delivered

The number of pups in each litter was determined upon delivery. Any pups delivered dead, resulting from still births, were added to the number of pups delivered. Upon calculation of the results, leptin treated dams gave birth to significantly more pups compared to control rats (Table 2 and Figure 8).

Table 2: Raw data representing the number of pups delivered from leptin treated (n=5) and control (n=5) groups.

Number of Pups Delivered	
Leptin Treated	**Control**
13	9
12	7
13	12
14	13
13	10

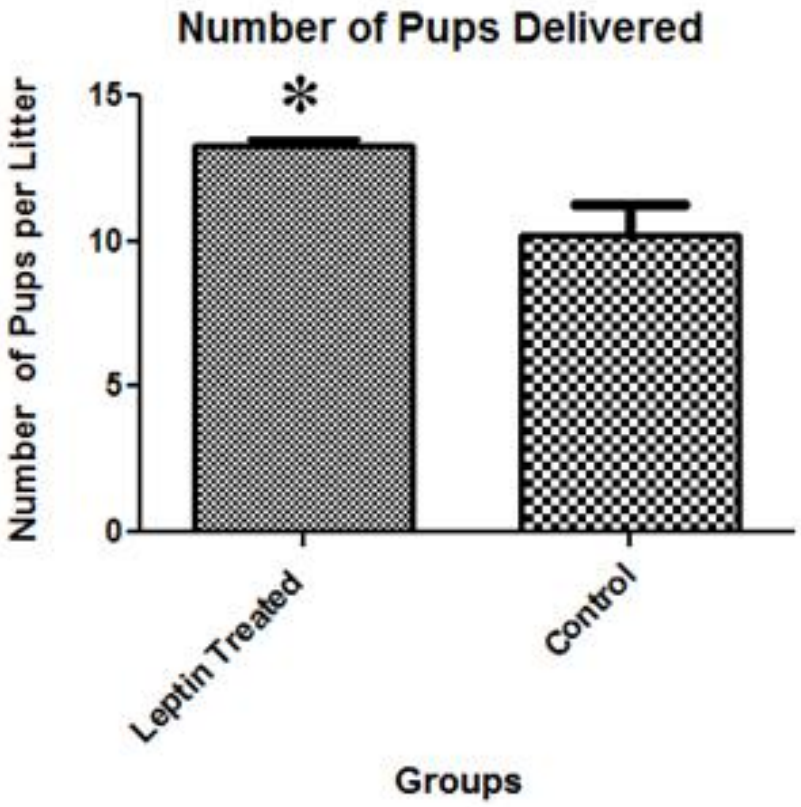

Figure 8: Effects of intracerebroventricular leptin on litter size. The number of pups delivered was significantly ($p<0.05$) higher in the leptin treated group (n=5) compared to the control (n=5), which suggests that leptin improves reproduction.

Leptin Does Not Decrease Individual Pup Birth Weight

The birth weight of the entire litter was measured after delivery. Individual pup birth weight was calculated by dividing the litter BW by the number of pups present. The birth weight of pups from leptin treated dams did not differ from that of control offspring (Table 3 and Figure 9). Therefore, leptin did not alter the birth weights of the offspring.

Table 3: Average individual pup birth weight was measured by dividing the litter birth weight by the number of pups in the litter. This table represents the raw data averages of leptin treated (n=5) and control (n=5) litters collected in grams.

