EFFECTS OF RECOMBINANT ADENO-ASSOCIATED VIRUS ENCODING LEPTIN ON BODY WEIGHT REGULATION AND ENERGY HOMEOSTASIS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2000
ACKNOWLEDGMENTS

This dissertation has been made possible through the support and encouragement I have received from faculty, friends and family here in Gainesville and around the world. I first want to thank my mentor, Dr. Pushpa Kalra, for all the time she has made for me over the years. Dr. Kalra has helped me become independent; I thank her for having faith in me and taking me on as her graduate student. Dr. Kalra has been a perfect role model, a mentor and a friend. I also want to acknowledge Dr. Satya Kalra for unofficially co-mentoring me in my Ph.D. endeavors. He has been good humored and kind at all times, going well out of his way to help me in times of personal crisis. My committee members Dr. Streit and Dr. Schultz have assisted me to grow intellectually and I thank them. Dr. Streit has also been part of the stress relief operation at the Market Street Pub when occasion demanded.

I am very grateful to my colleagues in the lab; in particular Dr. Michael Dube, who taught me the ropes when I started, and has always been ready with a witty remark to lighten things up. Dr.s Elena Beretta and Michela Bagnasco, "the Italians" who have been there when I just did not have enough hands, and who in addition to scientific input introduced me to the pleasures of (almost) all things Italian. Erin Rhinehart, my fellow graduate student, and dear friend, has been a calm rational voice of reason that helped me keep things in perspective many times. I want to thank Laura Dixon, a new member of the lab and a new friend, who has been extremely helpful and supportive in every way possible.
I thank Drs. Zolotukhin, Scarpace, and Moldawer for allowing me access to their laboratory facilities. I am grateful to Dr. Bill Farmerie for introducing me to the "fun" in molecular biology, Drs. Gerry Shaw, Colin Sumners, Mohan Raizada, Charlie Wood for their support over the years, and all my friends for lending me their ears all along.

I want to thank my husband from the bottom of my heart. Without his love, support and chauffeuring I would never have reached this far and lastly I thank my parents, my mother- in- law, my sister and brother who have been wonderful and supportive at all times.
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EFFECTS OF RECOMBINANT ADENO-ASSOCIATED VIRUS ENCODING LEPTIN ON BODY WEIGHT REGULATION AND ENERGY HOMEOSTASIS

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December 2000

Chair: Pushpa S Kalra
Major Department: Neuroscience

Leptin is a weight reducing hormone synthesized by white adipose tissue. Administration of leptin reduces body weight (BW) in obese and normal rodents. However, obesity is not a result of leptin deficiency, instead excess leptin accompanies human obesity suggesting resistance to leptin actions. We hypothesize that leptin resistance is due to insufficient availability of leptin at target sites within the brain.

We employed recombinant adeno-associated virus encoding leptin (rAAV-leptin) gene therapy to enhance leptin production. An intravenous injection of rAAV-leptin to leptin deficient ob/ob mice increased blood leptin levels and reduced BW in a dose dependent manner. When administered intracerebroventricularly (icv), it increased leptin mRNA in the hypothalamus, and suppressed BW gain without decreasing food intake (FI) in adult lean male and female Sprague-Dawley rats. A single injection icv of rAAV-leptin regulated BW for six months without any evidence of leptin resistance. Our data show a dose-dependent dichotomy in the response to icv rAAV-leptin. While rats
receiving 5 $10^{10}$ particles of rAAV-leptin icv maintained their pre-injection BW, a 2 fold higher dose caused a 10-15% decrease in BW accompanied by a significant reduction in FI. UCP-1 mRNA in brown adipose tissue (BAT) was enhanced with rAAV-leptin with both the high and low dose, indicating that increased leptin production in the hypothalamus enhanced energy expenditure via increased thermogenesis. Analysis of body composition revealed a marked decrease in body fat without altering lean mass. Serum leptin and insulin levels were reduced, however, blood glucose levels were normal. Hypothalamic expression of the appetite regulating pro-opiomelanocortin (POMC) and Neuropeptide Y (NPY) genes were altered with the higher dose only.

Thus, we show for the first time effective use of gene therapy for long term BW regulation. A single central injection of rAAV-leptin reduced BW without development of leptin resistance. Whereas lower levels of centrally produced leptin reduce BW by increasing energy expenditure, higher levels reduce BW both by increasing energy expenditure and by decreasing FI via an increase in hypothalamic POMC and decrease in NPY signaling.
CHAPTER 1
LITERATURE REVIEW

The survival of higher organisms, including mammals, depends upon the maintenance of adequate BW. In order to maintain a stable BW over an extended period of time it is essential that the amount of energy intake is matched by the energy expenditure. Regulation of energy expenditure for all the energy requiring processes in the body is a complex and tightly controlled process. The physiological control of energy homeostasis though effective is not perfect often resulting in obesity which is on the rise in the western world. In the US, obesity has reached epidemic proportions (Wilding et al., 1998) with nearly half the adult population of the US being clinically obese. It is now generally believed to be caused by dysregulation in the balance between food intake and energy expenditure rather than a loss of control over the amount of food ingested. Obesity is a major contributing factor for medical conditions such as non-insulin dependent diabetes mellitus (NIDDM), infertility, hypertension and cardiovascular disease. Failure to contain the obesity epidemic now will only further burden the health care system in the US.

Neuroanatomy of Appetite Regulation

Studies involving twins, adoption studies, analysis of familial aggregation as well as studies from several animal models of obesity, point towards obesity being a result of both genetic and environmental factors (Stunkard et al., 1990; Coleman, 1978) and not
just a lack of will power. Current literature supports the hypothesis that both hyperphagia and anorexia are brought about by the induction of environmental, genetic or hormonal changes in neurochemical signaling within the hypothalamus (Kalra SP, 1997; Kalra SP et al., 1998). Over half a century ago lesions of the ventromedial nucleus (VMH) of the hypothalamus were shown to result in increased BW and obesity (Anand and Brobeck, 1951). Based on the early lesion studies, the VMH was termed the “satiety” center. In contrast, lesions of the lateral hypothalamic nucleus (LHA) lead to aphagia; this nucleus was thus termed the “feeding” center (Hetherington and Ranson, 1940). It is now known that there are discrete sites in the hypothalamus associated with appetite.

There is an increasing awareness that expression of appetite is chemically coded in the hypothalamus in the central nervous system (reviewed in: Kalra SP, 1997; Kalra SP et al.,1999; Morley, 1987). An array of neurotransmitters and neuromodulators localized in the central nervous system (CNS) forms a distinct circuitry of pathways comprised of both appetite stimulating (orexigenic) and appetite inhibiting (anorexigenic) signals (Kalra SP 1997; Kalra SP and Kalra PS 1996). Appetite is thus regulated by a complex network of interconnected over-lapping pathways of neuromodulators in the brain, however, these central regulatory mechanisms are complex and are not yet fully understood.

**Molecules Involved in the Regulation of Appetite**

CNS neurons involved in the production of orexigenic and anorexigenic signals are regulated both by peripherally secreted hormones that cross the blood brain barrier, and by other neurotransmitters within the brain. A change in availability of neural
orexigenic molecules such as Neuropeptide Y (NPY) and Agouti related peptide (AGRP) or anorexigenic molecules such as alpha-melanocyte stimulating hormone ($\alpha$MSH) and cocaine and amphetamine regulated transcript (CART) precede the onset of feeding (Kalra SP et al., 1998). Disruption of the neural microenvironment through either changes in the amount or signaling capability of these molecules may lead to hyperphagia and subsequent obesity on the one hand or to anorexia and weight loss on the other (Kalra SP et al., 1998).

**Table 1-1: List of major appetite regulating neuropeptides**

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<th>Anorexigenic peptides</th>
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<td>Proopiomelanocortin (POMC), alpha melanocyte stimulating hormone ($\alpha$MSH, Lu et al., 1994))</td>
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<td>Agouti related peptide (AGRP, Ollmann et al., 1997)</td>
<td>Cocaine and amphetamine regulated transcript (CART, Kristensen et al., 1998)</td>
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<td>Orexins (Sakurai et al., 1998)</td>
<td>Leptin (Zhang et al., 1994)</td>
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<tr>
<td>Melanin Concentrating Hormone (MCH, Qu et al., 1996)</td>
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Neuropeptide Y

NPY is the most potent stimulant of appetite and feeding behavior currently known (Kalra SP, 1997). Our laboratory has shown that NPY injected intracerebroventricularly (icv) causes increased feeding in satiated rats (Clark et al., 1984). An important physiologic role for NPY in the regulation of appetite was suggested by the demonstrations that NPY mRNA is up-regulated in food deprived rats (Sahu et al., 1992), NPY secretion increases in the paraventricular nucleus (PVN) of the hypothalamus in association with increased appetite for food and NPY antibodies block feeding in fasted rats (Kalra SP et al., 1991). NPY neurons are localized in highest density in the arcuate nucleus (ARC) of the hypothalamus, and project to the PVN where NPY is released from terminals into the extracellular compartment (Kalra SP et al., 1998). Five NPY receptors Y1, Y2, Y4, Y5 and Y6 have been cloned (Larsen et al., 1993; Kanatani et al., 1990; Schaffhauser et al., 1997; Hu et al., 1996) however, feeding receptor has not been unequivocally identified. Both Y1 and Y5 receptors have been implicated in separate studies as being involved in feeding behavior (Larsen et al., 1993; Kanatani et al., 1990, Schaffhauser et al., 1997; Hu et al., 1996).

Orexins

Orexins, two recently discovered peptides localized in the lateral hypothalamus, also stimulate feeding behavior (Sakurai et al., 1998). Both Orexin A and B stimulate food intake, although Orexin A, a 33 amino acid peptide is the more active form (Sakurai et al., 1998). There are reciprocal synaptic contacts between orexin producing cells in the LHA and NPY neurons as well as POMC producing neurons in the ARC, thus, raising the possibility that orexins may be involved in the regulation of feeding. Our laboratory has identified the sites of action of Orexin A to be the LHA, PVN and perifornical area.
(PFH) of the hypothalamus (Dube et al., 1998). Although not as potent as NPY, orexins elicit an increase in food intake

**Melanocortins**

Melanocortins are peptides cleaved from the pro-opiomelanocortin (POMC) precursor molecule that exert their effects by binding to members of a family of melanocortin receptors. POMC is differentially processed in various neurons to produce a variety of peptides including the orexigenic peptide, β-endorphin, and the anorexigenic peptide, αMSH. Disruption of proopiomelanocortin processing leads to obesity (Yaswen et al., 1999; Krude et al., 1998). αMSH, a melanocortin receptor 4 (MC-4R) agonist, reduces food intake (Lu et al., 1994). Its activity is antagonized by Melanin Concentrating Hormone (MCH, not derived from POMC), and by AGRP (Ollmann et al., 1997; Considine and Caro, 1997). Targeted disruption of the MC-4 R in mice leads to hyperphagia and subsequent obesity (Huszar et al., 1997).

AGRP is found primarily in the ARC nucleus of the hypothalamus and also acts at the MC-4R to cause an increase in feeding. An icv injection of AGRP increases feeding (Rossi et al., 1998) and over expression of AGRP leads to morbid obesity (Ollman et al., 1997). Pharmacological blockade of the MC-4R reduces the anorectic effects of leptin, suggesting that the melanocortin system is an important downstream target of leptin action (Seeley et al., 1997). This concept is supported by the recent finding that in humans obesity is strongly linked to a region of chromosome 2 near the POMC gene locus (Comuzzie et al., 1997). Defect in MC-4R is the most common cause of monogenic obesity seen in humans and is responsible for 3-4% of the cases of severe early onset obesity (Farooqi et al., 2000).
Leptin

The presence of a weight reducing hormone had been suspected for many decades based on the study of mouse models of genetic obesity. Many of these models are characterized by single gene mutations as seen in the obese ob/ob mice, the diabetic db/db mice, Zucker fatty fa/fa rats and tubby mice (reviewed by Spiegelman and Flier, 1996; Levine and Billington, 1998). The obese ob/ob mice are characterized by severe obesity, hyperinsulinemia and hyperglycemia that resemble diabetes mellitus, as are the diabetic, obese db/db mice. Parabiosis experiments were conducted in the 1970’s by Coleman in which two strains of mice were surgically joined together in a manner such that they shared their blood circulation. When an ob/ob mouse shared circulation with a wild type mouse it became lean leading Colman to argue for the possibility of a blood borne factor that was missing in the obese mouse. In similar experiments when the db/db mouse shared circulation with a wild type mouse, it failed to become lean. The wild type mouse in this experiment however, was aphagic, became hypoglycemic, lost weight and died of starvation. Coleman also conducted a parabiosis experiment with ob/ob and db/db mice. In this case the ob/ob mouse became lean without any effects seen in the db/db mouse. Based on these experiments Coleman hypothesized that the db/db mice produced a weight reducing factor but were unresponsive to it, possibly because they lacked the receptor for it, while the ob/ob mice lacked the factor itself.

This factor was finally identified in 1994 and named leptin (Greek, *leptos* = thin). Leptin was discovered by positional cloning, an approach not used before in the discovery of a major hormone (Zhang et al., 1994). Leptin is the product of the obese
gene and is also referred to as the obese protein. Leptin is a 16 kD secreted protein hormone produced primarily in white adipose tissue (Auwerx and Staels, 1998, Campfield et al., 1996; Campfield et al., 1995; Caro et al., 1996). Other minor sites of leptin production include the gastric mucosa (Bado et al., 1998), placenta (Senaris et al., 1997), kidney, mammary epithelium (Smith-Kirwin et al., 1998), skeletal muscle, brown adipose tissue (Wang et al., 1998) and most recently leptin mRNA and protein have recently been localized in the brain (Morash et al., 1999). The amount contributed from each of these sites to the plasma leptin pool and their physiological significance awaits further studies. One report suggests that the brain is a significant, non-adipose source of leptin contributing up to 40% of the circulating leptin in women (Wiesner et al., 1999). These authors also suggest that there is a gender bias towards the release of leptin from the brain, with female brains releasing more leptin than males.

Leptin has a circulating half life of approximately 30 minutes, is released in a pulsatile manner from adipose tissue and demonstrates a circadian rhythm with a nighttime elevation in the circulating concentration (Caro et al., 1996; Lewis-Higgins et al., 1996). In rodents as well as in humans, leptin circulates in the blood bound to several plasma proteins. In obese subjects, there is a decrease in the amount of bound leptin and an increase in free leptin in the circulation (Houseknecht et al., 1996). As with other hormones, the potential functions of these binding proteins could be to change the rate of clearance of leptin, increase or decrease its biological activity, as well as to affect the transport of leptin.

Injection of recombinant leptin into obese ob/ob mice (Halaas et al., 1995), which have now been identified to have a nonsense mutation at codon 105 of the obese gene
(Zhang et al., 1994), leads to BW loss due to decreased food intake and increased energy expenditure (Pelleymounter et al., 1995). Leptin also decreases BW in normal mice and in mice with diet induced obesity when administered at supra-physiological doses (Campfield et al., 1996; Caro et al., 1996; Flier, 1997). Such observations have led researchers to dub it the peripheral satiety factor.

Circulating leptin levels are positively correlated with body fat mass and are elevated in several models of rodent and human obesity (Considine et al., 1995; Friederich et al., 1995; Maffei et al., 1995). Steady state levels of leptin are elevated in a variety of rodent models of obesity (Maffei et al., 1995; Mizuno et al., 1996; Friederich et al., 1995). Fasting causes a dramatic down-regulation while increased caloric intake results in up-regulation of leptin mRNA in adipocytes (Ahima et al., 1996; Friedman 1996; Mizuno et al., 1996; Saladin et al., 1995). These observations have led to the proposal that leptin serves as an adipostat informing the brain of the status of energy storage in the adipose tissue so that appropriate changes in appetite, metabolism and nutrient partitioning can be signaled. Leptin crosses the blood brain barrier (BBB) through a non-linear saturable transport mechanism, in a unidirectional manner (Banks et al., 1996). It is transported intact across the BBB as well as through "leaks" at circumventricular organs where the BBB is not as tight. Uptake of leptin is reported in the choroid plexus, ARC region of the hypothalamus and in the median eminence (Banks et al., 1996). It is not known how leptin gets access to specific areas in the brain that are not directly peri-ventricular in location. Interestingly, diet induced obesity in rodents is associated with a decrease in the amounts of leptin transported across the BBB (Burguera et al., 2000).
Leptin reduces BW in *ob/ob* mice; however, the effects of leptin in normalizing the metabolism of obese *ob/ob* mice do not stem solely from a decrease in caloric intake. This is illustrated by the report that *ob/ob* mice that are pair fed to wild type lean littermates lose less weight than do *ob/ob* mice administered leptin (Levin et al., 1996). Leptin selectively reduces adipose tissue mass unlike that observed in times of starvation when there is also a loss in lean body mass (Pelleymounter et al., 1995). Further, the elevation in free fatty acids and ketones observed in times of energy crunch is not seen following leptin treatment of wild type rats (Shimabukuro et al., 1997). An important difference in leptin administration vs. decreased energy input mediated decrease in BW is that leptin does not decrease energy expenditure as is observed with a decrease in food intake. On the contrary, there is an increase in energy expenditure with leptin administration has been reported (Halaas et al., 1997). Leptin thus exerts its larger role in energy metabolism through increased energy expenditure as well as reduced caloric intake.

Although the effects of leptin given intracerebroventricularly (*icv*) or peripherally are similar (Halaas et al., 1997), *icv* leptin is more potent in reducing food intake and enhancing metabolism, thus suggesting that leptin modulates its effects on energy balance by action at the level of the CNS (Campfield et al., 1995; Halaas et al., 1997). How leptin mediates its effects and through which modulators is not fully understood. The primary site of leptin action is thought to be the hypothalamus and involves hypothalamic nuclei associated with the regulation of feeding such as the ARC, VMN, PVN, etc. The neural connections involved in the action of leptin on energy metabolism were elucidated by analyzing *fos* like immunoreactivity as an indicator of neuronal activation. These
studies indicate that leptin stimulates *fos* like immunoreactivity in the dorsomedial VMH, the dorsomedial nucleus (DMN) of the hypothalamus, the ventral and parvicellular subdivisions of the PVN, the premammillary nucleus as well as the superior lateral parabrachial nucleus of the hypothalamus (Yokasuka et al., 1998; Elmquist et al., 1998, 1999). Tract tracing studies have revealed major anatomical links between the DMN and PVN (Swanson and Swachenko, 1983). The PVN, an important nucleus for regulation of appetitive behavior has descending axons to autonomic preganglionic neurons within the spinal cord and the medulla. Thus, leptin activation of nuclear groups in the PVN and ARC may regulate neuroendocrine function and energy balance possibly through the sympathetic nervous system.

**Leptin Receptors**

Leptin affects BW and reproduction via binding to receptors in the CNS. The leptin receptor was first isolated from mouse choroid plexus using an expression cloning strategy (Tartaglia et al., 1995). The 894 a leptin receptor belongs to the class 1 cytokine receptor family and is a single transmembrane spanning receptor (Tartaglia et al., 1995). It is closely related to the gp130 transduction unit of the cytokine receptors such as those for interleukin-6 leukemia inhibitory factor, granulocyte colony stimulating factor and cilliary neurotrophic factor. It has a short intracellular domain that contains a Janus kinase (JAK) binding site; the extracellular domain binds its ligand leptin (Tartaglia et al 1995). Six different isoforms (ob-R a-f) of the leptin receptor exist (Tartaglia, 1997, White et al., 1997, Reviewed in Ahima et al., 2000). These leptin receptor isoforms share a common extracellular ligand binding domain at the amino terminus, but differ at the intracellular carboxy-terminal domain. Only the long form, ob-Rb, contains all the
intracellular motifs needed for activation of the JAK/STAT signaling pathway. The biological actions of leptin following binding to ob-Rb are mediated through activation of the JAK/STAT signaling pathway (Tartaglia 1997; White et al., 1997). Ob-Ra, Ob-Rc, Ob-Rd and ob-Rf can activate JAK but this activation is weak and does not led to the activation of STAT, these isoforms thus are incapable of signaling (Ghilardi et al., 1996; Bjorbaek et al., 1997) Ob-Re lacks both the transmembrane and the intracellular domain and circulates as a soluble leptin receptor, the function of this receptor is not clear (Halaas and Friedman, 1997). The short form of the receptor ob-Ra, has a truncated intracellular domain and is not capable of signaling (Tartaglia, 1997). It is highly localized in the choroid plexus and is speculated to play a role in the transport of leptin across the BBB (Tartaglia, 1997).