Average Individual Pup Birth Weight (g)	
Leptin Treated Dam (offspring)	**Control Dam (offspring)**
5.95	6.44
6.21	7.16
6.33	5.60
5.99	6.49
5.86	5.61

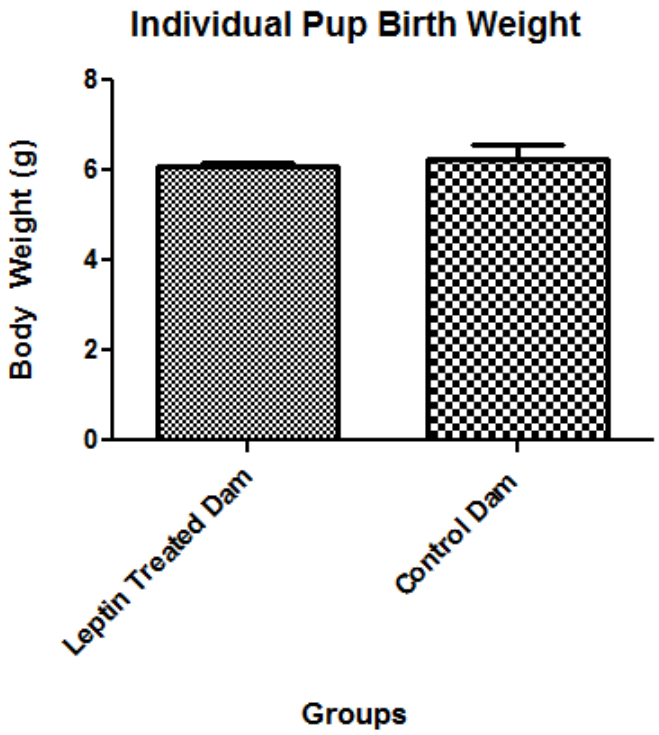

Figure 9: The effects of intracerebroventricular leptin on pup BW. The pups' birth weight did not differ between leptin treated (n=5) and control (n=5) groups, indicating that leptin did not alter the healthy weight of the pup.

Leptin Increases Pup Brain Weight Throughout Development

Pups were sacrificed on day 12 of development. The whole brains of each individual pup were dissected and weighed using a digital scale. Pup brain weights were used as comparison between the groups to distinguish leptin actions on fetal development. Litter weights were also measured at time of sacrifice. Individual pup weight was found by dividing the litter weight by the number of pups present. To ensure non bias, average brain weights were divided by average body weight for each litter. Pups from leptin treated dams had a significant increase in the relative brain weight when compared to pups from the control dams (Table 4 and Figure 10).

Table 4: Pup brain weights were gathered at time of sacrifice, day 12 of development, from each pup (9 per litter). To determine a relative brain weight, the brain weights were averaged for each litter and divided by the average pup body weight, leptin treated (n=5) and control (n=4). The table describes the raw data averages in grams.

Offspring from Treated Dam			**Offspring from Control Dam**		
Brain Wt	***Body Wt***	***Relative Brain Wt***	***Brain Wt***	***Body Weight***	***Relative Brain Wt***
0.785	17.656	0.044	0.628	14.9	0.046
0.873	19.111	0.045	0.22	22.078	0.01
0.809	18.444	0.044	0.161	19.33	0.008
0.693	14.278	0.049	0.158	21.578	0.007
0.525	10.183	0.052			