There are genetic rodent models of obesity attributed to leptin receptor defects. In *db/db* mice, there is a premature stop codon in the 3’ end of the ob-Rb transcript, resulting in the synthesis of a receptor with a truncated intracellular domain resembling ob-Ra (Coleman, 1978; Chen et al., 1996; Tartaglia, 1997; Chua et al., 1996). The *db/db* mice are completely insensitive to the effects of leptin and are morbidly obese. In the Zucker *fa/fa* rats there is a Gln-to-pro substitution at amino acid 269 in the extracellular domain of the leptin receptor leading to a decreased cell surface expression of the receptor and hence decreased leptin signaling (Phillips et al., 1996, da Silva et al., 1998). The Zucker *fa/fa* rats are consequently obese, but unlike the *db/db* mice, the *fa/fa* rats are capable of responding to very high doses of leptin administered *icv* (Cusin et al., 1996). Another model of rodent obesity associated with defective leptin receptors is the obese Koletsky rat. A point mutation at amino acid 763 leads to a stop codon in the
extracellular domain of the receptor so that these rats do not express any leptin receptor (Wu-peng et al., 1997; Takaya et al., 1996). In humans a few rare cases of obesity associated with mutations in the leptin receptor have been reported (Clement, et al., 1998). These patients are hyperphagic, obese and insensitive to exogenous leptin administration as in the case of the db/db mice.

The long form of the leptin receptor has been localized in heart, lung, kidneys, ovaries, uterus, testes, pancreas, adipose tissue and brain (Couce et al., 1997; Mercer et al., 1996; Tartaglia, 1997; White et al., 1997; Zomorano et al., 1997) with the highest levels of ob-Rb in the hypothalamus of the brain (Elmquist et al 1998). More specifically ob-Rb has been found in the VMH, DMH, ARC, PVN, and LHA in the hypothalamus (Elmquist et al., 1998). Leptin receptors are colocalized in NPY and POMC producing neurons in the ARC, suggesting that the CNS actions of leptin include modulation of orexigenic and anorexigenic molecules (Cheung et al., 1997; Kalra SP et al., 1998; Leibowitz and Hoebel, 1998).

**Leptin interaction with appetite regulating molecules**

**Leptin and NPY**

NPY producing neurons are found in the ARC nucleus of the hypothalamus. NPY mRNA levels are significantly elevated in ob/ob mice as well as in db/db mice (Sanacora et al., 1990; Wilding et al., 1993). Administration of leptin to ob/ob mice returns NPY mRNA levels to wild type levels and corrects the obese phenotype (Stephens et al., 1995). It has also been shown that icv leptin decreases NPY mRNA expression and release in the hypothalamus (Schwartz et al., 1996). However, NPY
knockout mice, which are normal in most aspects, are sensitive to the actions of leptin (Baraban et al., 1997; Ericson et al., 1996; Ericson et al., 1996). In the absence of NPY (NPY -/-) ob/ob mice were still responsive to exogenously administered leptin, although the obese phenotype was not corrected. Thus, even though leptin and NPY have reciprocal effects on feeding and leptin reduces NPY gene expression, it seems unlikely that leptin induced decrease in feeding and increase in energy expenditure occurs solely through the NPY system.

**Leptin and the Melanocortins**

Several lines of evidence suggest a connection between leptin and the melanocorticergic pathways. The long form of the leptin receptor Ob-Rb and POMC mRNA are co-expressed neurons in the ARC of the hypothalamus (Cheung et al., 1997). POMC mRNA is reduced in leptin deficient animals, and leptin administration causes increased expression of POMC mRNA (Mizuno et al., 1997; Thornton et al., 1997; Schwartz et al., 1997). Leptin delivered icv increases POMC mRNA expression as does fasting (Schwartz et al., 1997). αMSH, a product of the POMC gene, decreases feeding when injected icv (Poggioli et al., 1986) by binding to the MC-4R (Lu et al., 1994). Thus, αMSH is an important player in mediating leptin's effects on inhibiting appetite.

Obesity in another rodent model, the lethal yellow mouse (A^y/a) mouse, is caused by constitutive expression of the agouti peptide (Miller et al., 1993). Agouti is a potent antagonist of the hypothalamic MC-4R and thus increases feeding in mice (Ollmann et al., 1997). AGRP is homologous to agouti and antagonizes both MC-3R and MC-4R (Fong et al., 1997). Mice lacking leptin have elevated hypothalamic AGRP mRNA. Reinstatement of leptin in these mice decreases AGRP mRNA expression
Further, the levels of AGRP are elevated 8-10 fold in leptin receptor mutant db/db mice. (Ollmann et al., 1997). Administration of a synthetic brain melanocortin receptor antagonist blocks the effect of leptin on feeding (Seeley et al., 1997). In the fed state leptin suppresses AGRP expression, but during fasting this restraint is lowered resulting in upregulation of AGRP and increased drive towards ingestive behavior (Wilson et al., 1999). Because POMC derived melanocortins are MC-4R agonists and AGRP is an antagonist, the net result of leptin action is decreased signaling through the MC-4R. Thus, leptin may possibly mediate its weight reducing actions by altering/modulating melanocortin neurochemistry within the hypothalamus.

**Leptin and Insulin**

The relationship between leptin and insulin levels in the circulation is very complex. In the absence of leptin as seen in the leptin deficient ob/ob mice there is hyperinsulinemia as well as hyperglycemia. Leptin deficiency causes severe insulin resistance in these mice which is reversed upon administration of leptin. Exogenous administration of leptin to ob/ob mice leads to a decrease in the plasma levels of insulin as well as glucose to the normal range (Halaas et al., 1995; Campfield et al., 1995; Weigle et al., 1995; Schwartz et al., 1996). Interestingly, these changes precede any changes in food intake or BW. Leptin administration also reverses the severe insulin resistance and hyperglycemia in lipodystrophic mice that lack white adipose tissue and hence have very low levels of circulating leptin. Thus, leptin has effects on insulin secretion and action independent of its actions on food intake. It is not known whether leptin exerts its effects on insulin secretion directly in the periphery via its actions on the pancreatic β-cells or through targets in the central nervous system. In the literature, data
on the exact influence of leptin on insulin secretion are controversial, although there is some evidence of a negative effect of leptin on insulin secretion (reviewed in Casaneuva and Dieguez, 1999).

Insulin, on the other hand, has a positive effect on the synthesis and secretion of leptin. Blood leptin levels are elevated after peak insulin secretion during the course of the feeding cycle (Saladin et al., 1995; Sinha et al., 1996). Insulin directly stimulates leptin mRNA synthesis in white adipocytes in vitro (Rentsch et al., 1996). Intravenous injection of insulin in rodents leads to an increase in plasma leptin levels accompanied by increases in adipose tissue leptin mRNA expression (Saladin et al., 1995). Slowing of leptin production is rapidly reversed by insulin administration (McDougald et al., 1995).

Some reports suggest that in humans the levels of insulin may predict the levels of leptin. In the plasma leptin levels increase with insulin administration, and conversely, the lowered levels of insulin in the fasting state, coincide with lowered leptin in the plasma (Boden et al., 1997; Segal et al., 1996; Boden et al., 1996). There is a positive relationship between hyperleptinemia and insulin resistance (Cusin et al., 1995).

How insulin mediates its effects in energy regulation is not completely understood. Like leptin, insulin acts on the hypothalamus to decrease food intake (Woods SC et al., 1996). Ahima and Flier (2000) suggested that the effects of leptin on nutrition are mediated in part by insulin. There is a prevalent view that leptin facilitates the actions of insulin in the CNS. Leptin possibly plays an important role in enhancing insulin sensitivity in the periphery. There is further debate as to whether it is the central action of leptin that enhances insulin sensitivity or whether this is accomplished by the direct action of leptin on target organs outside of the CNS.
Fat tissue and Uncoupling Proteins in Energy Metabolism

There are two different types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT). White adipose is the major source of stored energy in the form of triglycerides and is found throughout the animal kingdom while BAT is restricted to mammals. WAT is broken down and its triglyceride stores used in times of energy deprivation. The mass of WAT in the periphery reflects the balance between energy expenditure and intake (Klaus, 1996). It plays a key role in signaling energy availability to the hypothalamus through production of leptin. WAT and BAT are localized in anatomically distinct sites, with BAT found mainly in the interscapular, subscapular, axillary and the suprasternal regions (Klaus, 1996). BAT is well established as a thermoeffector organ (Reviewed in Cannon et al., 1997). It is a source of non-shivering thermogenesis and plays an important part in the regulation of body temperature in small animals such as rodents. In humans, however, BAT is found only through early infancy.

BAT is extensively innervated by the sympathetic nervous system (SNS, reviewed in Himms-Hagen, 1991; Bartness et al., 1999). In response to inputs such as cold exposure, overfeeding and acute feeding the hypothalamus transmits signals to the BAT via the SNS. The SNS acts on the BAT through $\beta_3$ adrenergic receptors to elicit heat production. Bartness et al., (1999) using pseudorabies virus, a transneural viral tract tracer, showed a direct connection between magnocellular neurons of the PVN and interscapular BAT (iBAT), as well as between some brainstem regions such as the caudal raphe region of the brain and iBAT. Substantial innervations to iBAT from the SCN as well as the MPOA are also seen but the functional implications for this innervation are not known. BAT is diffused in adipose tissue of non-human primates but lacks $\beta_3$
adrenergic receptors required for sympathetic activation. Mice with genetic ablation of BAT are obese, have reduced energy expenditure and are prone to diet-induced obesity (Hamman et al., 1998). These studies suggest that intact BAT is necessary for protection from diet induced obesity.

BAT cells have a unique mitochondrial machinery that allows them to uncouple oxidative phosphorylation from ATP synthesis. The thermogenic action of brown adipocytes is due to the presence of a mitochondrial protein known as uncoupling protein 1 (UCP-1). UCP-1 is a proton translocator present in the inner mitochondrial membrane and functions by diverting protons from ATP synthesis towards the dissipation of heat (Klingenberg 1990; Garlid et al., 1998). Five types of UCP’s (UCP-1-5) have been identified. UCP-2 as well as UCP-3 can also uncouple mitochondrial respiration (Fleury et al., 1997; Gong et al., 1997). UCP-1 is expressed only in BAT, and is primarily responsible for cold induced thermogenesis (Boss et al., 1998). UCP-2 is expressed in several tissues such as liver, skeletal muscle, white adipose tissue and brain areas involved in homeostasis, specifically hypothalamic nuclei such as the SON, SCN, PVN and ARC (Fleury et al 1997; Gimeno et al., 1997). UCP-3 is expressed primarily in skeletal muscle and heart (Vidal-Puig et al., 1997). UCP-4 is a brain specific uncoupling protein (Mao et al 1999), of yet unknown function.

UCP-1 levels are sensitive to stimuli such as fasting, cold exposure and thyroid hormone levels in the blood in a tissue specific manner. Fasting decreases UCP-1 mRNA expression in BAT while cold exposure leads to an increase in UCP-1 mRNA in this tissue (Boss et al., 1998) as do rising thyroid hormones (Nicholls and Rial 1999). The primary stimulus for enhancing UCP-1 is norepinephrine action via the β1 and β3
adrenergic receptors (Boss et al 1998). Targeted disruption of the UCP-1 gene leads to mice that are cold intolerant but not obese mice (Enerback et al., 1997).

UCP-2 expression on the other hand is unchanged in BAT upon fasting, but is increased in muscle. Cold exposure increases UCP-2 expression in muscle, BAT and heart. There is increased expression of UCP-2 in WAT in obesity resistant strains of mice, (Collins et al., 1997; Surwitt et al., 1998) but it remains unchanged in diet induced obese mice. Similarly, there is no change in BAT UCP-2 in response to a high fat diet. Therefore, there are tissue specific differences in mechanisms regulating UCP-2 expression in response to increased dietary fat. Generally, UCP-2 is modulated by diet but is not regulated by the SNS.

UCP-2 and 3 are 56% homologous to UCP-1. UCP-3 is 73% similar in structure to UCP-2, but its functions are more closely related to UCP-1. Sympathetic innervation is necessary to maintain basal mRNA levels of UCP-3. As with UCP-1, UCP3 is upregulated by cold exposure, sympathetic stimulation and thyroid hormones. Genetic knockouts for UCP-3 result in mice that are not obese (Vidal-Puig et al., 2000), while mice over-expressing UCP-3 are lean yet surprisingly hyperphagic (Clapham et al., 2000).

**Leptin and Uncoupling Proteins**

There are reports that support the view that BAT helps regulate BW after hyperphagia with non-shivering thermogenesis (Himms-Hagen, 1990). Thermogenesis by BAT may be one mechanism by which leptin regulates BW. Immunohistochemical methods have localized leptin in BAT although to a much lesser degree than in WAT (Cinti et al., 1997). Leptin increases sympathetic activity in ob/ob mice with increases in
BAT UCP-1 mRNA (Collins et al., 1996). Peripheral delivery of leptin leads to increased energy expenditure as measured by oxygen consumption and UCP-1 mRNA expression in BAT (Scarpace et al., 1997). Leptin given *icv* along with food restriction prevents the food restriction induced decrease in UCP-1 and UCP-3 mRNA levels without an effect on UCP-2 expression (Scarpace et al., 2000). Denervation of BAT prevents upregulation of UCP1 by leptin (Scarpace et al., 1999). UCP-3 mRNA increases with leptin treatment in skeletal muscle of *ob/ob* mice (Liu et al., 1998). There appears to be a strain specific effect on UCP-2 induction by leptin in mice. Leptin induces UCP-2 mRNA in *wt* mice (Zhou et al. 1997). However, there is no effect of leptin on UCP-2 mRNA in diet induced obesity resistant strains of *A/J* or B6 mice (Surwitt et al., 1998). In summary, it is clear that leptin acts to modulate the different subtypes of UCP’s in different ways (Scarpace et al., 2000). Whereas UCP-1 is modulated via changes in sympathetic activity in the BAT, UCP-2 and 3 are regulated by leptin independently of the SNS via unknown mechanisms.

**Adenoassociated Virus and Recombinant Adenoassociated Virus**

Adenoassociated virus (AAV) is a single stranded DNA virus and a member of the parvovirus family. AAV requires the presence of a helper virus for viable transfection (Berns and Bohenzky, 1987). The most important feature for the use of AAV is safety. AAV is non-pathogenic, non-immunogenic and thus ideal for use *in vivo*. AAV exists as a latent infection in humans and is not associated with the etiology of any disease (Berns and Bohenzky, 1987). Most significantly, this virus is capable of infecting non-dividing cells (Flotte et al., 1995; Klein and Peel, 2000). The site of integration of
the wild type AAV is human chromosome 19 (Samulski, 1983). rAAV vectors are derived from AAV but unlike their wild type parent AAV are incapable of site specific integration and thus integrate randomly into the host genome in the presence of a helper virus (Xiao et al., 1997). Recombinant AAV is made up of a simple capsid with a single stranded DNA genome that has short viral inverted terminal repeats (ITR’s) but no viral coding sequences (Hermonat et al., 1984; McLaughlin et al., 1988). This removal of all viral sequences except the ITR’s further enhances the safety of AAV for in vivo studies. This is achieved by eliminating the generation of wt helper virus as well as reducing the probability of an immune response to rAAV delivery (Xiao et al., 1996). One limiting factor in the use of this virus as a vehicle for gene delivery is the limitation of the insert size, up to 4.7 kb. There are, however, recent publications that have “expanded” the size of the insert with the use of heterodimer vectors (reviewed in Samulski, 2000).

rAAV vectors have great potential for delivery of genes to the CNS. AAV can transduce non-dividing neurons over extended periods of time in different areas of the adult rat brain (Kaplitt et al., 1994). Other tissues successfully transduced by rAAV include the spinal cord, eye, muscle, lung, heart and liver without any detectable cellular immune responses (reviewed in Xiao et al., 1997). Of all the different types of viral vectors used in gene therapy, rAAV makes up only 1.1%. This was mostly because in the past it was difficult to produce high titre AAV vectors for in vivo delivery, and due to the comparatively slower transduction and lower number of cells transduced. At the University of Florida Gene Therapy Center and elsewhere there are now production paradigms in place that have resulted in higher titre virus production. Due to its excellent
safety features and ability to transduce a variety of tissues, rAAV will likely be the virus of choice for gene therapy applications in the future.

**Study Design and Rationale**

Leptin is a fundamental component of the energy regulatory system. Deficiency of leptin or mutations in its receptor leads to morbid obesity in a variety of rodent models (Halaas and Friedman, 2000). However, most human obesity is not genetic in origin and does not occur due to mutations such as those described for the *ob/ob* or *db/db* mice (Campfield et al., 1996). Human obesity is accompanied by hyperleptinemia and often hyperinsulinemia. Leptin levels increase with increasing fat mass resulting in a positive correlation between adiposity and serum leptin levels in humans (Caro et al., 1995). In human obesity leptin gene expression and increased leptin secretion are increased (Considine et al., 1996). Obese humans resemble *db/db* mice in that they have an impaired response to the high leptin levels in their system. The principal site of action of leptin is the brain, but in obese patients the brain does not respond to the peripherally elevated leptin levels. This unresponsiveness to endogenous elevated leptin is termed "leptin resistance".

The etiology of leptin resistance is little understood. There are several possible causes of leptin resistance. The first hypothesis is that there is a defect in the transport of leptin across the BBB (Van Heek et al., 1997), possibly due to a saturated transport system across the BBB attributable perhaps to excess leptin availability as seen in hyperleptinemia. This view is supported by experiments in obese rodents that were insensitive to peripherally administered leptin but responded by losing weight when leptin was injected *icv* (Van Heek et al 1997). Also, the CSF:serum ratio of leptin is
greatly reduced in obese humans (Caro et al., 1996). Since the CSF leptin concentration of obese humans is 30% higher than normal controls, it is also likely that a part of leptin resistance seen in obesity could be due to "reduced leptin sensitivity " within the CNS.

Defects in the leptin receptors implicated in facilitating the transport of leptin across the BBB may be another means of disruption of leptin transport. However, this possibility is refuted by the presence of leptin in the CSF of Koletsky rats which due to a mutation in their leptin receptor gene lack any form of the leptin receptor (Wu-peng et al., 1997) yet can transport leptin into the CSF.

Aging is another a factor associated with leptin resistance. Aged rats are leptin resistant and have increased adiposity with an increase in serum leptin levels (Hua Li et al., 1998). Fasting-induced regulation of serum leptin as well as leptin mRNA expression are impaired in aged rats (Hua Li et al 1998).

Deciphering the nature of leptin resistance is of paramount importance to the understanding of human obesity. Currently there is no direct evidence to support any of the above mentioned possible causes of leptin resistance. In common forms of obesity there are neither structural nor functional defects in leptin receptors in the hypothalamus, thus in the absence of defects in either the availability of leptin in the CSF, its receptor protein or the ability of its receptors to function correctly, we propose that leptin resistance stems from insufficient availability of leptin at the site of its target neurons in the hypothalamus. Leptin resistance is likely due to loss of leptin availability at specific sites within the brain especially the appetite regulating nuclei within the hypothalamus. The effects of elevated, site directed, levels of leptin in the hypothalamus is not known.
There are a few studies that have used viral vectors to deliver the leptin gene into rodent systems. In 1996, Chen et al. generated sustained hyperleptinemia for a period of 28 days in male Wistar rats via carotid artery infusion of an adenovirus vector encoding leptin. The animals lost BW and displayed reduced appetite in response to the 8ng/ml plasma leptin achieved. In the same year Muzzin et al., injected an adenovirus encoding leptin through an injection into the tail vein in ob/ob mice and corrected their obesity and other associated symptoms such as hyperinsulinemia and diabetes. Similar results were seen in another study (the only study reported with AAV) where leptin was delivered via rAAV encoding leptin into the skeletal muscle. BW and food intake were reduced in ob/ob mice for a period of 15 weeks post injection. There are no reports in the literature that explore the long-term outcome of central delivery of leptin using gene therapy methods.