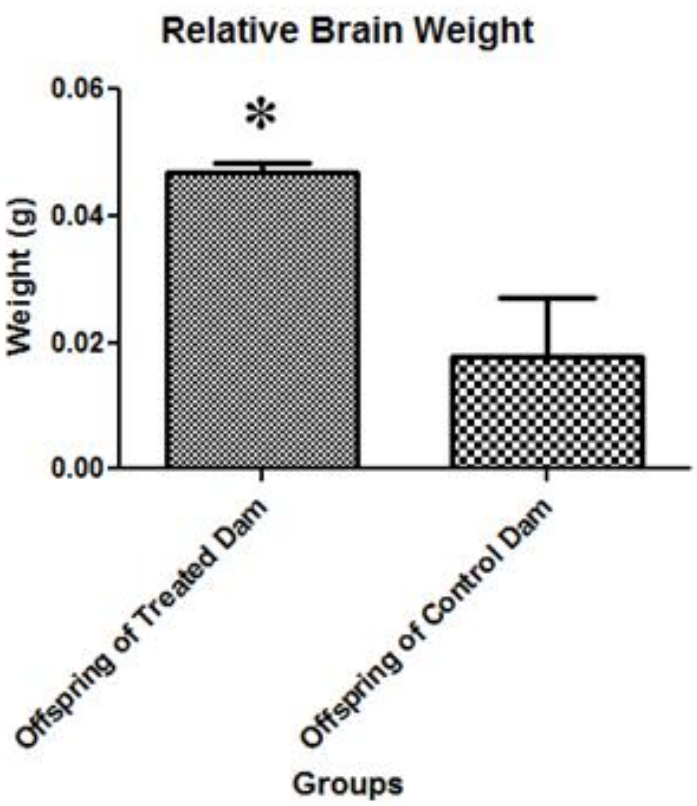

Figure 10: The average pup brain weights and body weights were calculated to determine leptin action on fetal development. Pups from leptin treated dams (n=5) showed a significant ($p<0.05$) increase in relative brain weight when compared to control groups (n=4).

Leptin Improves Maternal Behavior

Maternal behavior of each dam was observed throughout the study. Acceptance and rejection of the litter was noted during the first four days of pup development. Acceptance was observed as caring for the litter by allowing them to suckle. Rejection was classified as negligence or cannibalistic actions of the dam within the first 4 days of development.

All leptin treated dams accepted their young right away after recovery from delivery. Two control dams rejected their young, not cleaning nor allowing them to suckle. Therefore,

leptin may have improved maternal behavior (Figure 11). The control dams that rejected their litter during the first four days of development were removed from the study and the remaining pups were sacrificed.

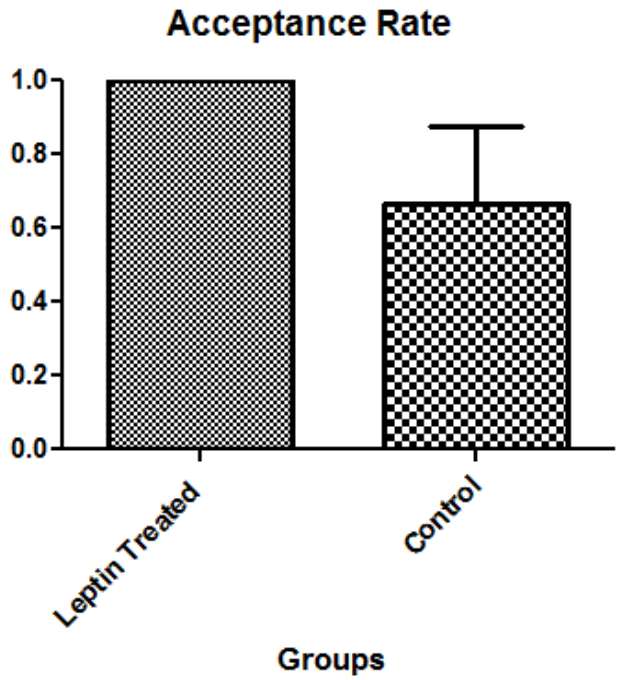

Figure 11: The effects of intracerebroventricular leptin on maternal behavior. The leptin treated group accepted 100% of their young while 2 out of 6 (33%) control dams rejected their young.

Milk Transfer

Beginning four days after delivery, the pups (9 per litter) were separated overnight from their dam. They were reunited the following morning to suckle for one hour. To quantify the amount of milk transferred from the dam to the offspring, the body weights (BW) of both dam and litter were taken before and after this one hour suckling session. Weight gains in the litter after this period of suckle represents the total milk transferred to the offspring.

Leptin Increases Milk Transfer

The amount of milk transferred in a one hour observation was averaged over the experiment duration of 7 days. Dams injected with leptin transferred significantly more milk to their offspring when compared to control dams (Table 5 and Figure 12). This observation indicates that intracerebroventricular leptin improves reproduction by increasing milk yield during lactation.