The recent clinical trial (Heymsfield et al., 1999) to study the potential therapeutic role of leptin in reducing obesity yielded maximum reductions in BW (7.1 kg in treated humans vs. 1.7 kg in the placebo group) with recombinant leptin administered subcutaneously at a dose of 0.3 mg/kg BW. Direct delivery of leptin into the CSF is more potent than peripherally administered leptin. The main concern, however, has been safety in the delivery of leptin into the CSF. With the rAAV encoding leptin the safety concerns can be addressed. Since it is our hypothesis that leptin resistance stems from a loss of leptin availability within the central nervous system, in the studies presented in this dissertation I sought to understand the long term consequences and viability of central leptin delivery using a rAAV vector encoding leptin in lean Sprague-Dawley rats. The objective of these studies was to deliver leptin into the third ventricle of the brain in
order to achieve targeted increase at specific hypothalamic sites involved in the regulation of energy balance and BW regulation. We propose that leptin resistance is not due to chronic over-exposure at its site of action but conversely due to an insufficiency at critical hypothalamic nuclei.

As summarized earlier, several neural and peripheral systems are implicated in mediating the action of leptin. In order to elucidate which of these known modulating systems is implicated, I examined the hypothalamic neuropeptidergic and blood hormonal changes in response to central delivery of rAAV encoding leptin. This research will likely provide information useful for developing targeted therapies for some forms of human obesity by determining the efficacy and functioning of site specific increases in leptin concentrations in the brain over the long term.
CHAPTER 2
GENERAL METHODS

Experimental Animals

Adult male and female Sprague-Dawley rats (200-250 g) were purchased from Harlan (Indianapolis, IN). All rats were housed individually in an air-conditioned room (22-25°C) with lights on from 0500-1900 hrs. Food and water were available ad-libitum to all animals. Rats were sacrificed at the end of the experiment by decapitation. Leptin mutant ob/ob mice were purchased from Jackson Laboratories. Mice were housed four per cage in a specific pathogen free environment. Mice were sacrificed by cervical dislocation. The animal protocols were approved by the Institutional Animal Care and Use Committee.

Third Ventricle Cannulation and Injection

Permanent cannulae were stereotaxically implanted in the third cerebroventricle of male and female Sprague-Dawley rats, under anesthesia (Ketamine 100mg/kg BW + Xylazine 15 mg/kg BW) according to the rat stereotaxic atlas (Palkovits and Brownstein, 1988). The nose bar was set 5 mm above the horizontal zero. The cannula was placed at the midline, 6.4 mm anterior to the interaural line and 8 mm deep and cemented in place with dental cement. The cannulae were observed for cerebrospinal fluid (CSF) flow and then closed with a stylet. This flow of CSF served as an indicator of accuracy of the placement of the cannulae. Rats were allowed to recover from surgery for 7-10 days before the experiment was initiated. The stylets were removed from the cannulae 30 mins before the time of injection. Injectors were constructed to fit the implanted cannulae. PE-
50 tubing was attached to the injector on one end and a Hamilton syringe on the other. Vectors were injected in a 5 µl volume to unanesthetized rats over a period of 30 seconds using the Hamilton syringe. The injector was retained in the cannula for an additional 30 seconds in order to prevent backflow.

**Blood Collection**

Blood samples from rats were collected from the jugular vein at different time points during the course of the long-term studies. Rats were anesthetized with Ketamine (100 mg/kg BW) and Xylazine (15 mg/kg BW). The jugular vein was exposed by an incision in the skin at the level of the jugular vein. A 20 gauge needle coated with heparin was inserted through muscle into the vein and 1.0 ml blood was slowly withdrawn into a heparinized syringe. The incision wound was closed with metal clips. The blood was centrifuged at 3000 rpm for 3 mins, plasma was removed and stored at -20°C until analysis of blood hormone levels. Blood samples from mice were collected by retro-orbital puncture and processed as for rats.

**Cerebro-spinal Fluid Collection**

Cerebro-spinal fluid (CSF) was collected as previously described (Stein et al., 1983) using a stereotaxic apparatus. Rats were maximally ventroflexed, a 22 gauge needle attached to PE 50 tubing mounted on the stereotaxic apparatus was then inserted horizontally into the cisterna magna at 6.4-6.6 mm below the occipital crest. Flow was initiated with gentle suction using a Hamilton syringe, following which CSF was collected by gravity drainage. CSF was centrifuged to remove blood and debris; only
clear CSF was used for leptin analysis. CSF was collected on ice and stored at -20°C until use.

**Food Intake Measurement**

Twenty four hour food consumption was monitored on a weekly basis for the duration of each experiment. Pre-weighed food pellets were placed in specially designed feeders that were placed inside the rat cages. Food was weighed at the end of the 24 hour period along with any spillage collected at the bottom of the feeders. Food intake was calculated to the nearest 0.1 g as the difference between initial and final food weight over a 24 hour period.

**Urine Collection**

Two 24 hour urine samples were collected on consecutive days after placing animals in metabolic cages. The animals were placed in the metabolic cages for three days prior to collection of samples. The average volume collected over a 24 hour period was 15-20 ml. 10 ml of 0.1N HCl was added to the urine sample post collection. The samples were stored at –20°C until assayed for norepinephrine.

**Organ Collection**

Rats were sacrificed by decapitation. Trunk blood was collected in 15 ml polypropylene collection tubes. The brains were rapidly removed from the skull and snap frozen in powdered dry ice. In experiments where hypothalami were used for analysis, the brains were removed and the hypothalami were carefully dissected out and snap frozen in 2 ml sterile RNase free tubes. White adipose tissue was removed from the
epididymal area and snap frozen directly in 2 ml RNase free tubes. Interscapular BAT was dissected with surgical scissors. The BAT was deposited into petri dishes containing cold normal saline. While in cold saline the WAT was trimmed off as well as any muscle tissue. This was done as quickly as possible in order to maintain the integrity of the RNA in BAT. Once cleaned the BAT was snap frozen and stored in 2 ml tubes. All collected tissues were stored at –80°C until used for analyses.

**Carcass Fat and Protein Estimation**

Carcass water, fat and fat-free dry mass were determined gravimetrically (Fong, 1989). Carcasses were weighed immediately after killing the rats, then frozen in liquid nitrogen and pulverized with solid carbon dioxide in a commercial blender. Pulverized carcasses were dried for 2-4 days to a constant mass at 80°C. Lipid content was determined by sequential chloroform-methanol (1:1), ethanol-acetone (1:1), and petroleum ether extractions. Carcass protein content was measured from dried carcass aliquots after NaOH extraction with a routine Bradford protein assay.

**Oxygen Consumption**

Oxygen consumption was assessed as previously reported (Scarpace et al., 1992). Briefly, oxygen consumption was measured in three rats simultaneously with an Oxyscan analyzer (OXS-4; Omnitech Electronics, Columbus, OH). Flow rates were 21/min with a 30 second sampling time at 5 min intervals. The temperature was maintained in the thermoneutral zone at 26°C. Results were normalized to BW and expressed as ml.min⁻¹.Kg⁻⁰.⁶⁷.
RNA Isolation

Total RNA was isolated from tissue using an RNA isolation kit (STAT-60, Teltest Inc, Friendswood, TX). This method is a modification of the Chomscynski and Sacchi RNA isolation procedure. Briefly, 30-100 mg tissue was homogenized using a Polytron homogenizer in STAT-60 solution. The samples were extracted with chloroform and precipitated in isopropanol. The precipitate was washed in ethanol, and reconstituted in DEPC treated water. The samples were read in a spectrophotometer at an absorbency of 260 as well as 280 nm. Ratios of the two reading equal to 1.8 and not greater than 2.0 was an indicator of good RNA yield. Integrity of the RNA samples was verified by running aliquots of the sample on 1% agarose gels. All RNA samples were stored at -80°C until use.

Leptin mRNA Expression using RT-PCR

Leptin mRNA expression was analyzed using reverse transcriptase-PCR (RT-PCR). Briefly, total RNA was extracted from hypothalami using the RNA STAT 60 RNA isolation kit (Tel test Inc, Friendswood, TX). First-strand cDNA was synthesized using 1 ug total RNA with a RNA PCR kit. All reagents were purchased from PE Biosystems, Foster City, CA. Primers were designed to the rat leptin gene to encompass a 308 bp region of the coding sequence. (Gen bank Accession code D49653), Sense: 3’ CCC ATT CTG AGT TTG TCC, Antisense: 3’ GCA TTC AGG GCT AAG GTC. Primers were designed for cyclophilin (internal control) to generate a 470 bp product (Gene bank accession code M19533). Sense: 3’ GAC AAA GTT CCA AAG ACA GCA GAA A, Antisense: 3’ CTG AGC TAC AGA AGG AAT GGT TTG A. The PCR products generated by these primers were sequenced and independently verified and
found to match rat leptin and rat cyclophilin completely. Linearity of the PCR was tested by amplification for 20-45 cycles for leptin and cyclophilin. The linear range was found to be between 25 and 40 cycles.

Five microliters of the first-strand cDNA was amplified for 30 cycles for leptin and 26 cycles for cyclophilin. Each gene was amplified in a separate PCR reaction from a single RT reaction by using the following parameters:

**Leptin:** Denaturation @ 95°C, 1 min, annealing @ 56°C, 1 min, extension @ 72°C, 1 min, 30 cycles, 10 min final extension 72°C.

**Cyclophilin:** Denaturation @ 94°C for 50 s, annealing @ 55°C for 45 sec, extension @ 72°C for 2 min, 26 cycles.

PCR products were analyzed using agarose gel electrophoresis. Twenty microlitres of the PCR products were separated on a 2% agarose gel stained with ethidium bromide and placed on an UV illuminator equipped with a camera connected to a gel documentation system (BIORAD). The gel image was analyzed using an image analysis program (Image Quant system BIORAD laboratories Inc). The relative expression of the mRNA levels were derived from a comparison of the intensity of the target and simultaneously run internal controls (cyclophilin). All PCR products were run on a single gel in order to control for inter gel variation.

**Dot blot analysis for UCP-1 and UCP-3**

Total cellular RNA was extracted as described above. The integrity of the isolated RNA was verified using 1% agarose gels stained with ethidium bromide. The RNA was quantified by spectrophotometric absorption at 260 nm as well as 280 nm using multiple dilutions of each sample.
The full-length cDNA clone for uncoupling protein-1 (UCP1) was kindly provided to Dr Phillip Scarpace by Dr. Leslie Kozak, Jackson Laboratory, Bar Harbor, ME and verified by Northern analysis, as previously described (Scarpace et al., 1997). Full length UCP-3 cDNA was kindly supplied by Dr Olivier Boss and used as previously described (Boss et al. 1997). All probes were random prime labeled using Prime-A-Gene kit (Promega, Cat # U 1000) according to manufacturer's instructions. The labeled probes were purified by filtering through a Nick Column (Pharmacia).

For dot-blot analysis, multiple concentrations of RNA were immobilized on nylon membranes (Gene Screen Plus, Dupont, NEN) using a dot-blot apparatus (Bio-Rad, Richmond, CA). The membranes were pre-wet in 20X SSC for 10 mins before the diluted samples were applied. After applying the samples, the membranes were baked at 80°C for 2 hours. The baked membranes were warmed in 40°C water for 2 mins, and then pre-hybridized for 30-60 mins at 65°C while rotating in Hybaid Quikhyb solution. The labeled probe was added in a concentration of 1.5 X 10^6 cpm/ml of the hybridization solution. The membranes in hybridization solution were hybridized for 2 hours at 65°C. After hybridization, the membranes were washed in 2X SSC/0.1% SDS at 50°C for 15 mins with two changes of solution. The membranes were further washed in 0.1X SSC/0.1% SDS for 15 mins. The blots were removed from the hybridization bottles, wrapped in saran wrap and exposed to a phosphor imaging screen for 24-48 h. Care was taken to minimize folds in the saran wrap. The latent image on the phosphor imager screen was scanned using a Phosphor Imager (Molecular Dynamic, Sunnyvale, CA) and analyzed by Image Quant Software (Molecular Dynamics). Intensities were calculated per µg total RNA for each animal. Control as well as treated animal samples were applied.
on the same blot to minimize variability. All samples from one experiment were run on the same blot.

**In Situ Hybridization (ISH)**

**Construction of Riboprobes**

The POMC probe was constructed by cloning a 478 bp cDNA fragment (5' psn 220, 3’ psn 697, GenBank Accession No J00759) into pGEM-T vector (Promega Corp., Madison, WI). The NPY probe was constructed using a plasmid containing a 511 bp rat NPY fragment kindly provided by Dr S.L. Sabol (NIH, Bethesda, MD). The 396 bp complete AGRP cDNA fragment (GenBank U89484) used to construct the probe, a generous gift of Dr Roger Cone (Oregon Health Science University, Portland, OR), was inserted into pBSK+/- vector.

Antisense riboprobes were transcribed in the presence of $^{35}$S-UTP (Amersham Life Sciences Inc., Arlington Heights, IL) using T7 (AGRP, POMC) or T3 (NPY) RNA polymerase.

**Table 2-1. Riboprobes for in situ hybridization**

<table>
<thead>
<tr>
<th>Target</th>
<th>Probe Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGRP</td>
<td>396 bp complete mouse AGRP cDNA</td>
</tr>
<tr>
<td>NPY</td>
<td>511 bp spanning 1-511 of rat preproNPY mRNA</td>
</tr>
<tr>
<td>POMC</td>
<td>478 bp spanning psn150-608 of rat POMC mRNA</td>
</tr>
</tbody>
</table>
Tissue Sectioning

The brain tissue used for ISH was snap frozen in dry ice and stored at –80°C until used. The brains were coronally sectioned at –20°C in a cryostat into 16 µm sections. The sections covered areas of the brain starting at the level of the anterior commissure and ending past the ventral pre-mammillary nucleus of the hypothalamus, thus effectively covering the entire hypothalamus. The sections were thaw-mounted on microscope slides coated with Poly-L-Lysine. The sections were collected in four series; slides were stored at –80°C until use.

Tissue Processing

On the day of the experiment, one series of sections was removed from the freezer and allowed to warm up to room temperature (RT). They were post-fixed in 3 % paraformaldehyde for 10 min at RT followed by two washes in phosphate buffered saline (PBS, pH 7.4) for 5 min each. Next, the slides were incubated in 0.1M triethanolamine (pH 8.0) for 10 min at RT, dehydrated through 70%, 80%, 95% and 100 % alcohol for 2 min each and air-dried. 100µl hybridization buffer containing $^{35}$S labeled antisense probe containing 1 X 10$^6$ cpm was applied to each slide. The sections were covered with parafilm and incubated at 50°C overnight in a humidified oven.

On day two of the procedure, the parafilm was carefully removed from the sections. The slides were washed in 2 X SSC for 5 min at RT with two changes of solution, 0.2 X SSC for 30 min at 55°C, 30 min in 0.1X SSC at 60°C and 2X SSC at 37°C for 5 min. Next the sections were incubated in 20 µg/ml RNase solution in 2 X SSC for 30 min at 37°C while shaking. After another wash in 0.1 X SSC at 60°C the slides were
air dried and exposed to Kodak Biomax MR autoradiography film for 3-5 days. Slides were dipped in Kodak NTB2 emulsion, dried and stored at 4°C for 2-4 weeks. The slides were counterstained with 0.1 % cresyl violet after developing. Slides from control and treated animals were processed together to eliminate any variability that might be introduced by processing.

**Analysis of ISH data**

For semi-quantitative analysis, the relative optical density (ROD) calculated as total target area multiplied by the integrated optical density for AGRP, NPY, and POMC were estimated from autoradiograms with the MCID image analysis system (Imaging Research, St. Catherine, Ontario, Canada). Twelve sections from each brain were matched anatomically and analyzed. The background optical density in an area adjacent to each target was subtracted from the target. The ROD of 12 sections in the same brain were added and expressed relative to the average ROD from the control group.

**Immunohistochemistry for Green Flourescent Protein (GFP)**

Immunohistochemistry was performed as described earlier (Peel et al., 1997, Klein et al., 1998). Briefly, animals were anesthetized with 100mg/kg sodium pentobarbital ip and perfused with 300 ml 0.9% saline followed by 300-600 ml cold 4 % paraformaldehyde in 1 X PBS. Brains were removed and kept in 4% paraformaldehyde/1XPBS overnight, then in 20 % sucrose solution, followed by 30 % sucrose until the tissue submerged. The brains were sectioned coronally in a cryostat into 40 μm slices. Immunohistochemistry was performed on floating sections. The sections were blocked in 0.01M PBS/ 1% bovine serum albumin /0.3% Triton-X 100 for one hour at room temperature, followed by incubation in a GFP polyclonal antibody
(Clontech Laboratories, Palo Alto, CA, 1:2000 dilution) at 4°C for 24-48 hrs. After rinsing in high salt buffer sections were incubated for 24 hrs at RT in secondary antibody conjugated to biotin followed by incubation in Extravidin (Sigma) for 1 hour. Sections were then stained in diaminobenzidine (Sigma), mounted on slides, dried overnight, dehydrated through alcohol and coverslipped.

**Radioimmunoassays**

**Leptin**

Serum and plasma leptin was assayed in glass tubes using a rat leptin RIA kit (Linco Research, Inc., St Charles, MO) according to manufacturer's instructions. The sensitivity of this assay is 0.5 ng/ml and the range of detection is 0.5 ng/ml to 50 ng/ml. All samples were assayed in duplicate to minimize variability. Leptin in the CSF was measured in polystyrene tubes using a more sensitive rat/mouse leptin RIA kit (ALPCO, Windham, NH). The sensitivity of this assay is 6 pg/ml and the range of detection is 12.5-800 pg/ml.

**Insulin**

Insulin was measured in polystyrene tubes with a rat insulin RIA kit (Linco Research, Inc., St. Charles, MO) according to manufacturer's instructions. The sensitivity of the assay is 0.1 ng/ml and the range of detection is 0.1 ng/ml to 10 ng/ml. All samples from one experiment were analyzed in a single assay.
Norepinephrine

The NE RIA was performed in polystyrene tubes performed using a kit from ALPCO (Windham, NH) according to manufacturer's instructions. In this assay NE is first extracted from the urine sample using a cis-diol specific boronate affinity gel, simultaneously acylated to N-acylnorepinephrine and then converted enzymatically into N-acylnormetanephrine. The extracted sample is run in the RIA. The analytical sensitivity of this assay is 135 pg/ml and the range of detection is 0.15 to 0.5 ng/ml.

Thyroid hormones (T3 and T4)

The thyroid hormones T3 (tri iodo-thyronine) and T4 (tetra iodo-thyronine) were analyzed in serum and plasma samples using solid phase T3 and T4 RIA kits (ICN Pharmaceuticals, Inc. Costa Mesa, CA). The assays were conducted separately for the two hormones in tubes coated with either T3 (polyclonal) or T4 (monoclonal) antibody. The analytical sensitivity of the T3 RIA is 6.7 ng/dL, while that of the T4 RIA is 0.76 µg/dL.

Free Fatty Acid Analysis

Serum non-esterified fatty acid (FFA) levels were measured by an in vitro enzymatic colorimetric method for quantitation of non-esterified fatty acids (free fatty acids) using a NEFA-C kit (Wako Chemicals USA Inc. Richmond, VA) according to manufacturer's instructions. In this assay there is acylation of coenzyme A (CoA) by the fatty acids in the presence of additional acyl-CoA synthetase (ACS). The acyl-Co-A thus made is oxidized by the addition of acyl-CoA oxidase with generation of hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase leads to the oxidative
condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline with 4-aminoantipyrine to form a purple adduct that is measured colorimetrically at 550 nm.

**Glucose Measurement**

Serum/plasma glucose was measured using Sigma Diagnostics Glucose (Trinder) reagent. This is a quantitative, enzymatic determination of glucose. It is a colorimetric reaction, read at 505 nm.

**Recombinant AAV Production**

To produce a recombinant AAV, DNA from two plasmids are transfected into a host cell in culture. One of these plasmids consists of an AAV vector containing the AAV terminal repeat sequences (ITR’s) and the promoter along with the gene of choice. The other plasmid is a helper plasmid that contains both the rep and cap genes needed for packaging the DNA flanked by the ITR’s (Hauswirth et al., 2000) as well as Ad helper genes required for AAV infection. A plasmid construct, pTR-leptin, containing 640 bp rat leptin cDNA (a kind gift from Dr Roger Unger, Southwestern Medical center, Dallas, TX) under the control of a hybrid chicken β actin (CBA) promoter with a cytomegalovirus (CMV) enhancer and AAV terminal repeats was generated. To produce rAAV-leptin, rAAV vector plasmid was co-transfected with pTR-leptin. The product was then co-transfected with the helper plasmid pDG carrying the AAV rep and cap genes, as well as Ad helper genes, required for rAAV replication/packaging. Plasmid DNA used in the transfection was purified by conventional alkaline lysis/CsCl gradient protocol. Before transfection the presence of the two flanking ITR’s in the plasmid DNA isolated
from *Escherichia coli* was confirmed with digestion of 0.5 µg plasmid DNA with *Sma I*. The transfection was carried out by incubation of HEK-293 cells (low passage number, passage 29-40) at 37°C for 48 hrs in the presence of calcium phosphate precipitate of rAAV and pDG plasmids. Forty-eight hr post-transfection, cells were harvested by centrifugation for 10 min at 1,140 g. Cells were then lysed in 15 ml of 0.15 M NaCl - 50 mM Tris HCl, pH 8.5 by 3 freeze/thaw cycles in dry ice-ethanol/37°C baths. Benzonase (Nycomed Pharma A/S, pure grade) was added to the mixture (50 µg/ml final concentration) and the lysate was incubated for 30 min at 37°C. The crude lysate was clarified by centrifugation at 3,700 g for 20 min and the virus-containing supernatant was further purified by iodixanol (Nycomed) density gradient centrifugation in a Type 70 Ti rotor at 350,000 g for 1 hr at 18°C. Each gradient consisted of (from the bottom up): 60%, 40%, 25%, and 15% iodixanol. The last density step contained 1 M NaCl. After centrifugation, the 40% iodixanol fraction containing virus was applied to heparin agarose Type I column (Sigma, St Louis, MO) equilibrated with PBS-MK (1xPBS-1 mM MgCl₂, 2.5 mM KCl). The rAAV was eluted with the same buffer containing 1 M NaCl. The virus was concentrated by centrifugation through the BIOMAX 100 K filter (Millipore) according to the manufacturer's instructions. The control vector, rAAV-UF5 was produced using a similar protocol after generation of a construct plasmid containing green flourescent protein (GFP) cDNA. The virus thus produced was titred using two methods, the Infectious Center Assay and Quantitative PCR (Hauswirth et al., 2000). The ratio of physical-to-infectious particles was less than 100. rAAV vectors, purified using iodixanol gradient/heparin-affinity chromatography, were 99% pure as judged by the PAAG/silver-stained gel electrophoresis (not shown). Since mini-Ad helper plasmid
pDG was used to produce the vectors, there was no Ad or wtAAV contamination in the rAAV stocks used in these studies.

**Statistical Analysis**

Weekly BW and 24 hr food intake in rats were compared between groups using a two way ANOVA with treatment and time as variables. The p value was set at p<0.05 to attain significance. In experiments with three experimental groups, BW and FI were compared using one way ANOVA followed by post hoc analysis using Neuman Keul's multiple comparison test.

Circulating leptin, insulin, glucose, T3, T4, and NEFA and urinary NE levels between two treatment groups were compared using Students ‘t’ test with p<0.05 considered significant. Circulating serum/plasma leptin, insulin and glucose levels were compared across time were done so using a one way ANOVA followed by Neuman Keul's multiple comparison test post-hoc.

Body fat, body protein and total oxygen consumption were compared using Students 't' test. UCP-1, UCP-3, POMC, AGRP, NPY and leptin mRNA levels were compared using Students 't' test. p<0.05 was considered significant in all analyses.

In ob/ob mice average FI data and serum/plasma leptin levels for each group were compared by ANOVA and Tukey’s post hoc test. For BW data, least squares means for each group were calculated for the change from initial values. These means were compared by 2-way ANOVA with time and treatment as variables followed post hoc by Tukey’s multiple comparison test for comparisons within a group and among groups at each time point. Level of significance was set at p<0.05.
All analyses were performed using Graph Pad Prism Software Version 3.00 for windows (Graph Pad Software, San Diego, CA, USA, www.graphpad.com).
CHAPTER 3
LEPTIN GENE THERAPY REVERSES OBESITY IN OB/OB MICE

Introduction

Leptin, a 16-kD secreted protein, is secreted predominantly by the adipose tissue (Zhang et al., 1994). Serum levels of leptin correlate directly with body-fat mass (Considine et al., 1996). Leptin exerts its effects on appetite and thermogenesis via receptors located in the central nervous system (Spiegelman and Flier, 1996) and is thought to act mainly on the hypothalamus, although leptin receptors are also present in peripheral tissues. There are several genetic models of obesity in rodents, one of which is the \textit{ob/ob} mouse which bears a mutation in the gene encoding leptin, resulting in lack of leptin and increased food consumption. This is the underlying cause of the increased mass of adipose tissue and development of a syndrome that resembles morbid obesity and non insulin dependent diabetes mellitus in humans (Halaas et al., 1995). Additional abnormalities associated with the \textit{ob} mutation are hypothermia, lethargy, hyperglycemia, glucose intolerance and hyperinsulinemia. These abnormalities are alleviated by administration of recombinant leptin which reduces food intake, increases energy expenditure and leads to a loss of BW and fat mass (Pelleymounter et al., 1995; Halaas et al., 1995; Maffei et al., 1995). However, for leptin to be effective there is a need for continuous administration of the recombinant leptin protein; withdrawal results in reversal to the obese phenotype (Giese et al., 1996). The delivery of genes via gene
therapy, especially in diseases that are a result of missing or mutated genes, is especially useful since it offers the potential for sustained delivery of the gene in question. Adenoassociated virus is a non-immunogenic, non-pathogenic virus (Berns and Bohenzky 1987). Long term expression of AAV vectors has been demonstrated in the lung, liver, muscle, heart as well as the brain (Kaplitt et al., 1994; Flotte et al., 1996). In order to achieve long-term sustained delivery of leptin in ob/ob mice we constructed a recombinant AAV vector encoding leptin cDNA. There were three objectives of this study (1) to enhance leptin levels in the blood for extended periods of time (2) to test the efficacy of intravenously delivered rAAV-leptin on reducing BW and food intake (FI) and (3) to determine whether enhanced leptin levels sustained over an extended period would result in leptin resistance in obese leptin deficient ob/ob mice.

Materials and Methods

Animals

Leptin mutant ob/ob mice were purchased from Jackson Laboratories (Bar Harbor, Maine). The animals were housed 4 per cage, in a temperature (23°C) and light controlled (14 hr light, 10 hr dark), specific pathogen free environment; standard mouse chow (Teklad, Madison, WI) and water were available ad libitum. The animal protocols were approved by the Institutional Animal Care and Use Committee.

Recombinant AAV Production

To produce a recombinant AAV, DNA from two plasmids are transfected into a host cell in culture. One of these plasmids consists of an AAV vector containing the AAV terminal repeat sequences (ITR’s) and the promoter along with the gene of choice. The other is a helper plasmid that contains both the rep and cap genes needed for
packaging the DNA is flanked by the ITR’s (Hauswirth et al., 2000) as well as Ad helper genes required for AAV infection. A plasmid construct, pTR-leptin, containing 640 bp rat leptin cDNA (a kind gift from Dr Roger Unger, Southwestern Medical center, Dallas, TX) under the control of a hybrid chicken β actin (CBA) promoter with a cytomegalovirus (CMV) enhancer and AAV terminal repeats was generated (Fig. 3-1). To produce rAAV-leptin, the rAAV vector plasmid was co-transfected with pTR-leptin. The product was then co-transfected with the helper plasmid pDG carrying the AAV rep and cap genes, as well as Ad helper genes required for rAAV replication/packaging. Plasmid DNA used in the transfection was purified by conventional alkaline lysis/CsCl gradient protocol. Before transfection the presence of the two flanking ITR’s in the plasmid DNA isolated from Escherichia coli was confirmed with digestion of 0.5 µg plasmid DNA with Sma I. The transfection was carried out by incubation of HEK293 cells (low passage number, passage 29-40) at 37°C for 48 hrs in the presence of calcium phosphate precipitate of rAAV and pDG plasmids. Forty-eight hrs post-transfection, cells were harvested by centrifugation for 10 min at 1,140 g. Cells were then lysed in 15 ml of 0.15 M NaCl - 50 mM Tris HCl pH 8.5 by 3 freeze/thaw cycles in dry ice-ethanol/37°C baths. Benzonase (Nycomed Pharma A/S, pure grade) was added to the mixture (50 µg/ml final concentration) and the lysate was incubated for 30 min at 37°C. The crude lysate was clarified by centrifugation at 3,700 g for 20 min and the virus-containing supernatant was further purified by iodixanol (Nycomed) density gradient centrifugation in a Type 70 Ti rotor at 350,000 g for 1 hr at 18°C. Each gradient consisted of (from the bottom up): 60%, 40%, 25%, and 15% iodixanol. The last density step contained 1 M NaCl. After centrifugation, the 40% iodixanol fraction containing virus
was applied to heparin agarose Type I column (Sigma, St Louis, MO) equilibrated with PBS-MK (1xPBS-1 mM MgCl₂, 2.5 mM KCl). The rAAV was eluted with the same buffer containing 1 M NaCl. The virus was concentrated by centrifugation through the BIOMAX 100 K filter (Millipore) according to the manufacturer instructions. The control vector, rAAV-UF5 was produced using a similar protocol after generation of a construct plasmid containing green fluorescent protein (GFP) cDNA (Fig. 3-1). The virus thus produced was titred using two methods, the Infectious Center Assay and Quantitative PCR (Hauswirth et al., 2000).

The titre of rAAV-leptin used in the study was 3×10¹³ physical particles/ml. The ratio of physical-to-infectious particles was less than 100. rAAV vectors, purified using iodixanol gradient/heparin-affinity chromatography, were 99% pure as judged by the PAAG/silver-stained gel electrophoresis (not shown). Since mini-Ad helper plasmid pDG was used to produce the vectors there was no Ad or wtAAV contamination in the rAAV stocks used in this study.

**Study Design**

Male ob/ob mice were randomly divided into 4 groups (n=7-8 per group). Mice were injected with one of 3 different doses (6×10⁹, 6×10¹⁰, 6×10¹¹ particles) of rAAV-leptin or a single dose of rAAV-UF5 (control) in a 50 µl volume via the tail vein. BW was monitored weekly for the duration of the experiment (75 days). Average food consumed per week by each mouse was calculated by placing pre-weighed food pellets in the cages and re-weighing after 7 days. Food intake was first recorded at day 18 post-injection when a difference in BW was apparent and was monitored weekly until Day 62 of the experiment. Blood samples were collected by retro-orbital puncture on Days 54
and 75 post-injection for analysis of serum leptin levels with a rat leptin radioimmunoassay kit (Linco Research, Inc, St Louis, MO). On Day 75 post-injection, animals were sacrificed by cervical dislocation.

Data Analyses

Average FI data and serum leptin levels for each group were compared by ANOVA and Tukey’s post hoc test. For BW data, least squares means for each group were calculated for the change from initial values. These means were compared by 2-way ANOVA with time and treatment as variables followed post hoc by Tukey’s multiple comparison test for comparisons within a group and among groups at each time point. Level of significance was set at p<0.05.

Results

There was a clear dose dependent effect of rAAV-leptin on BW in ob/ob mice. Analyses of the BW data showed significant effects of treatment and time (p<0.001). Administration of 6x10^{11} particles/ml rAAV-leptin elicited a steady loss in BW beginning on Day 19 until Day 75 post-injection (p<0.001 vs. initial BW, Fig.3-2). On Day 75 this represented a loss of 32% from the pre-injection weight (Fig. 3-2) and was 44% lower than the BW of obese control mice at this time (p<0.001). This loss in BW was accompanied by a 24% decrease in FI throughout the experiment (p<0.01 vs. rAAV-UF5 treated controls, Fig 3-3). In contrast, injection of a one order lower magnitude titre (6x10^{10}) of rAAV-leptin prevented BW gain, resulting in maintenance of BW in the pre-injection range for the duration of the experiment (Fig.3-2). This maintenance effect was apparent from Day 26 post-injection when the BW of controls and 6x10^9 particles rAAV-
leptin injected animals displayed a progressive increase in BW. Interestingly, BW maintenance in the 6x10^{10} particles-treated mice was not accompanied by any significant change in FI. The BW and FI of mice injected with the lowest rAAV-leptin dose (6x10^9 particles) were indistinguishable from those of controls.

Plasma leptin levels assayed on Days 54 and 75 post-injection are shown in Fig.3-4. Leptin levels in control, 6x10^9 and 6x10^{10} particles rAAV-leptin injected mice were at the lower end of sensitivity of the rat leptin RIA. However, mice injected with 6x10^{11} rAAV-leptin particles, displayed detectable circulating leptin concentrations in the range of 2-3ng/ml on both Days 54 and 75 post-injection.
Fig. 3-1. Schematic diagrams of the rAAV vector constructs used in the study. TR: 145-bp AAV terminal repeat sequence; CMV-β–act: cytomegalovirus (CMV) enhancer linked to chicken β-actin promoter; pA: polyadenylation site, GFP: green fluorescent protein, leptin: rat leptin cDNA, IVS: intervening sequence.

Fig. 3-2. Dose dependent change in body weight in ob/ob mice post iv rAAV-leptin injection. Body weight was significantly (p<0.001) reduced by a single 50 µl intravenous injection of rAAV–leptin in obese male ob/ob mice in a dose dependent manner, the highest dose was the most effective.
Fig. 3-3. Effect of different doses of rAAV-leptin on food intake in ob/ob mice. Food intake in ob/ob mice was decreased by the highest dose (p <0.01 vs. control). n = 6-8 per group.

Fig. 3-4. Serum leptin levels post iv injection of rAAV-leptin. Serum leptin levels in ob/ob mice were elevated significantly after intravenous injection of 6 X 10^{11} particles of rAAV-leptin (p<0.05). n = 4-6 per group.
Fig. 3-5. Representative *ob/ob* mice. rAAV-UF5 (left) vs. rAAV-Leptin treated (right) 6 weeks post injection.
Discussion

The present results demonstrate the successful transfer of leptin using a recombinant AAV vector in a dose-dependent manner. These results confirm earlier reports of the use of AAV for gene transfer with long lasting effects (Samulski et al., 1997; Mandel et al., 1998; Murphy et al., 1997). Gene transfer with intravenous injection of AAV has been demonstrated in liver, lung and muscle (Samulski et al., 1997). In the studies presented in this chapter we did not examine the tissues transduced by AAV. A single injection of rAAV-leptin to ob/ob mice via the tail vein resulted in an elevation in blood leptin levels for over 10 weeks. In leptin deficient ob/ob mice, the high dose rAAV-leptin produced circulating leptin levels of 2-3 ng/ml. Mice injected with the lower dose had almost undetectable serum leptin levels. Basal leptin levels detected also in the leptin deficient control vector injected ob/ob mice. These baseline values probably represent non-specific interference in the plasma of ob/ob mice and has been reported by other researchers previously (Murphy et al., 1997).

The effects of rAAV-leptin on BW were recorded for the entire duration of the experiment. There was a dose dependent change in BW in the leptin deficient ob/ob mice with rAAV-leptin injection. The highest dose of rAAV-leptin (6x10^{11} particles) caused a significant decrease in BW vs. initial BW starting at 19 days post injection and was maintained for the duration of the study. Mice given the middle dose (6x10^{10} particles) maintained their initial BW and failed to display the BW gain characteristic of ob/ob mice, suggesting that the small amounts of leptin measured in the circulation was adequate to affect BW gain. There was no difference in the BW of the lowest dose of rAAV-leptin (6x10^{9} particles) injected mice as compared to that of controls.

Administration of the leptin protein for short periods leads to decreased BW through
reduction in caloric consumption accompanied by an increase in energy expenditure in
ob/ob mice (Zhang et al., 1994; Halaas et al., 1995; 1997; Pellemounter et al., 1995;
Harris et al., 1998). Intriguingly, only the highest dose of the vector elicited a change in
food intake. While the weight loss in the high dose group can be attributed to decreased
energy intake, the observation that the middle dose blunted BW gain is the most
interesting. These data confirm the super-sensitivity of ob/ob mice in their response to
leptin, as previously reported (Ioffe et al., 1998). Although this study did not examine the
parameters to measure energy expenditure, it is well documented in the literature that
leptin enhances energy expenditure in ob/ob mice (Halaas et al., 1995, 1997;
Pellemounter et al., 1995). This highlights a dichotomy in the action of leptin based on
the dose administered. Whereas, the high dose affects energy expenditure in conjunction
with energy intake thus affecting both aspects of the energy homeostasis equation, the
lower effective dose retards the rate of BW gain by affecting energy expenditure only.

Interestingly, reinstatement of leptin via rAAV-leptin injection in ob/ob mice did
not lead to development of leptin resistance during the 75 days of observation.
Resistance to leptin action is usually associated with the presence of higher than normal
peripheral levels of leptin. In this experiment, serum leptin levels even in the highest
dose group did not exceed those of normal wild type mice and do not constitute
hyperleptinemia as seen in obese rodents. This could be one possible basis for the lack of
resistance to leptin in these rAAV-leptin treated animals. Also, since ob/ob mice are
supersensitive to leptin administration (Pellemounter et al., 1995; Ioffe et al., 1998)
much higher peripheral leptin levels would likely be necessary to induce resistance.
In summary, rAAV is able to effectively introduce leptin into leptin deficient ob/ob mice. This reinstatement of leptin has a dose dependent effect on decreasing body mass along with reduction in energy intake and apparent enhancement of energy expenditure without induction of leptin resistance. This initial demonstration of the successful use of rAAV to deliver leptin was extended in later chapters to study the central effects of leptin in lean rodents.
CHAPTER 4
REGULATION OF BODY WEIGHT WITH LEPTIN GENE THERAPY IN LEAN SPRAGUE-DAWLEY RATS

Introduction

Since its discovery in 1994 by Zhang et al., leptin's integral role in the maintenance of energy homeostasis has been firmly established. Leptin is a 16 kD protein secreted primarily by adipocytes (Zhang et al., 1994). Genetically obese ob/ob mice lack functional leptin and are hyperphagic, hyperinsulinemic, and manifest a syndrome resembling non insulin dependent diabetes mellitus (NIDDM, Zhang et al., 1994). The obese phenotype in ob/ob mice can be reversed with recombinant leptin treatment, along with normalization of the hyperinsulinemia, hyperglycemia and low basal metabolic rate characteristic of this model (Pellemounter et al., 1995; Halaas et al., 1995; Campfield et al., 1995). In Chapter 3 , I have reported correction of obesity in these mice using viral vectors to deliver the leptin gene. Although the ob/ob mice are grossly obese due to the lack of leptin, this is not the case in obese humans. Morbid obesity associated with leptin deficiency has been identified in very few humans (Montague et al., 1997). Most human obesity is not genetic in origin; it is in fact associated with markedly elevated serum leptin levels that correlate positively with body mass (Considine et al., 1996). These observations have led to the speculation that obesity may be a consequence of resistance to the weight reducing actions of leptin. However, the site and mechanism of leptin resistance is not known. A likely possibility to account for the lack of leptin action despite high circulating levels is inadequate availability of leptin at
its site of action. This concept is supported by the demonstration of decreased cerebrospinal fluid to blood leptin level ratio in obese humans which implies decreased availability of leptin in the CNS of these patients (Caro et al., 1996). Diet induced obesity in rodents is also linked to a decrease in the amount of leptin transported into the CSF (Bruguera et al., 2000).

The localization of leptin receptors suggests that the primary site of action of leptin is the hypothalamus. Leptin receptors are localized in critical nuclei of the hypothalamus such as the ARC, PVN, VMH and the DMN that form the neural substrate for the appetite regulating network (Elmquist et al., 1997). Recombinant leptin administered intracerebroventricularly (icv) in rodent models of obesity is effective in reducing food intake and enhancing metabolism (Halaas et al., 1997).