Table 5: Litter weight gains after a 1h period of suckling were taken over 7 days. These gains were averaged for each dam, leptin treated (n=5) and control (n=4). The raw data averages are represented in the table above with gains in grams.

Average Litter Weight Gain in 1h Suckle Period (g)	
Leptin Treated (offspring)	**Control (offspring)**
5.2	1.3
4.2	2.7
3.7	3.3
3.8	2.3
4.0	-

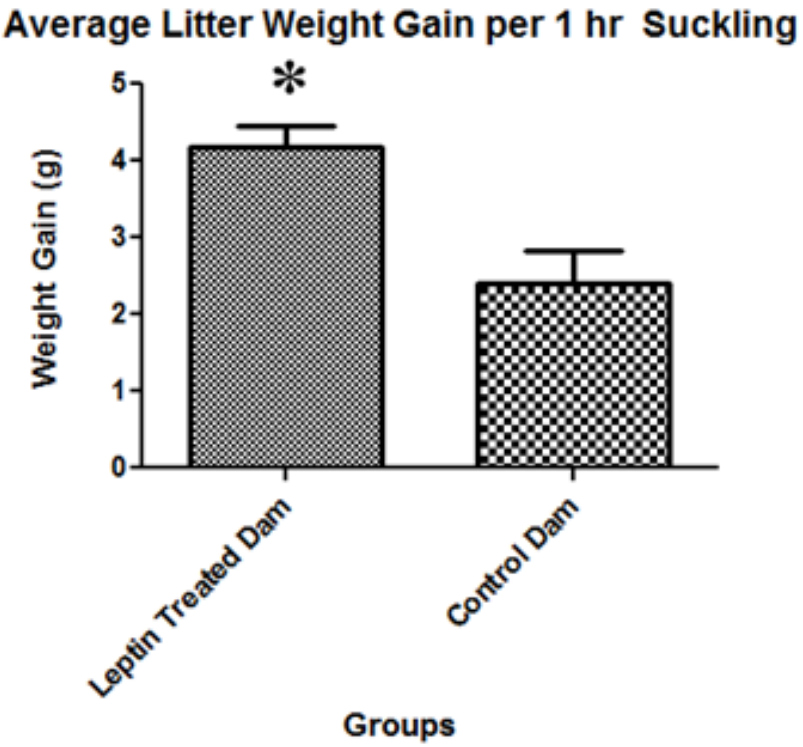

Figure 12: Effects of intracerebroventricular leptin on the amount of milk transferred from dam to litter. There was a significant difference ($p < 0.05$) in the litter weight gain of pups belonging to the leptin treated dams (n=5) after 1-hour of suckling when compared to the pups of control dams (n=4).

Gene Expression

Leptin is Expressed in the Hypothalamus

Gel electrophoresis was employed to validate leptin mRNA expression in our cDNA samples. Samples were run on a 1.5% agarose gel in Tris/Borate/EDTA (TBE) buffer. The size of the leptin transcript is 180 base pairs (bp). A 1,000bp ladder was used for DNA size determination (Fisher Scientific, CA). Expression of leptin was noted across all lanes/samples (Figure 13).