Evidence from rodent studies that diet induced obesity associated with peripheral hyperleptinemia can be attenuated with central but not peripheral leptin treatment (Halaas et al., 1997) and centrally administered leptin has a greater potency than either intravenous infusion or intramuscular injection confirm that leptin’s effects on energy balance occur at the level of the CNS (Halaas et al., 1997; Campfield et al., 1995). These results raise the question whether continuous elevation of leptin at its site of action would also result in leptin resistance as seen with peripheral hyperleptinemia, or whether leptin resistance is a consequence of insufficient availability at the site of action, as alluded to above. In order to address this question we generated an adenoassociated viral vector (rAAV) encoding leptin. AAV is ideally suited for use in in vivo experiments in that it is non-immunogenic and non-pathogenic (Berns and Bohenzky 1987) and can transduce non-dividing neurons in a variety of brain regions (Kaplitt et al., 1994). When delivered
into the CNS AAV transfects neurons preferentially and has been shown to have stable, long-term expression lasting for up to 18 months post injection (Mandel et al., 1998). In order to test the hypothesis that resistance to endogenously high leptin levels is not due to resistance to leptin action within the CNS but rather to insufficiency at its target sites, we examined the long-term effects of leptin delivery in the central nervous system at its site of action in wild-type lean male and female rats. If there is central resistance to hyperleptinemia then chronic enhancement of leptin levels within the CNS would be expected to result in obesity. The data from this experiment support the hypothesis that obesity may be due to inadequate availability of leptin at its target sites.

**Methods**

**Study Design**

Male and female lean Sprague-Dawley rats received permanently cannula in the third ventricle and were randomly divided into two groups each (n= 6-8 per group) so that the average BW of the groups was identical. One group each was injected icv with a single injection of $6 \times 10^{10}$ particles rAAV-leptin in a 5 µl volume. Control groups received 5 µl or rAAV-UF5 icv. BW and 24 hour food intake were recorded weekly for a period of 12 weeks (n= 6-8 per group). At 12 weeks post-injection the animals were sacrificed by decapitation, blood was collected for subsequent analysis of leptin by RIA.

A second experiment was performed to characterize the expression of rAAV post icv injection. Female Sprague-Dawley rats (9-12 per group) were implanted with permanent third ventricle cannulae and injected with either rAAV-UF5 or rAAV-leptin, as above. Animals were sacrificed 6 weeks post injection. Three animals per group were
used for body fat and protein determination. Three of the rAAV-UF5 treated animals were perfused and their brains were collected and sectioned for immunohistochemical localization of GFP. Hypothalami were rapidly excised from the brains of six rAAV-leptin and six rAAV-UF5 injected rats for RT-PCR determination of leptin mRNA expression.

**Carcass Fat and Protein Estimation**

Carcass water, fat and fat-free dry mass were determined gravimetrically (Fong, 1989). Carcasses were weighed immediately after killing the rats, then frozen in liquid nitrogen and pulverized with solid carbon dioxide in a commercial blender. Pulverized carcasses were dried for 2-4 days to a constant mass at 80°C. Lipid content was determined by sequential chloroform-methanol (1:1), ethanol-acetone (1:1), and petroleum ether extractions. Carcass protein content was measured from dried carcass aliquots after NaOH extraction with a routine Bradford protein assay.

**Immunohistochemistry for Green Flourescent Protein (GFP)**

Immunohistochemistry was performed as described earlier (Peel et al., 1997, Klein et al., 1998). Briefly, animals were anesthetized with 100mg/kg sodium pentobarbital ip and perfused with 300 ml 0.9% saline followed by 300-600 ml cold 4% paraformaldehyde in 1 X PBS. Brains were removed and kept in 4% paraformaldehyde/1XPBS overnight, then in 20% sucrose solution, followed by 30% sucrose until the tissue submerged. The brains were sectioned coronally in a cryostat into 40 μm slices. Immunohistochemistry was performed on floating sections. The sections were blocked in 0.01M PBS/ 1% bovine serum albumin /0.3% Triton-X 100 for
one hour at room temperature, followed by incubation in a GFP polyclonal antibody (Clontech Laboratories, Palo Alto, CA, 1:2000 dilution) at 4°C for 24-48 hrs. After rinsing in high salt buffer sections were incubated for 24 hrs at RT in secondary antibody conjugated to biotin followed by incubation in Extravidin (Sigma) for 1 hour. Sections were then stained in diaminobenzidine (Sigma), mounted on slides, dried overnight, dehydrated through alcohol and coverslipped.

**Leptin mRNA Expression using RT-PCR**

Leptin mRNA expression was analyzed using reverse transcriptase-PCR (RT-PCR). Briefly, total RNA was extracted from hypothalami using the RNA STAT 60 RNA isolation kit (Tel test Inc, Friendswood, TX). First-strand cDNA was synthesized using 1 ug total RNA with a RNA PCR kit. All reagents were purchased from PE Biosystems, Foster City, CA. Primers were designed to the rat leptin gene to encompass a 308 bp region of the coding sequence. (Gen bank Accession code D49653), Sense: 3’ CCC ATT CTG AGT TTG TCC, Antisense: 3’ GCA TTC AGG GCT AAG GTC. Primers were designed for cyclophilin (internal control) to generate a 470 bp product (Gene bank accession code M19533). Sense: 3’ GAC AAA GTT CCA AAG ACA GCA GAA A, Antisense: 3’ CTG AGC TAC AGA AGG AAT GGT TTG A. The PCR products generated by these primers were sequenced and independently verified and found to match rat leptin and rat cyclophilin completely. Linearity of the PCR was tested by amplification for 20-45 cycles for leptin and cyclophilin. The linear range was found to be between 25 and 40 cycles.
Five microliters of the first-strand cDNA was amplified for 30 cycles for leptin and 26 cycles for cyclophilin. Each gene was amplified in a separate PCR reaction from a single RT reaction by using the following parameters:

**Leptin**: Denaturation @ 95 °C, 1 min, annealing @ 56 °C, 1 min, extension @ 72 °C, 1 min, 30 cycles, 10 min final extension 72 °C.

**Cyclophilin**: Denaturation @ 94 °C for 50 s, annealing @ 55 °C for 45 sec, extension @ 72 °C for 2 min, 26 cycles.

PCR products were analyzed using agarose gel electrophoresis. Twenty microlitres of the PCR products were separated on a 2% agarose gel stained with ethidium bromide and placed on an UV illuminator equipped with a camera connected to a gel documentation system (BIORAD). The gel image was analyzed using an image analysis program (Image Quant system BIORAD laboratories Inc). The relative expression of the mRNA levels were derived from a comparison of the intensity of the target and simultaneously run internal controls (cyclophilin). All PCR products were run on a single gel in order to control for inter gel variation.

**Statistical Analysis**

Weekly BW and food intake data were compared between groups using a two way ANOVA with treatment and time as variables. Serum leptin levels, body fat content, body protein content and hypothalamic leptin mRNA were compared between treatment groups using Students ‘t’ test. The p value was set at p<0.05 to attain significance.
Results

Hypothalamic leptin mRNA RT-PCR

Leptin gene expression in the hypothalamus of rAAV-leptin injected rats was confirmed by relative RT-PCR. As shown in Fig 4-1, a faint band of leptin mRNA is seen in rAAV-UF5 rats. In the rats injected with rAAV-leptin this band was very prominent. Leptin mRNA levels expressed relative to cyclophilin mRNA were significantly higher in the hypothalami of rAAV-leptin treated rats ( p<0.05 Fig.4-1 ).

Immunohistochemical localization of GFP

Gene expression following viral vector therapy was verified by immunohistochemical localization of GFP in the brain of rAAV-UF5 injected control rats. No GFP positive cells were observed in the negative controls which were not incubated with the GFP antibody. At 6 weeks post-injection, GFP-positive cells were distributed in mid-line structures along the site of injection in the third cerebroventricle, and the lateral ventricles extending from the anterior commissure to the posterior hypothalamus (Fig. 4-2, 4-3). Almost all these cells displayed neuron-like morphology with GFP immunoreactivity also evident in dendritic and axonal fibers. Prominent clusters of immunopositive cells were localized in the bed nucleus of the anterior commissure, ventrally in the preoptic area and in the suprachiasmatic nucleus. Caudally, GFP-positive cells were seen in the anterior hypothalamic area, paraventricular nucleus, dorsomedial hypothalamus and arcuate nucleus (Fig 4-3). Occasionally, small groups of GFP-positive cells were seen in remote areas near the hippocampus.
Body Weight

In female rats BW was significantly decreased (p<0.05) following the icv rAAV-injection vs. the rAAV-UF5 treated control group (Fig 4-4). The first significant decrease in BW was observed at 2 weeks post injection and was maintained until 7 weeks with a return to initial BW from weeks 8 to 12 post-injection. These rats exhibited no gain in BW over the 12 week period. In contrast, the control rats exhibited an overall gain of 17% over their initial BW so that the rAAV-leptin treated females weighed 17% less than the controls at the end of the experiment.

The response of male rats to rAAV-leptin was different to that of females. The rAAV-leptin treated male rats continued to gain weight, albeit at a slower rate than the control animals (Fig 4-5, Controls 408.6 + 7.2 g vs. 363.7 + 2.3 rAAV-leptin at the end of 12 weeks). At the end of the experiment at 12 weeks post-injection the rAAV-UF5 treated control male rats had gained 30% BW compared with 17% in the rAAV-leptin treated group (p<0.05).

Food Intake

There was no difference in 24 hr food intake (FI) in either the female (overall average, 19.5 ± 1.0 g controls vs. 18.6 ± 1.2 g rAAV-leptin group, Fig 4-6) or male (Overall average, 24.6 ± 0.5 g controls vs. 24.4 ± 0.5 g rAAV-leptin, Fig. 4-7) rats administered rAAV-leptin vs. their respective control groups.

Serum Leptin Levels

As expected, blood leptin levels in female control rats were significantly higher than in male control rats (p<0.05, Fig 4-8 and 4-9). Circulating levels of leptin in both males and females were markedly suppressed when measured at 12 weeks following a
central rAAV-leptin injection. In rAAV-leptin treated female rats serum leptin levels were significantly lower than in rAAV-UF5 treated female rats (0.8 ± 0.2 ng/ml vs. 3.7± 0.6 ng/ml, p<0.05). Similarly, the rAAV-leptin treated male rats displayed significantly lower leptin levels than the rAAV-UF5 treated group (0.5± 0.1 vs. 1.98 ± 0.2 ng/ml).

**Carcass Fat and Protein Analysis**

Body composition analysis at 6 weeks post-injection indicated that weight reduction was due solely to a loss of fat depots. There was a significant depletion in fat in rAAV-leptin treated rats (p<0.05). Total body fat was 12.5% in controls vs. 5.4% in the rAAV-leptin treated group (Fig 4-10 A and Fig 4-11) there was with no change in lean mass with rAAV-leptin treatment. (Fig. 4-10, A and B.).
Fig 4-1. Effect of a single injection of rAAV-leptin on leptin mRNA expression in the hypothalamus of female Sprague-Dawley rats. There was a significant induction in hypothalamic leptin mRNA expression in rAAV-leptin treated rats vs. controls (p<0.05). n= 6 per group.
Fig. 4-2. Photomicrograph (4X) of representative hypothalamic sections showing GFP positive cells around the site of icv rAAV-UF5 injection. The panel on the left (A) represents a GFP antibody -ve control with no positively stained cells, the panel on the right (B) is positively stained for GFP, transduction of neurons is visible along the third ventricle. OC = optic chiasm 3V= third ventricle, AC = anterior commissure.
Fig. 4-3. Photomicrograph (20 X) of a representative hypothalamic section showing GFP immunoreactivity in neurons and fibres transduced by rAAV-UF5. Groups of GFP positive cells are seen in the ARC, extending along the third ventricle towards the PVN. ARC = Arcuate nucleus of the hypothalamus, 3V = third ventricle, PH = posterior hypothalamus.
Fig 4-4. Effect of rAAV-leptin on BW in female Sprague-Dawley rats. BW was significantly decreased in rAAV-leptin treated rats vs. control rAAV-UF5 treated rats (p<0.05) over a period of 12 weeks following a single injection of rAAV-leptin. n = 6-8 per group.

Fig 4-5. Effect of a single injection of rAAV-leptin on BW in male Sprague-Dawley rats. BW was significantly lower in rAAV-leptin treated rats vs. rAAV-UF5 treated control rats (p<0.05) for the 12 week period of the study. n=6 per group.
Fig 4-6. Twenty four hour food intake in female Sprague-Dawley rats injected icv rAAV-UF5 (control) or rAAV-leptin. Food intake was unchanged with treatment. n = 6-8 per group.

Fig 4-7. Twenty four hour food intake in male Sprague-Dawley rats injected icv with rAAV-UF5 (control) or rAAV-leptin. Food intake was unchanged with treatment. n = 6 per group.
**Fig 4-8.** Serum leptin levels in female Sprague-Dawley rats. Leptin levels were significantly decreased (p<0.05) in rAAV-leptin vs. the rAAV-UF5 treated control rats at 12 weeks post-injection. n=6-8 per group.

**Fig 4-9.** Serum leptin levels in male Sprague-Dawley rats. Leptin levels were significantly decreased (p<0.05) in rAAV-leptin vs. the rAAV-UF5 treated control rats at 12 weeks post-injection. n= 6 per group.
Fig 4-10. Body composition analysis post rAAV-leptin injection. Body fat was significantly decreased (A) while lean mass as depicted by protein composition was unaltered (B) at 6 weeks after a single injection of rAAV-leptin.
**Fig. 4-11. Effect of rAAV-leptin on body fat.** Abdominal fat is depleted in the rAAV-leptin. Left = rAAV-UF5, Right = rAAV-leptin
Discussion

In order to test the hypothesis that leptin resistance is due to insufficient availability at the site of action the objective of this study was to determine the long term efficacy of sustained leptin elevation in the CNS. Leptin was delivered into the CSF of wild type (wt) lean male and female rats via a rAAV viral vector encoding leptin and BW and FI were measured for 12 weeks. We demonstrate here a successful transduction of neurons in the CNS at the site of action of leptin. We observed several GFP positive cells in and around the hypothalamic nuclei associated with the regulation of appetite. We believe we have enhanced local leptin production in the hypothalamus by rAAV mediated central leptin gene delivery. This idea is supported by the demonstration of increased leptin mRNA in the hypothalamus in addition to the presence of several groups of GFP positive neurons in the hypothalamus. A single icv injection of rAAV-leptin was effective in maintaining BW in Sprague-Dawley rats for 12 weeks post-injection.. We observed a significant decrease in BW with leptin treatment in both male as well as female rats as compared to the control rats. However, there was a sex-specific difference in the response to rAAV-leptin treatment. Whereas the males continued to grow, albeit at a slower rate than the controls (17 % vs. 30 % total weight gain), the female leptin treated rats maintained BW at or below the pre-injection level. One possible explanation for this sex difference in BW response could be the effective dose of rAAV-leptin. Since both the male and female rats received the same actual dose, the males with a 30 % higher initial BW received a 30% lower relative dose. It is possible that increasing the relative dose in male rats would produce the same pattern of changes in BW as displayed by the female rats. It is also likely that this divergence may be due to the "fat melting" action of leptin (Chen et al ., 1996). As females have a higher fat mass compared with males, thus, this
may account for part of the difference in response to rAAV-leptin. We observed a striking reduction in body fat post icv rAAV-leptin injection without any change in muscle mass. These results confirm previous observations that leptin acts to reduce BW by specifically targeting the adipose mass in the body. Because peripheral leptin levels were markedly suppressed with icv rAAV-leptin injection, this fat melting effect is likely due to the central effect of leptin at its hypothalamic sites of action rather than peripheral action directly at the level of the adipocytes.

A large body of experimental evidence shows that leptin acts on the hypothalamic appetite regulating network to inhibit FI (Reviewed in Kalra SP et al., 1998). However, contrary to these reports, in our study the reduction in BW was not accompanied by a decrease in caloric consumption. FI in neither the male, nor the female, leptin treated groups differed from their respective controls. This brings up an interesting question as to how the weight loss is mediated by leptin in these animals and argues against the hypothesis that leptin is predominantly an appetite suppressing hormone. Evidence suggestive of a selective effect of leptin on energy expenditure, independent of an effect on FI, has been previously reported (Harris et al., 1998; Breslow et al., 1999). Leptin has been shown to actively stimulate the sympathetic nervous system to enhance energy expenditure by way of increased thermogenesis by activation of uncoupling proteins in BAT (Scarpace et al., 1997). Indeed, the location of GFP positive cells in this study establish a neuroanatomical basis for links with the SNS as well as BAT. It is possible that the loss or decrease in the rate of weight gain in the rAAV-leptin treated animals may primarily be attributable to enhanced energy expenditure. This possibility will be addressed in later chapters.
An interesting observation was the drastic decrease in peripheral leptin levels in rats injected centrally with rAAV-leptin. The primary role of leptin in metabolic homeostasis is to provide information to the hypothalamus on the status of body fat, thereby modulating CNS functions that regulate energy balance (reviewed in Ahima et al 2000; Schwartz et al., 2000). Thus, it would be intuitive that direct injection of leptin into the CNS falsely serves as a signal to the brain of the presence of adequate fat reserves and thus initiate a negative feed-back loop that would deplete body fat as seen in these rats and this would secondarily result in lower peripheral levels of leptin.

At the end of the 12 week experiment we did not observe the onset of leptin resistance in either sex, although, in female rats there was a return to initial BW. None of the rAAV-leptin injected animals displayed any inclination towards developing obesity. This is an important observation in that it directly supports our hypothesis that leptin resistance is not a result of hyperleptinemia or saturation of leptin receptors in the CNS, but due to insufficiency of leptin at its target sites. Thus, leptin resistance may be due to diminished availability of leptin in the interstitial fluid bathing the leptin receptors at specific hypothalamic sites involved in the regulation of appetite and energy balance. By sustained enhanced production of leptin with an AAV vector we have chronically increased production and availability at the critical sites.

Cumulatively, these results document the efficacy of rAAV-leptin gene therapy in transducing neurons and reveal a sex difference as well as a dichotomy in BW regulatory mechanisms by leptin that is independent of caloric consumption. This issue is further explored in later chapters. These data also highlight the use of rAAV as an effective gene delivery vehicle intracerebroventricularly. Due to its excellent safety features and the
ability to transduce a wide variety of tissues AAV will likely be the virus of choice for gene therapy applications in the future.
CHAPTER 5
LONG TERM EFFECTS OF LEPTIN GENE THERAPY

Introduction

Leptin, a 16 kD protein hormone is an afferent signal from the periphery to the brain in a homeostatic feedback loop that regulates adipose tissue mass (Zhang et al., 1994). Obese ob/ob mice that are leptin deficient have a reversal of the obese phenotype when given recombinant leptin. Leading to the early hypothesis that obesity in humans maybe a result of either absolute or a relative deficiency of leptin (Friedman and Halaas 1998; Rosenbaum N and Liebel 1998). However, a consistent feature of obesity is the presence of high levels of circulating leptin (Friedman and Halaas 1998). Recent studies have suggested that reduced sensitivity to rising endogenous leptin levels, as seen with increasing adiposity, may play a significant role in the development of obesity. Both diet induced obesity as well as several genetic models of obesity appear to be associated with resistance to the anorexic effects of leptin (Friedrich et al., 1996; Halaas et al., 1997). Thus, while the experimental elevation of leptin, within physiological levels, produces a transient decrease in food intake and weight loss (Halaas et al., 1997; Ahima et al 1996), the normal physiological context in which leptin acts as a negative adipostatic signal limiting weight gain in times of nutritional excess, remains to be defined. The primary site of action of leptin has been determined to be the brain, where its receptors are localized in critical nuclei of the hypothalamus such as the ARC, PVN,
VMH and the DMN (Schwartz et al., 1996; Elmquist et al 1997; Elmquist et al 1998).

The exact mechanism by which leptin mediates its weight reducing function is uncertain.

A decrease in the cerebrospinal fluid to serum leptin level ratio in obese individuals, implies decreased availability of leptin in the CNS, the primary site of leptin action (Considine et al 1995). Further, the fact that obese rodents respond to central leptin in the face of resistance to peripheral leptin supports a role for reduced leptin transport to the CNS (Van Heek et al., 1997). Thus, leptin resistance seems to be associated with insufficiency of leptin at its main site of action, the brain. Data presented in Chapter 4 of this dissertation support this assumption and demonstrate that leptin resistance does not occur with chronic elevation of leptin at its site of action. A sustained increase in leptin levels in the CNS with rAAV encoding leptin for a period of 12 weeks prevented normal weight gain. The attenuation of normal weight gain, however, was not accompanied by any decrease in food intake. An imbalance of metabolic energy (energy absorbed and available for metabolism) compared with energy expenditure yields either a loss or a gain of body mass. Therefore, one possible explanation for the attenuation in the rate of weight gain, despite normal food intake could be enhanced energy expenditure.

The experiments discussed in this chapter are designed to explore the underlying mechanism for retarded BW gain in the presence of normal food intake. In order to assess energy expenditure in rats with sustained long term elevation of leptin in the CNS we examined the hormones and other factors associated with enhanced energy expenditure. While a number of neuroendocrine afferent signals are implicated in BW homeostasis, the major efferent pathway is the sympathetic nervous system (SNS), which affects both energy expenditure and substrate utilization. Leptin increases central
sympathetic outflow (Mantzoros et al., 1996). A single leptin injection icv increases plasma norepinephrine (NE) in a dose dependent manner (Satoh et al., 1999). In rhesus macaques, icv leptin increased circulating NE levels by approximately 50% within an hour of administration (Tang-Christensen et al., 1999). Circulating catecholamines affect glucose and lipid metabolism (Nonogaki, 2000) and NE has been shown to increase thermogenesis in BAT via activation of the β3 adrenergic receptors (Landsberg and Young, 1992). In skeletal muscle NE augments glycogenolysis and thereby promotes energy utilization (Nonogaki, 2000).

Thyroid hormones have important thermogenic function (Krotkiewski, 2000) and leptin alters thyroid hormone levels. Thyroid hormones stimulate resting metabolic rate and, therefore, enhance energy expenditure in rodents (Jekabsons et al., 1999). Circulating thyroid hormone levels decrease during fasting and, leptin administration prevents this drop in plasma triiodo-thyronine (T3) and tetraiodo-thyronine (T4) levels (Ahima et al., 1996; legradi G et al., 1997). Recent data suggest that central leptin stimulates T3 production via enhanced conversion of T4 to T3 (Cusin et al., 2000). Thyroid hormones could thus be important mediators of the effect of leptin on energy expenditure.

Other candidates involved in regulation of energy homeostasis include the metabolic regulators, the uncoupling proteins (UCP's, reviewed in Adams, 2000). UCP's are found in the inner mitochondrial membrane and uncouple protons from ATP synthesis leading to generation of heat as opposed to ATP (Lin and Klingenberg, 1980). Uncoupling protein-1 (UCP-1) is abundant in BAT, an effector organ for adaptive thermogenesis in rodents. In rodents, leptin administration upregulates UCP-1 mRNA
expression through sympathetic activation of \( \beta_3 \) adrenergic receptors in BAT (Scarpace et al., 1997). This increase in UCP-1 is an important means by which leptin may regulate energy expenditure. In humans, the major thermogenic "organ" is the skeletal muscle (Rolfe and Brand, 1986). UCP-3, a homologue of UCP-1, is expressed in skeletal muscle and brown fat (Vidal-puig et al., 1997). UCP-3, like UCP-1, is a thermogenic protein (Boss et al., 1996; Vidal-puig et al., 1997). Indeed UCP-3 expression in skeletal muscle increases in response to thyroid hormone administration (Vidal-puig et al., 1998) and is modulated by leptin (Scarpace et al., 1998). Interestingly mice overexpressing UCP-3 are hyperphagic and lean (Clapham et al., 2000) and have a striking reduction in adipose tissue mass. Thus it is likely that in our experimental rats with sustained elevation of leptin in the CNS, body weight restraint without lowered food intake might be due to activation of UCP-1.

Age is a contributing factor to the development of obesity. An age related increase in body adiposity leads to increases in peripheral leptin and often times is accompanied by obesity, possibly due to development of leptin resistance. In humans and rodents visceral fat or deep abdominal fat levels increase with aging (Brazilai et al., 1998; Shimokata et al., 1989). Leptin levels increase significantly with age in rodents (Rasmussen et al., 1999; Wolden-Hanson et al., 1999) as does leptin mRNA (Maffei et al., 1995). Another factor accompanying aging is the increase in plasma insulin levels (Rasmussen et al., 1999; Wolden-Hanson et al., 1999), which may contribute to insulin resistance and NIDDM. We propose that the obesity associated with increasing age is not due to lack of peripheral leptin or insulin rather is due to decreased availability of leptin at its target sites. Thus, sustained central availability of leptin should prevent the
adverse effects of aging on obesity, insulin and related factors. The long term studies
detailed in this chapter detail the biochemical basis of BW maintenance without a
reduction in food intake.

**Materials and methods**

**Study Design**

Male and female lean Sprague-Dawley rats were permanently cannulated in the
third ventricle, randomly divided into two groups each (n= 6-8 per group) so that the
average BW of the groups was identical. Each group was injected icv with a single
injection of either rAAV-leptin or rAAV-UF5 (controls), a dose of 6 X10$^{10}$ particles in
5 µl. BW and food intake were recorded weekly for 24 weeks. An additional control
group of untreated un-operated females was simultaneously monitored. At 6 and 16
weeks post-injection blood samples were collected from the jugular vein for leptin,
insulin and glucose analyses. Urine samples were collected at week 16 for NE analysis.
At 24 weeks post-injection the animals were sacrificed by decapitation, blood, BAT and
skeletal muscle were collected for analyses.

**Radioimmunoassays**

**Leptin**

Serum and plasma leptin was assayed in glass tubes using a rat leptin RIA kit
(Linco Research, Inc., St Charles, MO) according to manufacturer's instructions. The
sensitivity of this assay is 0.5 ng/ml and the range of detection is 0.5 ng/ml to 50 ng/ml.
All samples were assayed in duplicate to minimize variability. Leptin in the CSF was
measured in polystyrene tubes using a more sensitive rat/mouse leptin RIA kit (ALPCO,
Windham, NH). The sensitivity of this assay is 6 pg/ml and the range of detection is 12.5-800 pg/ml.

**Insulin**

Insulin was measured in polystyrene tubes with a rat insulin RIA kit (Linco Research, Inc., St. Charles, MO) according to manufacturer's instructions. The sensitivity of the assay is 0.1 ng/ml and the range of detection is 0.1 ng/ml to 10 ng/ml. All samples from one experiment were analyzed in a single assay.

**Norepinephrine**

The NE RIA was performed in polystyrene tubes performed using a kit from ALPCO (Windham, NH) according to manufacturer's instructions. In this assay NE is first extracted from the urine sample using a cis-diol specific boronate affinity gel, simultaneously acylated to N-acylnorepinephrine and then converted enzymatically into N-acylnormetanephrine. The extracted sample is run in the RIA. The analytical sensitivity of this assay is 135 pg/ml and the range of detection is 0.15 to 0.5 ng/ml.

**Thyroid hormones (T3 and T4)**

The thyroid hormones T3 (tri iodo-thyronine) and T4 (tetra iodo-thyronine) were analyzed in serum and plasma samples using solid phaseT3 and T4 RIA kits (ICN Pharmaceuticals, Inc. Costa Mesa, CA). The assays were conducted separately for the two hormones in tubes coated with either T3 (polyclonal) or T4 (monoclonal) antibody. The analytical sensitivity of the T3 RIA is 6.7 ng/dL, while that of the T4 RIA is 0.76 µg/dL.
Dot blot analysis for UCP-1 and UCP-3

Total cellular RNA was extracted as described above. The integrity of the isolated RNA was verified using 1% agarose gels stained with ethidium bromide. The RNA was quantified by spectrophotometric absorption at 260 nm as well as 280 nm using multiple dilutions of each sample.

The full-length cDNA clone for uncoupling protein-1 (UCP1) was kindly provided to Dr Phillip Scarpace by Dr. Leslie Kozak, Jackson Laboratory, Bar Harbor, ME and verified by Northern analysis, as previously described (Scarpace et al., 1997). Full length UCP-3 cDNA was kindly supplied by Dr Olivier Boss and used as previously described (Boss et al 1997). All probes were random prime labeled using Prime-A-Gene kit (Promega, Cat # U 1000) according to manufacturer's instructions. The labeled probes were purified by filtering through a Nick Column (Pharmacia).

For dot-blot analysis, multiple concentrations of RNA were immobilized on nylon membranes (Gene Screen Plus, Dupont, NEN) using a dot-blot apparatus (Bio-Rad, Richmond, CA). The membranes were pre-wet in 20X SSC for 10 mins before the diluted samples were applied. After applying the samples, the membranes were baked at 80°C for 2 hours. The baked membranes were warmed in 40°C water for 2 mins, and then pre-hybridized for 30-60 mins at 65°C while rotating in Hybaid Quikhyb solution. The labeled probe was added in a concentration of 1.5 X 10^6 cpm/ml of the hybridization solution. The membranes in hybridization solution were hybridized for 2 hours at 65 °C. After hybridization, the membranes were washed in 2X SSC/0.1% SDS at 50 °C for 15 mins with two changes of solution. The membranes were further washed in 0.1X SSC/0.1% SDS for 15 mins. The blots were removed from the hybridization bottles, wrapped in saran wrap and exposed to a phosphor imaging screen for 24-48 h. Care was
taken to minimize folds in the saran wrap. The latent image on the phosphor imager screen was scanned using a Phosphor Imager (Molecular Dynamic, Sunnyvale, CA) and analyzed by Image Quant Software (Molecular Dynamics). Intensities were calculated per µg total RNA for each animal. Control as well as treated animal samples were applied on the same blot to minimize variability. All samples from one experiment were run on the same blot.

**Statistical Analysis**

BW and food intake data were compared with one way ANOVA, followed by post hoc analysis using Neuman-Keuls test. The p value was set at p<0.05 to attain significance. Longitudinal leptin and insulin levels were compared using one way ANOVA as well. Norepinephrine, glucose, T3 and T4 levels were compared with Students 't' test as was BAT UCP-1 mRNA expression and skeletal muscle UCP-3 mRNA expression in treated vs. control groups. p<0.05 was considered significant in all analysis.

**Results**

**Body Weight**

There were significant increases in the BW of female SD rats treated with rAAV-UF5 (control) and untreated un-operated control rats over the 6 month period of the experiment (Fig. 5-1). The rAAV-UF5 treated group gained 25.6% BW at 24 weeks post injection, this was identical to the BW increase (25.8%) of untreated un-operated controls. On the other hand, there was no significant BW gain the in the rAAV-leptin
treated rats which maintained their pre-injection weight (<3% gain overall) over the six month period. At the end of the experiment, control females weighed 315.4 ± 7.2 g, untreated females weighed 310.4 ± 10.5 and rAAV-leptin treated rats weighed 256.3 ± 8.0 g, this represented a difference of 22% in leptin treated vs. control groups (average initial BW of the three groups was 249.3 ± 2.3 g).

Similarly, there was a significant difference in BW at 24 weeks post injection in rAAV-UF5 vs. rAAV-leptin treated male rats (controls, 433.6 ± 15.4 g vs. 392.5 ± 14.7 g with rAAV-leptin treatment, Fig. 5-2). The initial BW of the male rats in this experiment was 325.6 ± 11.2 g controls vs. 324.8 ± 7.1 g rAAV-leptin. The control animals gained 32% vs. their initial BW as opposed to rAAV-leptin treated rats that gained 12.1% BW during the six months post injection.

**Food Intake**

There were no significant differences in 24 hour food intake in the three groups in female rats, 20.8 ± 0.4 g in rAAV-UF5, 20.5 ± 0.4 g in untreated, and 19.6 ± 0.4 g in rAAV-UF5 treated rats (Fig. 5-3). Similarly, amount of food consumed by male Sprague-Dawley rats was not different between treatment groups. Food intake by both rAAV-UF5 control male rats and rAAV-leptin treated male rats averaged 24.0 ± 0.6 g (Fig. 5-4).

**Leptin levels**

Leptin levels were determined at three time points over the 24 week course of the study (Fig. 5-5). Age related increases were observed in both the untreated female controls (1.3 ± 0.1 wk 6 vs. 2.6 ± 0.5 wk 16 vs. 9.1 ± 0.5 wk 24 untreated) as well as in rAAV-UF5 treated females (1.9 ± 0.1 wk 6 vs. 4.2 ± 1.0 wk 16 vs. 8.6 ± 1.1 wk 24).
The blood leptin levels of rAAV-leptin injected rats did not increase with age (0.9 ± 0.1 wk 6 vs. 1.43 ± 0.7 wk 16 vs. 1.79 ± 0.8 wk 24) and were significantly attenuated vs. controls at 6, 16 and 24 weeks post injection (p<0.05).

Similarly, in male rats treated with rAAV-leptin the age related increase in circulating leptin levels vs. rAAV-UF5 controls was not seen (P<0.05, Fig. 5-6). The circulating leptin levels of male rats treated with rAAV-leptin were significantly decreased vs. controls at 8, 16 and 24 weeks post injection.

**Insulin levels**

Circulating insulin levels in rAAV-leptin treated females remained unchanged at week 6 and 16 post injection. As expected untreated as well as rAAV-UF5 treated controls exhibited an age-related increase in serum insulin levels which was apparent at 24 weeks (p<0.05). The rAAV-leptin treated rats failed to show this increase in insulin levels at week 24 post-injection (Table 5-1).

Similarly, control rAAV-UF5 treated male rats had significantly elevated circulating insulin levels at week 24 compared with week 16 or 8 post injection (p<0.05). In rAAV-leptin treated rats there was an increase in serum insulin levels at 24 weeks, vs. week 16 or 8 but these levels at week 24 were significantly lower than those of rAAV-UF5 treated rats at the same time point (Table 5-2). Thus rAAV-leptin treatment suppresses the age-related increase in insulin in both males as well as females.

**Glucose levels**

Despite differences in insulin levels, the rAAV-leptin treatment in females (Table 5-1) as well as males (Table 5-2) displayed normoglycemia at all time points examined. There were no significant differences in glucose levels between treatment groups.
Urinary Norepinephrine levels

NE levels were measured in urine collected at week 16 post injection in male and female rats. There was no significant difference in rAAV-UF-5 treated vs. rAAV-leptin treated female rats (15.9 ± 4 vs. 16.2 ± 4.6 ng/ml, Fig. 5-7). Similarly there were no changes in urinary NE in male rats treated with rAAV-leptin (Fig. 5-8).

Thyroid Hormone levels

Neither T3 nor T4 levels measured at 16 weeks post injection were altered by rAAV-leptin treatment in female (Fig. 5-9) or male rats (Fig. 5-10).

UCP-1 mRNA expression

UCP-1 mRNA expression was significantly elevated (p<0.05) in rAAV-leptin treated females vs. rAAV-UF5 treated controls. rAAV-leptin treatment resulted in a 2 fold increase in BAT UCP-1 mRNA expression in females (Fig. 5-11). Similarly, a significant increase in UCP-1 mRNA expression was observed in male rats treated with rAAV-leptin vs. rAAV-UF5 controls at 24 weeks post injection (Fig. 5-12).

UCP-3 mRNA expression

Although there was a tendency for skeletal muscle UCP-3 mRNA expression to decrease in rAAV-leptin treated male and female rats, these differences were statistically not significant. (Fig's 5-13 and 5-14).
Fig. 5-1. Effect of long term leptin gene therapy on body weight in female Sprague-Dawley rats. There was a significant decrease in body weight (g) with rAAV-leptin treatment (p<0.05) vs. untreated and rAAV-UF5 treated groups. (n=6-8 per group).

Fig. 5-2. Effect of long term leptin gene therapy on body weight in male Sprague-Dawley rats. There was a significant decrease in body weight (g) with rAAV-leptin treatment (p<0.05) vs. rAAV-UF5 treated rats. (n = 6 per group).
Fig. 5-3. Effect of rAAV-leptin on food intake in female Sprague-Dawley rats. There was no difference in 24 hour food intake between the three treatment groups. n=6-8 per group.

Fig. 5-4. Effect of rAAV-leptin on food intake in male Sprague-Dawley rats. There was no difference in 24 hour food intake between the treatment groups. n=6-8 per group.
Fig. 5-5. Effect of central rAAV-leptin injection on circulating leptin levels in female Sprague-Dawley rats. Circulating leptin increased significantly over time in both untreated and rAAV-UF5 treated control rats, while the levels of leptin in rAAV-leptin treated rats were significantly lower vs. the other two groups at each time point studied (p<0.05). The age related increase in serum leptin was suppressed with rAAV-leptin treatment. n=6-8 per group.

Fig. 5-6. Effect of central rAAV-leptin injection on circulating leptin levels in male Sprague-Dawley rats. Circulating leptin increased significantly over time in rAAV-UF5 treated control rats, while the levels of leptin in rAAV-leptin treated rats were significantly lower vs. controls at each time point studied (p<0.05). The age related increase in serum leptin was suppressed with rAAV-leptin treatment. n = 6-8 per group.
Table 5-1. Time related changes in circulating leptin, insulin and glucose levels in female Sprague-Dawley rats. (*= p<0.05, vs. rAAV-UF5, a=p<0.05 vs. untreated, 1 =p<0.05, vs. week 6 and 16)

<table>
<thead>
<tr>
<th>Female</th>
<th>week</th>
<th>Untreated</th>
<th>rAAV-UF5</th>
<th>rAAV-leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (ng/ml)</td>
<td>6</td>
<td>0.36 ± 0.07</td>
<td>0.61 ± 0.11</td>
<td>0.45 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0.50 ± 0.05</td>
<td>0.33 ± 0.04</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.88 ± 0.2(^1)</td>
<td>1.18 ± 0.1(^1)</td>
<td>0.54 ± 0.07(^*)</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>6</td>
<td>179.8 ± 8.8</td>
<td>136.4 ± 8.4(^a)</td>
<td>123.3 ± 6.8(^a)</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>151.4 ± 12.5</td>
<td>141.3 ± 7.3</td>
<td>122.3 ± 4.8</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>133.9 ± 4.3</td>
<td>131.4 ± 9.1</td>
<td>135.0 ± 12.0</td>
</tr>
</tbody>
</table>

Table 5-2. Time related changes in circulating leptin, insulin and glucose levels in male Sprague-Dawley rats. (* = p<0.05 vs rAAV-UF5, 1= p<0.05 vs weeks 8 and 16)

<table>
<thead>
<tr>
<th>Male</th>
<th>week</th>
<th>rAAV-UF5</th>
<th>rAAV-leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (ng/ml)</td>
<td>8</td>
<td>0.50 ± 0.1</td>
<td>0.49 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0.46 ± 0.1</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.82 ± 0.3(^1)</td>
<td>0.84 ± 0.11(^*,1)</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>8</td>
<td>183.3 ± 7.4</td>
<td>177.2 ± 10.7</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>152.3 ± 7.7</td>
<td>138.4 ± 9.7</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>167.2 ± 12.9</td>
<td>161.5 ± 18.3</td>
</tr>
</tbody>
</table>
Fig. 5-7. Effect of central rAAV-leptin injection on urinary norepinephrine levels in female Sprague-Dawley rats at week 16 post-injection. There was no difference in the urinary NE levels between the treatment groups. n=6 per group.

Fig. 5-8. Effect of central rAAV-leptin injection on urinary norepinephrine levels in male Sprague-Dawley rats at week 16 post-injection. There was no difference in the urinary NE levels between the treatment groups. n=6 per group.
Fig. 5-9. Effect of rAAV-leptin on thyroid hormones in female Sprague-Dawley rats at 16 weeks post injection. There were no significant changes in either plasma T3 (A) or T4 (B) with rAAV-leptin treatment. n = 6 per group.

Fig. 5-10. Effect of rAAV-leptin on thyroid hormones in male Sprague-Dawley rats at 16 weeks post injection. There were no significant changes in either plasma T3 (A) or T4 (B) with rAAV-leptin treatment. n = 6 per group.
Fig. 5-11. Effect of rAAV-leptin on UCP-1 mRNA expression in BAT at 24 weeks post injection in female Sprague-Dawley rats. There was a significant increase in UCP-1 mRNA levels in rAAV-leptin treated rats vs. rAAV-UF5 treated controls (*= p<0.05). n =6 per group.