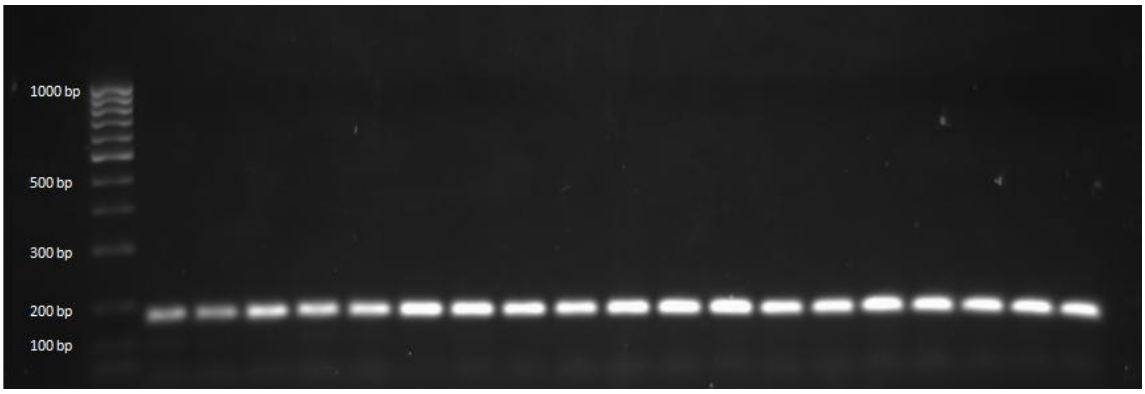

Figure 13: Effects of intracerebroventricular leptin on the expression of leptin in the hypothalamus of leptin treated and control dams and pups. Leptin mRNA was expressed in each sample at a size of 135 bp.

Leptin mRNA Levels are Increased in Treated Dam and Offspring

q-PCR techniques led to a normalized expression of leptin mRNA in the hypothalamus. Differences can be noted between leptin treated dams and control dams as well as the litter from leptin treated dams and the litter from control dams (Table 6 and Figure 14). There was a significant ($p<0.05$) difference between the litter groupings. This observation of leptin inheritance in the hypothalamus of offspring is novel.

Table 6: Normalized leptin mRNA values in the hypothalamus of leptin treated dams (n=3), control dams (n=3), offspring of leptin treated dams (n=6), and offspring of control dams (n=6). The value is shown in pg/µl.

Normalized Leptin mRNA values in the Hypothalamus (pg/µl)			
Leptin Treated	**Control**	**Leptin Treated (offspring)**	**Control (offspring)**
.00004	.00003	.00172	.00011
.00136	.00034	.00211	.00014
.00075	.00005	.00015	.00001
		.00005	.00002
		.00048	.00002
		.00005	.00001