Fig. 5-12. Effect of rAAV-leptin on UCP-1 mRNA expression in BAT at 24 weeks post injection in female Sprague-Dawley rats. There was a significant increase in UCP-1 RNA levels in rAAV-leptin treated rats vs. rAAV-UF5 treated controls (*= p<0.05). n =6 per group.
Fig. 5-13. Effect of rAAV-leptin on UCP-3 mRNA expression in skeletal muscle 24 weeks post injection in female Sprague-Dawley rats. There was no significant difference in UCP-3 mRNA levels in rAAV-leptin treated vs. rAAV-UF5 treated control rats. n=6 per group.

Fig. 5-14. Effect of rAAV-leptin on UCP-3 mRNA expression in skeletal muscle at 24 weeks post injection in male Sprague-Dawley rats. There was no significant difference in UCP-3 mRNA levels in rAAV-leptin treated vs. rAAV-UF5 treated rats. n=5 per group.
Discussion

With advancing age there is an increase in the propensity towards adiposity in humans as well as in rodents (Wolden-Hanson et al., 1999). This increase in adipose mass brings about increases in peripheral leptin levels which in turn leads to the development of leptin resistance (Friedman and Halaas, 1998) followed often by BW gain. We demonstrate here the retardation in age-related weight gain via rAAV-leptin delivery into the CNS without any evidence of leptin resistance in either male or female rats for up to 24 weeks after icv rAAV-leptin treatment. This confirms and extends the 12 week efficacy study presented in Chapter 4. Our data demonstrate an attenuation in the rate of BW gain over a period of 24 weeks following a single icv rAAV-leptin injection.

The untreated and the rAAV-UF5 treated control female rats displayed the normal weight gain pattern resulting in a 25% increase in BW over the course of the 6 month study. This pattern of weight gain was completely abolished with rAAV-leptin treatment, six months after an icv rAAV-leptin injection the BW of female rats was unchanged from their initial pre-injection level. Interestingly, the response pattern was somewhat different in male rats. Male rats continued to gain BW, however, the rate of growth was significantly retarded. This is similar to the data presented in Chapter 4 in that there is a maintenance of BW in females at their initial range, while males continue to grow, albeit at a slower rate than that observed in control rAAV-UF5 treated rats. These sex-specific differences may possibly be attributed to the fact that leptin reduces BW by primarily reducing adipose tissue mass while leaving lean mass intact (Chen et al., 1996). Since males have a higher muscle mass and lower fat mass compared with females it could be argued that the male rats given rAAV-leptin do not display a more marked effect on BW compared to the controls due to this inherent difference in body composition. It is also
possible that the differences in the dose of rAAV-leptin may account for this sex-specific response to rAAV-leptin treatment. Due to limitations in the volume that can be injected into the third ventricle at one time both male as well as female rats were administered the same actual dose (6X $10^{10}$ particles), which does not translate to the same effective dose. Perhaps an increase in the effective dose in male rats may produce a response similar to that seen in female rats.

As anticipated, and in confirmation of data presented in Chapter 4, central rAAV-leptin treatment in males as well as in female rats resulted in suppression of peripheral leptin levels. Circulating leptin levels were significantly attenuated in rAAV-leptin treated rats male and female rats versus their respective controls at all time points examined. Interestingly, we observed that while leptin levels in control rats continued to increase in correlation with advancing age and adiposity, the rAAV-leptin treated rats of both sexes did not show this age related increase. This is probably a result of negative feedback due to enhanced leptin production within the CNS following icv rAAV-leptin delivery. These results suggest a protective effect of rAAV-leptin on the effects of aging. The idea that icv leptin may prevent some age related changes is further supported by our observation that circulating insulin levels were significantly lower at 6 months after icv rAAV-leptin injection. As with leptin, insulin levels in the blood increase with advancing age (Wolden-Hanson et al., 1999). Rising insulin levels in the periphery correlate with rising adiposity and leptin levels in the periphery. This increase is often associated with the development of insulin resistance and impaired glucose tolerance leading eventually to the development of NIDDM. It is noteworthy that in rAAV-leptin treated rats the decrease in insulin levels was not accompanied by hyperglycemia in
either sex, suggesting an increase in insulin sensitivity. A number of observations in the literature support a insulin sensitizing role for leptin (Porte, Jr., et al., 1998, Nonogaki, 2000). This retention of insulin sensitivity in rAAV-leptin treated rats may have resulted from lack of the direct effects of leptin due to reduced peripheral leptin levels on pancreatic β-cells and adipocytes (Chen et al., 1996). A more likely explanation, on the other hand, is that the site of leptin action to regulate insulin sensitivity resides within the hypothalamus. Hence, the enhanced insulin sensitivity in rAAV-leptin treated rats could be due to increased production of leptin in the hypothalamus (Chen et al., 1996).

The regualtion of BW by icv rAAV-leptin without any decrease in food intake suggested an effect on basal metabolism. However, we did not find any differences in urinary NE levels at 16 weeks after rAAV-leptin treatment. We also failed to detect any effect of rAAV-leptin on the release of thyroid hormones. T4 and its biologically active metabolite T3 were similar after 16 weeks of rAAV-leptin and rAAV-UF5 treatment. The lack of these responses may be explained by the chronic treatment with rAAV-leptin. Although, short-term leptin is reported to augment thyroid hormones (Cusin et al., 2000), this is the first report of chronic leptin treatment over a six month period. We examined these parameters at 16 weeks post-injection at a time when compensatory changes may have already occurred and thus normalized the thyroid hormone and NE responses, if any, to rAAV-leptin. Thus chronic leptin treatment does not enhance the resting metabolic rate as reflected by thyroid hormone levels or the levels of excreted NE.

In order to explore the possibility of increased energy expenditure through muscle thermogenesis, we examined skeletal muscle UCP-3 expression. We did not detect any difference in UCP-3 expression in skeletal muscle vs. controls of either sex. Although
UCP-3 expression in BAT is regulated by leptin possibly by a direct action (Scarpace et al., 2000). The effect of leptin on the relatively more abundant skeletal muscle UCP-3 is not clear. Gomez-Ambrozi et al., 1999 reported enhanced UCP-3 mRNA in skeletal muscle response to short term leptin treatment suggesting a direct effect of leptin. The dramatic decrease in peripheral leptin levels in our experiments may account for the lack of up-regulation of UCP-3 expression in these animals. It is also likely that the primary function of UCP-3 is not regulation of energy expenditure (Chung et al., 1999) but the regulation of ATP synthesis (Boss et al., 2000). Further, UCP-3 knockout mice are not obese and have a normal response to fasting (Vidal-puig et al., 1999). The induction of UCP-3 during starvation, at a time when energy expenditure is decreased (Leibel et al., 1995), does not support a role for UCP-3 in energy dissipation. However, increases in circulating free fatty acid levels associated with starvation suggest that UCP-3 could facilitate oxidation of free fatty acids (Argyropulos et al., 1998) and thus possibly play a role in fatty acid metabolism. Thus, the role of UCP-3 in the regulation of energy balance and body weight remains unclear.

BAT is the principal anatomic location of non-shivering thermogenesis (Himms-Hagen 1985). Although BAT constitutes only ~1% of the total body mass in the adult rodent, this tissue contributes as much as one third to thermogenesis during cold exposure (Foster and Frydman 1979). Leptin increases sympathetic outflow to BAT in ob/ob mice suggesting that the mechanism of increased energy expenditure may involve increased thermogenesis in this tissue (Collins et al., 1996). Further, leptin has been demonstrated to increase energy expenditure in non-mutant rodents by enhancing UCP-1 in the short term (Scarpace et al., 1997). Also, chronic central leptin infusion favors the expression
of UCP-1 in BAT (Cusin et al., 1998). The implications of chronic leptin administration, however, were not known. Therefore, in order to elucidate the basis for the weight loss in the absence of an obvious decrease in FI we examined UCP-1 expression in BAT. Our results demonstrate a 2 fold up-regulation in UCP-1 mRNA levels at 24 weeks post rAAV-leptin injection in male and female rats. The magnitude of induction of UCP-1 did not vary in a sex specific manner. Thus, a possible mechanism for the prolonged BW maintenance action of rAAV-leptin in male and female rats is the increase in non-shivering thermogenesis implied by the dramatic increase in UCP-1 expression in BAT.

In summary, AAV is an ideal vector for long term in vivo gene delivery. Long-term icv rAAV-leptin leads to a retardation in BW gain in male and female lean wt Sprague-Dawley rats. This lack of BW gain is not due to decreased FI. The studies presented here show that rAAV-leptin retards BW gain by enhancing energy expenditure in the absence of unchanged energy intake. A possible mechanism of enhanced energy expenditure in these rAAV-leptin treated rats is through a significant up-regulation of UCP-1mRNA in BAT which is known to cause dissipation of energy in the form of heat. Another significant outcome of this study is that icv rAAV-leptin causes a decrease in age related increases in peripheral leptin as well as insulin levels. This decrease in insulin is accompanied by normoglycemia implying enhanced peripheral insulin sensitivity. Thus central leptin action may be important for preventing the development of age-associated increases in insulin levels leading to insulin resistance and NIDDM. This important observation needs to be further evaluated in future studies. Although we did not perform experiments to study possible behavioral alterations, we did not observe any obvious deviations from normal behavior in rAAV-leptin treated animals. There was no
occurrence of leptin resistance in rAAV-leptin treated rats, lending further support to our hypothesis of leptin insufficiency at hypothalamic sites as a probable cause of obesity. It is intriguing that in our studies there was no effect of \textit{icv} leptin on FI. We next examined whether higher doses of rAAV-leptin would reduce FI and perhaps increase the incidence of leptin resistance. We have also sought to determine the central mechanism of leptin action in experiments comparing the effects of two doses of rAAV-leptin on the hypothalamic neuropeptides involved in appetite regulation. These will be discussed in Chapter 6.
CHAPTER 6
DOSE DEPENDENT REGULATION OF BODY WEIGHT WITH rAAV-LEPTIN

Introduction

Exogenous leptin administration to leptin deficient mice leads to a decrease in BW accompanied with restoration of normal feeding patterns and metabolism (Pelleymounter et al., 1995). Results from extensive studies indicate that the effect of leptin on appetite and energy balance are mediated mainly via neuronal targets in the hypothalamus and other brain regions (Reviewed in Ahima et al., 2000). Leptin delivered icv inhibits food intake and decreases BW and adiposity more potently than peripheral leptin administration (Campfield et al., 1995; Halaas et al., 1997). Peripheral leptin activates neurons in the ARC, VMN, DMH as well as brainstem neuronal circuits implicated in feeding behavior. Indeed, leptin receptors are abundant in these areas of the hypothalamus (Mercer et al., 1996).

Human obesity is accompanied by hyperleptinemia which is indicative of leptin resistance and possibly plays a role in the pathogenesis of obesity. Potential mechanisms that may mediate leptin resistance include impairment of brain leptin transport via abnormalities of leptin receptors and/or post-receptor signaling. The CSF: plasma leptin ratio is decreased in obese individuals and may be indicative of impaired brain leptin transport (Caro et al., 1996; Schwartz et al 1996). Several rodent models of diet induced obesity retain responsiveness to icv leptin while not responding to peripheral
hyperleptinemia (Van Heek et al., 1997). These investigators reported, that in diet induced obesity prone rodent strains (C57BL/6 and AKR mouse strain), young lean mice respond to peripherally administered leptin with a decrease in food intake and reduction in body weight. However, as obesity developed, plasma leptin levels increased and the mice became insensitive to peripherally administered leptin. The obese AKR mice, which were resistant to peripheral leptin treatment, remained highly responsive to leptin when it was administered centrally. The results of the rodent studies imply that obese humans, who appear to be hyporesponsive or resistant to their elevated endogenous leptin, may respond to a central nervous system penetrant leptin analogue.

Neurons involved in the production of orexigenic and anorexigenic signals are regulated by peripherally secreted hormones such as leptin, that cross the blood brain barrier, and by other neurotransmitters within the brain. A change in availability of neural orexigenic molecules such as Neuropeptide Y (NPY) and Agouti related peptide (AGRP) or anorexigenic molecules such as α-melanocyte stimulating hormone (αMSH) precedes the onset of feeding (Kalra SP et al., 1998). Disruption of the neural microenvironment through either changes in the amount or signaling capability of these molecules, leads to hyperphagia and subsequent obesity (Kalra SP et al., 1998).

There is extensive documentation in the literature on the effect of leptin on reducing food intake and altering the neuropeptide circuitry involved in the regulation of appetite. The active leptin receptor, ob-Rb, has been localized in NPY AGRP and POMC producing neurons in the ARC (Hakansson et al., 1996). In the ob/ob mouse and in normal wild-type rats, leptin regulates appetite by modifying the expression of orexigenic and anorexigenic genes in the hypothalamus. The orexigenic or appetite stimulating genes
are suppressed by leptin and include NPY and AGRP, while the genes involved in reducing appetite are stimulated by leptin and include proopiomelanocortin (POMC), the precursor peptide for the appetite suppressing αMSH.

We believe that leptin resistance could be due to insufficient leptin availability at its sites of action in the hypothalamus. Our results from Chapters 4 and 5 demonstrate that long term sustained delivery of leptin into the hypothalamus leads to retardation in weight gain. These animals did not develop resistance to leptin, thus supporting our hypothesis of insufficiency rather than unresponsiveness to leptin as a cause of leptin resistance. However, we observed a dichotomy in the effects of centrally delivered leptin. In rats injected icv with rAAV-leptin an intriguing and consistent observation was maintenance of BW without any attenuation in FI as compared to control rats that continued to gain BW.

These results suggested that the threshold of leptin action to enhance energy expenditure is lower than that required for reducing food intake. In the experiments described in this chapter we sought to establish the dose effects of icv rAAV-leptin on regulation of body weight. We hypothesize that whereas at lower doses leptin alters only energy expenditure in the form of altered UCP-1 expression, an enhanced dose would alter both energy expenditure, and also reduce food intake by altering POMC, AGRP and NPY expression in the hypothalamus.
Materials and Methods

Study Design

Experiment 1

Female Sprague-Dawley rats (200-250 g, 6-9 per group) were implanted with permanent cannulae in the third ventricle and divided into 2 weight matched groups. After surgery rats were injected with either rAAV-UF5 or rAAV-leptin (5 X 10^{10} particles in 5 µl). Body weight was recorded once a week until sacrifice at 6 weeks. FI was not recorded in this experiment due to lack of equipment. CSF was collected from the cisterna magna for measurement of CSF leptin levels from rAAV-UF5 and rAAV-leptin treated animals before sacrifice. Brain, BAT and skeletal muscle were collected. Serum from trunk blood collected at sacrifice was subsequently analyzed for leptin, insulin, glucose, free fatty acids, T3, and T4. BAT UCP-1 mRNA expression, skeletal muscle UCP-3 mRNA expression were assessed with dot blot analysis. Three brains per group were sectioned and NPY, AGRP and POMC mRNA were determined by in situ hybridization.

Experiment 2

Female Sprague-Dawley rats (200-250 g, 6-8 per group) were implanted with permanent cannulae in the third ventricle, weight matched into groups and injected twice (48 hours apart) with a 5 µl injection of either rAAV-UF5 or rAAV-leptin (5 X 10^{10} particles/ injection). Two additional groups of rats were included in this experiment; an un-operated, untreated group (n=6) and a group of rats pair fed to the amount consumed by rAAV-leptin treated rats (n=6). Body weight and 24 hour food intake were recorded once a week for 6 weeks. For leptin determination CSF was collected from the cisterna magna of untreated, rAAV-UF5 and rAAV-leptin treated animals before sacrifice at 6
weeks. Blood and tissues were collected and processed as described above (expt 1). The experiment with the pair fed rats was conducted three weeks after the rAAV-leptin rats according to the protocol used for rAAV-leptin treated rats.

**In situ Hybridization (ISH)**

**Construction of Riboprobes**

The POMC probe was constructed by cloning a 478 bp cDNA fragment (5’ psn 220, 3’ psn 697, GenBank Accession No J00759) into pGEM-T vector (Promega Corp., Madison, WI). The NPY probe was constructed using a plasmid containing a 511 bp rat NPY fragment kindly provided by Dr S.L. Sabol (NIH, Bethesda, MD). The 396 bp complete AGRP cDNA fragment (GenBank U89484) used to construct the probe, a generous gift of Dr Roger Cone (Oregon Health Science University, Portland, OR), was inserted into pBSK+/- vector.

Antisense riboprobes were transcribed in the presence of $^{35}$S-UTP (Amersham Life Sciences Inc., Arlington Heights, IL) using T7 (AGRP, POMC) or T3 (NPY) RNA polymerase.

**Tissue Sectioning**

The brain tissue used for ISH was snap frozen in dry ice and stored at −80°C till used. The brains were coronally sectioned at −20°C in a cryostat into 16 µm sections coronally. The sections covered areas of the brain starting at the level of the anterior commissure and ending past the ventral pre-mammilary nucleus of the hypothalamus, thus effectively covering the entire hypothalamus. The sections were thaw-mounted on microscope slides coated with Poly-L-Lysine. The sections were collected in four series; slides were stored at −80°C until use.
Tissue Processing

On the day of the experiment, one series of sections were removed from the freezer and allowed to warm up to room temperature (RT). They were post-fixed in 3 % paraformaldehyde for 10 min at RT followed by two washes in phosphate buffered saline (PBS, pH 7.4) for 5 min each. Next, the slides were incubated in 0.1M triethanolamine (pH8.0) for 10 min at RT, dehydrated through 70%, 80%, 95% and 100 % alcohol for 2 min each and air-dried. 100µl hybridization buffer containing $^{35}$S labelled antisense probe containing $1 \times 10^6 \text{cpm}$ was applied to each slide. The sections were covered with parafilm and incubated at $50^\circ \text{C}$ overnight in a humidified oven.

On day two of the procedure, the parafilm was carefully removed from the sections. The slides were washed in 2 X SSC for 5 min at RT with two changes of solution, 0.2 X SSC for 30 min at $55^\circ \text{C}$, 30 min in 0.1X SSC at $60^\circ \text{C}$ and 2X SSC at $37^\circ \text{C}$ for 5 min. Next the sections were incubated in 20 µg/ml RNase solution in 2 X SSC for 30 min at $37^\circ \text{C}$ while shaking. After another wash in 0.1 X SSC at $60^\circ \text{C}$ the slides were air dried and exposed to Kodak Biomax MR autoradiography film for 3-5 days. Slides were dipped in Kodak NTB2 emulsion, dried and stored at $4^\circ \text{C}$ for 2-4 weeks. The slides were counterstained with 0.1 % cresylviolet after developing. Slides from control and treated animals were processed together to eliminate any variability that might be introduced by processing.

Analysis of ISH data

For semi-quantitative analysis, the relative optical density (ROD) calculated as total target area multiplied by the integrated optical density for AGRP, NPY, and POMC were estimated from autoradiograms with the MCID image analysis system (Imaging
Research, St. Catherine, Ontario, Canada). Twelve matched sections from each brain of three rats per group were analyzed. The background optical density in an area adjacent to each target was subtracted from the target. The ROD of 12 sections in the same brain were added and expressed relative to the average ROD from the control group.

**Leptin**

Serum and plasma leptin was assayed in glass tubes using a rat leptin RIA kit (Linco Research, Inc., St Charles, MO) according to manufacturer's instructions. The sensitivity of this assay is 0.5 ng/ml and the range of detection is 0.5 ng/ml to 50 ng/ml. All samples were assayed in duplicate to minimize variability. Leptin in the CSF was measured in polystyrene tubes using a more sensitive rat/mouse leptin RIA kit (ALPCO, Windham, NH). The sensitivity of this assay is 6 pg/ml and the range of detection 12.5-800 pg/ml.