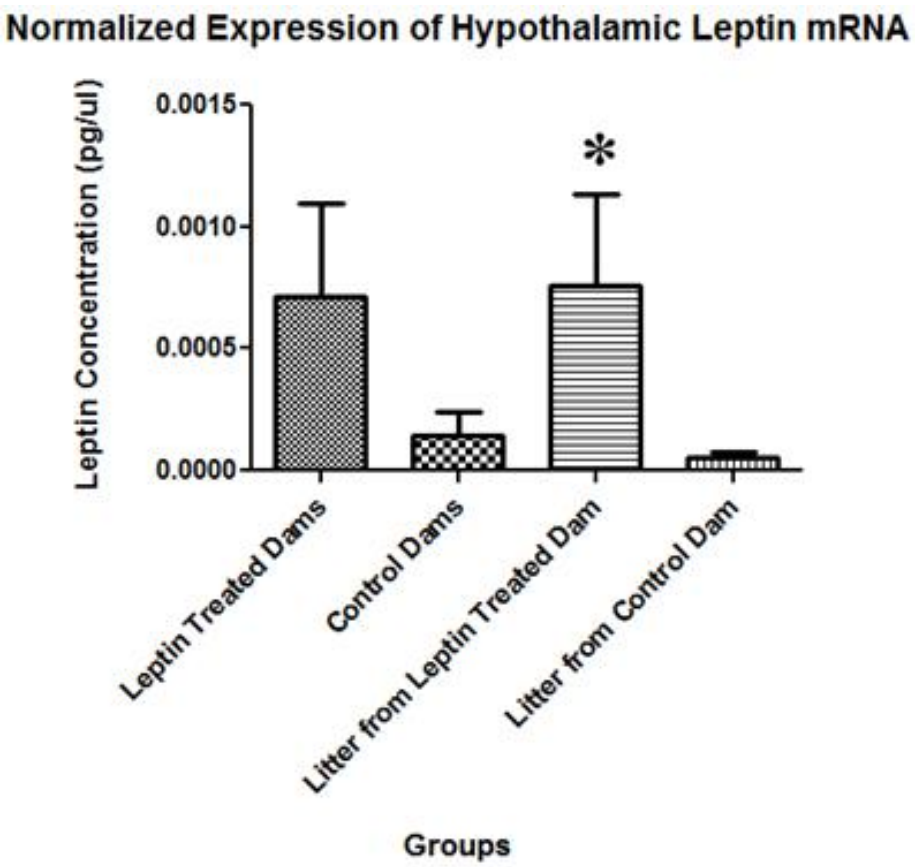

Figure 14: Leptin concentrations increased in leptin treated dams (n=3) when compared to the control (n=3). Pups from treated dams (n=6) also expressed significantly ($p<0.05$) higher levels of leptin mRNA in the hypothalamus compared to pups from control dams (n=6).

Serum Hormone Concentration

Prolactin Levels Increased in Leptin Treated Samples

Prolactin levels were assessed through use of a multiplex assay. Leptin treated dams showed an increase in prolactin serum levels compared to control dams (Table 7 and Figure 15).

Table 7: Concentration of prolactin found within dam serum of leptin treated (n=5) and control (n=4) groups (pg/ml).

Prolactin Levels in Dam Serum (pg/ml)	
Leptin Treated	**Control**
1005.56	589.84
1505.85	1174.05
4247.0	918.89
2197.4	520.1
2013.98	-

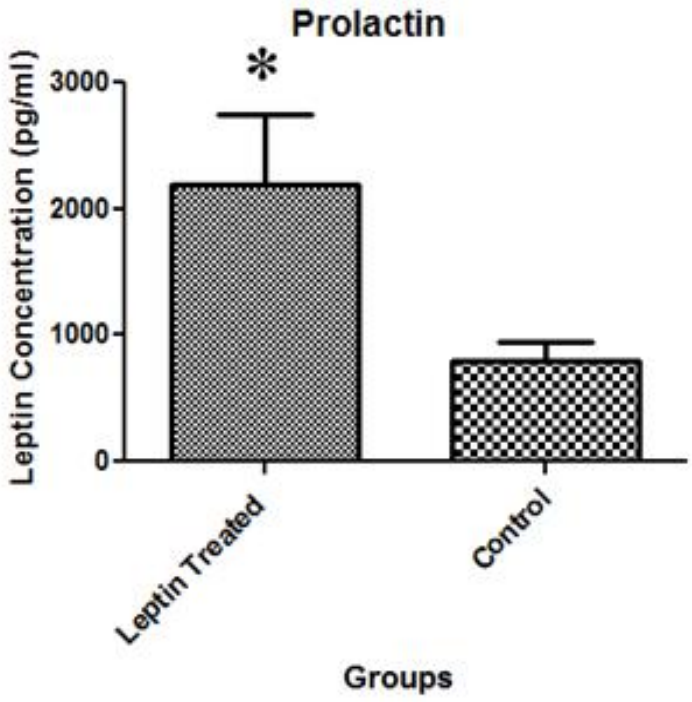

Figure 15: The effects of intracerebroventricular leptin on serum prolactin concentrations. Leptin treated dams (n=5) showed a significant ($p<0.05$) increase in prolactin levels compared to control dams (n=4).

Leptin Does Not Regulate Oxytocin

Oxytocin levels were measured through an ELISA assay. Leptin treated dams showed an increase in oxytocin serum levels compared to control dams (Table 8 and Figure 16); however, these results were not significantly different. Leptin did not down regulate nor up regulate oxytocin secretion. Therefore, leptin may have stabilized the normal secretion of oxytocin.

Table 8: Concentration of oxytocin in dam serum from leptin treated (n=5) and control (n=4) groups shown in pg/ml.

Concentration of Oxytocin in Dam Serum (pg/ml)	
Leptin Treated	**Control**
3.16	3.21
3.51	0
1.34	1.57
9.39	0.06
15.9	-

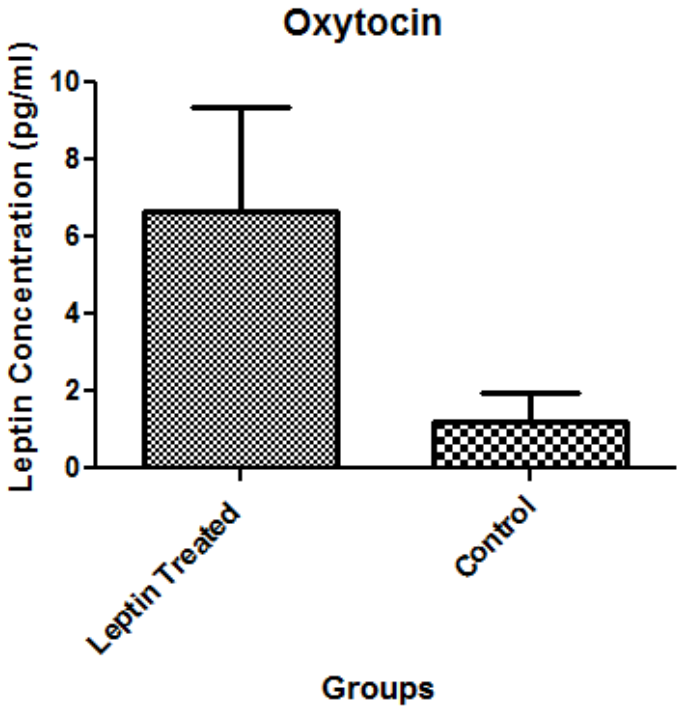

Figure 16: Effects of intracerebroventricular leptin on serum concentrations of oxytocin. Although the levels are increased in leptin treated dams, there is no significant difference compared to the control dams due to varying sample concentration.

CHAPTER 4

DISCUSSION

Leptin is a pleiotrophic hormone involved in many physiological functions throughout the body (Morrison 2009). Its role in reproduction alone is of great importance. The focus of this study revolved around the effect that leptin has on an important function of reproduction, lactation, specifically, the milk transfer from dam to pup. The combined results from this study indicate that leptin is an important regulator of many reproductive functions.

Our first observation in this study was the role of leptin in fertility. Previous research supports that leptin administration plays a positive role in fertility by correcting sterility (Barash et al. 1996; Hoggard et al. 1998). After surgery, control rats that did not receive leptin injections began having an irregular oestrus cycle. The continuance of the cycle ceased in diestrus and did not move, causing sterility, therefore indicating that leptin does increase fertility and the ability for the dam to become pregnant.

Due to a recent experiment with leptin and food intake, dam body weight was monitored daily throughout gestation to ensure proper fitness of the dam. Results showed that there was no difference in the amount of weight gained during gestation between leptin treated and control groups. Leptin did not decrease gestational weight of the treated dams. This was an important aspect to address because leptin is known to cause a decrease in appetite and food intake (Coll et al. 2007). This observation may be a result of the effect of increased estrogen levels during pregnancy. Estrogen works to stimulate appetite, while leptin works to decrease appetite. Therefore, at times of high estrogen levels, leptin's actions may be masked.

Upon delivery, the number of pups was recorded to quantify the reproductive abilities of intracerebroventricular (ICV) leptin. There was a distinct increase in the amount of pups delivered from leptin treated dams compared to the control group. Thus, leptin increases the ability of the dam to reproduce in quantity. What about quality? The individual pup birth weights were also calculated to address offspring fitness. There was no difference found between leptin treated and control groups. Therefore, leptin does not negatively affect the fitness profile of the offspring with regards to birth weight.

To determine leptin's action on developmental fitness of the pup, average weights of whole brain samples were weighed upon sacrifice. Research states that leptin does have the ability to affect the offspring of treated individuals (Henson and Castracane 2000). Because leptin can be found in breast milk (Feuermann et al. 2009) and transferred to the offspring, it can then have effect during lactation. Our results show that pups from leptin treated dams had larger brains with reference to weight (g). Therefore, leptin could act to increase fetal development even after delivery through milk transfer from the dam to the pup.

Maternal behavior was observed throughout the study. Remarkably, all leptin treated animals accepted their young within the first day of pup development. The untreated control rats displayed different patterns of maternal behavior. Negligent and cannibalistic behaviors were noted during the first 4 days of pup development. Two of the 6 control dams showed these behaviors from delivery to the fourth day of development, resulting in the sacrifice of their litter and removal from further participation in the study. This was an interesting observation that is indicative of leptin's ability to improve maternal behavior.

During quantification of milk transfer, the pups were moved away from their dam overnight to induce hunger. The next morning, the litter and dam were united to suckle. A 1-hour period of observation began as soon as the mother showed interest in the pups and suckling actually began. The amount of milk suckled in the 1-hour time frame was averaged over the experiment duration of 7 days. The results show that litters of leptin treated dams gained more weight as per 1-hour suckling period than litters of control dams. This value was significant, which implies that leptin increases milk availability and transfer from dam to pup.

Leptin mRNA expression in the hypothalamus was of great importance. Expectedly, the amount of mRNA in leptin treated dams was in fact higher than control dams, although it did not reach a significant level statistically. However, we did make an important observation involving the offspring that was quite novel. The pups from the leptin treated dams expressed increased levels of leptin mRNA in the hypothalamus compared to the pups of the control dams. Could there be a leptin inheritance factor here, or is this transfer based solely on the amount of leptin that could be transferred in breast milk? We believe that the up regulation of leptin mRNA in the pups is directly related to the amount of leptin mRNA in the dams. Previous research has shown leptin to be an important regulator of fetal brain development (Henson and Castracane 2000). This research linked leptin effect on GH. However, further studies should be conducted to look into this inheritable or transferrable factor or trait.

The effect that leptin has on other sex hormones has been acknowledged (Moschos et al.2002, Donato et al. 2009). It is known that prolactin levels should increase in a high leptin environment (Feuermann et al. 2009). Our result is in agreement with this research, as there was an increase of prolactin levels in the leptin treated dams. As expected, leptin increased prolactin secretion and improved milk yield during lactation.

The second hormone measured was oxytocin, which is known to aid in milk ejection. However, little is known about the relationship between leptin and oxytocin. We observed that even though the effects of ICV leptin seemed to increase the initial amounts of oxytocin in the serum, it did not have a statistically significant difference from the control. Therefore, leptin did not affect the normal secretion of oxytocin.

Future Directions

This experiment should be repeated on a much larger scale with emphasis on milk collection and analysis of the hormones found within. We know from research that leptin is found in breast milk (Feuermann et al. 2009); however, is this the only avenue that leptin is passed from dam to pup? Our research showed that leptin treated mothers gave birth to more pups. Moreover, on day 12 of development, these pups were more fit than the control litters. Was leptin acting through the transfer of milk from dam to pup, or is there some other factor involved in leptin action being transferred to the offspring? This is the scope of research for future study.

Conclusion

Overall, this research clearly supports leptin as an important reproductive hormone that regulates various reproductive functions. Leptin administration leads to increased fertility through actions on litter size and improved maternal behavior. Leptin also plays an important role in lactation by up regulating the amount of milk transferred from dams to pups. This may also play a role in the continuing development of the offspring as we observed in our study.

The result of this study for the first time indicates that maternal leptin increased leptin mRNA expression in the brains of the offspring and could also help in overall brain development of the offspring.

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