**Insulin**

Insulin was measured in polystyrene tubes with a rat insulin RIA kit (Linco Research, Inc., St. Charles, MO) according to manufacturer's instructions. The sensitivity of the assay is 0.1 ng/ml and a range of detection of 0.1 ng/ml to 10 ng/ml. All samples from one experiment were analyzed in a single assay. Insulin levels in the high dose group were not detectible with the above kit were assayed using a sensitive rat insulin RIA kit (Linco Research, Inc., St Charles, MO), the sensitivity of this sensitive assay assay is 0.02 ng/ml, the range of detection is 0.02- 1.0 ng/ml. The specificity of the antibody used in both insulin RIA's used is 100% to rat insulin.
**Statistical Analysis**

Weekly BW and FI were compared with one way ANOVA, followed by Neuman-Keuls test post hoc. The p value was set at p<0.05 to attain significance. Serum leptin, insulin, glucose, FFA, T3 and T4 levels were compared with Students 't' test in experiment 1 and with one way ANOVA in experiment 2. BAT UCP-1 mRNA expression and skeletal muscle UCP-3 mRNA expression, brain NPY, POMC, AGRP mRNA expression in treated vs. control groups were compared using Students 't' test in experiment 1 and One way ANOVA followed by post hoc analysis using Neuman-Keuls test. p<0.05 was considered significant in all analysis.

**Results**

**Body weight**

As described in Chapters 4 and 5 BW was significantly reduced in experiment 1, with a single (low dose, 5 X 10^{10} particles) injection of rAAV-leptin from two to six weeks post injection (Fig 6-1). The control rAAV-UF5 single injected rats gained 13% BW (235.6 ± 2.4 initial vs. 266.9 ± 5.6 g final) while the rAAV-leptin treated rats essentially maintained their pre injection BW's with a loss of 1.5± 2.8 %, (231.9 ± 3.5 g initial vs. 228.1 ± 6.1 g final).

In experiment 2, a significant difference in BW was first observed at one week post rAAV-leptin injections (high dose, 10 X 10^{10} particles) delivered 48 hours apart at the outset of the experiment. (Fig. 6-2). BW was significantly decreased vs. controls until week 6 post injection. These animals weighed 16.4% less than their initial weight (224.1 ± 5.4 initial vs. 188.4 ± 11.1 g final ), while the rAAV-UF5 injected controls displayed a
13.6% increase as seen also in the controls in experiment 1. This pattern of weight gain was similar to that of the untreated control group included in this experiment (Fig 6-2). The pair fed control (PFC) group were supplied a restricted amount to match the amount of food consumed by the rAAV-leptin injected rats. The PFC rats displayed a significant decrease in BW within one week of being placed on the reduced diet. This lower weight, similar to that of rAAV-leptin rats, was maintained until week 4 after which rats showed a tendency to gain BW. At week 6 their weight was 3% higher than their initial BW and 13% higher than the rAAV-leptin injected rats (p<0.05) despite the reduced FI.

**Food Intake**

Twenty four hour FI was significantly reduced with the high dose rAAV-leptin treatment (p<0.05, 16.6 ± 0.4 g control vs. 13.6 ± 0.8 g rAAV-leptin, Fig 6-3). The decrease in FI was observed at week one post-injection and maintained for the 6 week duration of the experiment. This average amount consumed by the rAAV-leptin treated rats over a 24 hour period at each week was supplied to the pair fed control group. The average FI in the rAAV-UF5 treated control rats did not differ from the untreated group (15.7± 0.3 g). Food intake in experiment 1 was not recorded, however, our previous data in Chapters 4 and 5 conclusively showed a lack of effect of this low dose of rAAV-leptin on FI. Since the BW loss in this experiment closely resembles that seen in data presented earlier in Chapters 4 and 5 we believe that this dose did not affect FI.

**Leptin**

Serum leptin levels were significantly reduced with the low dose rAAV-leptin injection vs. rAAV-UF5 treated controls (Fig 6-4 A, p<0.05). With the high dose rAAV-leptin the serum leptin levels were further depleted to almost non-detectable levels (Fig 6-4 B, p<0.001 vs. rAAV-UF5). These levels were significantly lower than the low dose
rAAV-leptin treated rats (p<0.05). Serum leptin levels of the rAAV-UF5 controls and PFC groups were similar to those of the untreated, ad-lib fed group.

**CSF Leptin and CSF:Serum Ratio's**

In contrast to the decrease in serum leptin levels, CSF leptin levels in neither the low nor the high dose rAAV-leptin treated rats differed from their respective controls (Fig 6-5 A, 6-6 A). These normal CSF levels in the presence of markedly depleted leptin levels resulted in a significantly higher CSF:serum leptin ratio in both groups of rAAV-leptin treated vs. their respective controls (Fig 6-5 B, 6-6 B).

**Insulin**

At week six, serum insulin levels in the low dose rAAV-leptin treated rats were somewhat lower than those of rAAV-UF5 control rats; this was statistically not significant (p>0.05, Fig. 6-7 A). However insulin levels were significantly attenuated with the high dose of rAAV-leptin (p<0.001, Fig 6-7 B). Serum insulin levels did not differ from the untreated control rats in either the rAAV-UF5 or the PFC groups (Fig 6-7B).

**Glucose**

Glucose levels were unchanged with low dose rAAV-leptin treatment vs. controls (Table 6-1). With the high dose rAAV-leptin there was a significant (p<0.05) 14% decline in serum glucose levels despite the decrease in insulin levels. The glucose levels in rAAV-UF5 and PFC rats were unaltered vs. untreated controls (Table 6-1).

**Thyroid Hormones**

Serum levels of thyroid hormones, T4 and T3, were not altered by in the low dose rAAV-leptin treated rats vs. rAAV-UF5 treated rats (Table 6-1). While T3 levels were unchanged with the high dose rAAV-leptin treatment, the levels of T4 were significantly
reduced in rAAV-leptin high dose treated rats (Table 6-1) and increased in the PFC controls. There were no differences in either T3 or T4 levels of rAAV-UF5 controls vs. the untreated control rats.

**Free Fatty Acid Levels**

FFA levels in rAAV-UF5 treated animals did not differ from those of untreated controls. However, FFA levels were significantly (p<0.05) decreased following either the low or high dose rAAV-leptin injection vs. their respective controls (Fig 6-8 A and B). A trend towards a decrease in FFA levels was seen in PFC rats but this was not significant (Fig 6-8 B).

**UCP-1**

UCP-1 mRNA in BAT was significantly elevated both with the low dose as well as high dose rAAV-leptin treatment (p<0.05) vs. controls (Fig 6-9). The magnitude of UCP-1 mRNA increase was approximately 2 fold following either dose of rAAV-leptin; there was no dose dependent effect of leptin on UCP-1 mRNA. There was a significant decrease in BAT UCP-1 mRNA expression in the pair fed controls vs both the rAAV-UF5 and rAAV-leptin high dose treated groups (p < 0.05).

**UCP3**

There was no significant change in skeletal muscle UCP-3 mRNA expression at 6 weeks post rAAV-leptin in either the low or high dose rAAV-leptin treated group. The trend towards decreased UCP-3 mRNA expression in the rAAV-leptin treated rats was statistically not significant (Fig 6-10).
**Brain NPY, POMC, AGRP mRNA expression**

Relative levels of neuropeptide gene expression were analyzed by in situ hybridization. Quantitative analysis of these genes was performed in the hypothalamic arcuate nucleus. POMC and AGRP were localized in the ARC nucleus alone, while NPY mRNA expression was observed in the DMN also. There were no significant changes in ARC NPY, POMC or AGRP mRNA expression in the low dose rAAV-leptin vs. rAAV-UF5 treated rats (Fig 6-14). This was in marked contrast to the changes in gene expression detected in the high dose rAAV-leptin treated rats. POMC mRNA in the ARC was significantly up-regulated (Fig 6-11 C and 6-15 A) while NPY mRNA was down-regulated in rAAV-leptin treated rats (Fig 6-12 C and 6-15 B). Surprisingly, AGRP mRNA, which is co-localized with NPY in ARC neurons, was not affected by rAAV-leptin treatment (Fig 6-13 C and 6-15 C). Neither POMC, NPY nor AGRP gene expression was altered in PFC rats.
Fig 6-1. Effect of low dose rAAV-leptin on body weight in lean female Sprague-Dawley rats. Body weight was significantly reduced with rAAV-leptin treatment (p<0.05). n=6-9 per group.

Fig 6-2. Effect of high dose rAAV-leptin on body weight in lean female Sprague-Dawley rats. Body weight was significantly reduced with rAAV-leptin treatment (p<0.05). The BW of diet restricted pair fed rats was significantly reduced vs. controls, and significantly higher than the rAAV-leptin treated rats. n=6-9 per group.
**Fig 6-3.** Effect of high dose of rAAV-leptin on food intake in female Sprague-Dawley rats. Food intake was decreased with the high dose ($p < 0.05$ vs. control). The pair fed animals were fed a diet to match the rAAV-leptin treated group and had FI significantly lower than controls. $n = 6-8$ per group.
**Fig 6-4.** Dose dependent effects of rAAV-leptin injection on serum leptin. (A) Low dose (B) High dose. Leptin levels were significantly decreased in rAAV-leptin treated rats at 6 weeks post injection with both high and low dose vs. control (p<0.05). Leptin levels were significantly lower with the high dose of rAAV-leptin vs. the low dose. * = p<0.05, ** = P<0.001
Fig. 6-5. Dose effects of *icv* rAAV-leptin on serum insulin levels in female SD rats 6 weeks post injection. (A) Low dose (B) High dose. Insulin was significantly decreased with rAAV-leptin high dose treatment (p<0.001), there was no effect of low dose rAAV-leptin on serum insulin levels. ** = P<0.001
Fig 6-6. Effect of low dose rAAV-leptin on cerebro spinal fluid levels of leptin (A) and CSF:serum leptin ratio (B). CSF leptin levels were unchanged with treatment while CSF:serum leptin level ratio was significantly increased (p<0.05). n=6 per group. * = p<0.05.
Fig 6-7. Effect of high dose rAAV-leptin on cerebrospinal fluid levels of leptin (A) and CSF:serum leptin ratio (B). CSF leptin levels were unchanged with treatment while CSF:serum leptin level ratio was significantly increased (p<0.05) in the rAAV-leptin treated group. n=4 per group. ** = P<0.001
Table 6-1. Serum Hormone and glucose profiles at week 6 post-injection.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Glucose (mg/dL)</th>
<th>T3 (ng/dL)</th>
<th>T4 (µg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rAAV-UF5 (5 X 10^10 particles)</td>
<td>136.4 ± 8.05</td>
<td>124.8 ± 4.8</td>
<td>3.3 ± 0.19</td>
</tr>
<tr>
<td>rAAV-leptin (5 X 10^10 particles)</td>
<td>123.3 ± 6.85</td>
<td>123.0 ± 7.7</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>Untreated</td>
<td>158.7 ± 20.8</td>
<td>110.1 ± 11.2</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>rAAV-UF5 (10 X 10^10 particles)</td>
<td>165.2 ± 7.06</td>
<td>114.4 ± 2.9</td>
<td>3.4 ± 0.14</td>
</tr>
<tr>
<td>rAAV-leptin (10 X 10^10 particles)</td>
<td>127.5 ± 3.3*</td>
<td>117.0 ± 5.7</td>
<td>2.5 ± 0.1*</td>
</tr>
<tr>
<td>Pair Fed</td>
<td>146.4 ± 6.01</td>
<td>121.1 ± 3.8</td>
<td>5.2 ± 1.7a</td>
</tr>
</tbody>
</table>
Fig 6-8. Effect of rAAV-leptin on serum free fatty acids. FFA's were significantly decreased with both low (A) and high (B) dose of rAAV-leptin. * = p<0.05
Fig 6-9. Change in UCP-1 mRNA in BAT with different doses of rAAV-leptin.
There was a significant upregulation in UCP-1 mRNA with both doses of rAAV-leptin, however there was no dose dependent difference in the induction of UCP-1 mRNA with rAAV-leptin. Pair Fed controls had significantly decreased UCP-1 mRNA vs controls as well as high dose rAAV-leptin treated animals. n=6-9 per group. * = p<0.05, a = p<0.05

Fig 6-10. Effect of rAAV-leptin on skeletal muscle UCP-3 expression. There were no significant effects on UCP-3 mRNA expression with either dose of rAAV-leptin treatment. n=6-9 per group.
Fig 6-11. Photomicrograph (10X) of the effects of high dose rAAV-leptin on POMC mRNA expression in the Arcuate nucleus of the hypothalamus. Panel A = rAAV-UF5, Panel B= Pair fed control, Panel C- rAAV-leptin high dose. There was a significant increase in POMC mRNA levels with rAAV-leptin treatment.
Fig 6-12. Photomicrograph (10X) of the effects of high dose rAAV-leptin on NPY mRNA expression in the Arcuate nucleus of the hypothalamus. Panel A = rAAV-UF5, Panel B= Pair fed control, Panel C- rAAV-leptin high dose. There was a significant decrease in NPY mRNA levels with rAAV-leptin treatment.
Fig 6-13. Photomicrograph (10X) of the effects of high dose rAAV-leptin on AGRP mRNA expression in the Arcuate nucleus of the hypothalamus. Panel A = rAAV-UF5, Panel B= Pair fed control, Panel C= rAAV-leptin high dose. There was no change in AGRP mRNA levels with rAAV-leptin treatment.
Fig 6-14. Effect of low dose rAAV-leptin on hypothalamic appetite regulating peptides. There were no significant changes in POMC (A) NPY (B) or AGRP (C) mRNA levels in the ARC nucleus of the hypothalamus.
Fig 6-15. Effect of the high dose of rAAV-leptin on hypothalamic appetite regulating neuropeptides. POMC was significantly elevated (A), NPY was significantly decreased (B) and AGRP was unchanged (C) by rAAV-leptin treatment. The expression was unchanged vs. controls in PFC rats.
Discussion

Leptin communicates information about the state of peripheral energy stores to the brain (Friedman and Halaas, 1998), specifically to the hypothalamus which is a major site of leptin action (Elmquist et al., 1998). A number of hypothalamic neuropeptides involved in the control of feeding behavior are direct targets of leptin action (Cone 1999; Elmquist et al., 1998; Xu et al., 1999). Human and rodent obesity is characterized by peripheral hyperleptinemia and leptin resistance. Possible underlying causes of leptin resistance include defective transport of leptin across the BBB or downregulation of leptin receptors in the hypothalamus in response to high leptin levels. However, there is little experimental evidence in support of either of these possibilities. Data presented in Chapters 4 and 5 demonstrated that chronically elevated leptin levels at its site of action the hypothalamus do not result in leptin resistance. This argues against the possibility of down regulation of leptin receptors to account for leptin resistance and suggests that leptin insufficiency at its target sites may account for the lack of leptin action seen in obese subjects. However, in these studies we did not observe the full spectrum of leptin action on energy expenditure and food intake. The rAAV-leptin treated rats displayed retardation in BW gain without the expected decrease in food intake. This was attributed largely to an increase in energy expenditure as evidenced by UCP-1 mRNA up-regulation in BAT with rAAV-leptin treatment. To test the possibility that the threshold of leptin level required to affect energy expenditure vs. FI may be different we conducted a dose escalation study. In this short-term six week experiment we observed maintenance of BW with a single injection of rAAV-leptin; the profile of BW regulation matched that seen in earlier experiments in Chapters 4 and 5. However, with a 2 fold increase in rAAV-leptin dose we observed a significant decline in BW. The final BW was 14%
below the initial BW while the controls gained 13%. Unlike our earlier experiments with the low dose there was a significant 15% decrease in FI with the high dose of rAAV-leptin which may account for the increased weight loss. Thus leptin affects FI in a dose dependent manner.

As anticipated, there was a decline in BW in the pairfed controls that were fed a restricted diet to match the caloric intake of the high dose rAAV-leptin treated rats. Interestingly, although the PFC rats lost weight initially, at week 4 post injection these animals appeared to rebound to their initial weight despite the still reduced caloric intake. This "rebounding" in BW may be explained by the compensatory mechanisms that exist in the body to maintain BW at a certain "set point".

Serum leptin levels were significantly decreased with the low dose of rAAV-leptin as seen earlier in Chapters 4 and 5. The depletion of serum leptin was greater in the higher dose group possibly due to the higher weight loss (fat loss) in this group. CSF leptin levels in either group of rAAV-leptin treated animals were not diminished as would be the case with the significantly depleted serum leptin levels. It is likely that secretion of leptin by the hypothalamic cells transduced with rAAV-leptin accounted for maintaining CSF leptin levels resulting in a significant elevation in the CSF:serum leptin ratio. We can thus state that chronic icv leptin availability does not lead to the development of leptin resistance. Thus one can infer that leptin resistance is not due to receptor downregulation or defects in intra-cellular signaling. Although we did not measure SOCS-3 (suppressor of cytokine signaling -3), it appears unlikely that a persistent increase in SOCS-3 following leptin binding to the Ob-Rb accounts for leptin resistance as proposed by Bjorbeck et al., 1998. Lack of leptin transport across the BBB
as a cause for leptin resistance was suggested by the observation of a decreased CSF: serum leptin ratio in obese individuals (Considine et al 1995). Our data provide direct experimental support for this hypothesis.

Serum insulin levels were unchanged with the single injection of rAAV-leptin, however, the higher dose significantly diminished insulin levels in the serum at 6 weeks post-injection. Surprisingly serum glucose levels were significantly reduced in these rats. The insulin sensitizing actions of leptin have been previously reported (Chen et al., 1997; Kahn and Flier 2000). It is, however, not certain whether this action occurs in the CNS or if leptin directly acts on target organs in the periphery to affect insulin sensitivity. In pancreatic β cells and in muscle leptin increases lipid oxidation and inhibits lipid synthesis, factors known to promote insulin sensitivity. Leptin could also affect insulin sensitivity by reducing nutrient influx via decreased appetite. Other ways that leptin may modulate insulin sensitivity is through modulation of neuroendocrine effectors such as glucocorticoids, which are increased by icv leptin and are known to enhance insulin sensitivity (Kahn and Flier 2000). In our model enhanced CNS leptin levels resulted in enhanced peripheral insulin sensitivity. In the presence of reduced peripheral leptin, this clearly supports the CNS as the site of leptin action to regulate insulin sensitivity. The molecular basis for the insulin sensitizing effect of leptin remains to be explored further.

To facilitate our understanding of the difference in the mechanism of action of the two doses we studied the effect of icv rAAV-leptin on serum thyroid hormones. Thyroid hormones are involved in the regulation of resting metabolic rate (Jekabsons et al., 1999). A restricted diet, as in the PFC group, requires conservation of energy and a decrease in resting metabolic rate. The elevated serum T4 levels in this group implying decreased
turnover of T4 to the more potent T3 supports this reasoning. Leptin has been reported to enhance metabolism by increasing the turnover of the less potent thyroid hormone T4 to the active form T3 (Cusin et al., 2000). As in our long-term study we saw no effect of a single injection of rAAV-leptin on either circulating T3 or T4 at week 6 post-injection. However, in the high dose group we detected a significant decrease in serum T4 levels with rAAV-leptin treatment suggesting enhanced turnover of T4, however, T3 levels were not increased. Thus, although there was a suggestion that leptin enhances energy expenditure by increasing turnover of T4 to T3 we did not obtain unequivocal evidence of this. This issue may need to be further explored at shorter time intervals after rAAV-leptin treatment. To conclusively determine the effects of leptin on the regulation of the thyroid hormones we may require determination of hypothalamic thyroid releasing hormone (TRH) mRNA levels, and plasma TRH levels in response to leptin.

In order to explore the effect of leptin on thermogenesis we examined the expression of UCP-3 which is abundantly expressed in skeletal muscle, and skeletal muscle is the main thermoeffector "organ" in humans. It is not clear how leptin affects skeletal muscle UCP3 where it is relatively more abundant than in BAT. There are some reports of enhanced UCP-3 mRNA in skeletal muscle in response to short term leptin treatment (Gomez-Ambrozi et al., 1999). However, in our studies there was no difference in skeletal muscle UCP-3 expression vs. controls in both the low and high dose group, although, there was a trend towards reduced expression in the latter. The marked decrease in blood leptin levels in our experiment may account for the lack of UCP-3 up-regulation in these animals. Another possible explanation is that the primary function of UCP-3 function is not the regulation of energy expenditure (Chung et al., 1999) but the
regulation of ATP synthesis (Boss et al., 2000). In fact, UCP-3 knockout mice are not obese and display a normal response to fasting (Vidal-puig et al., 1999). The induction of UCP-3 during starvation, at a time when energy expenditure is decreased (Leibel et al., 1995) does not support a role for UCP-3 in energy dissipation.

In rodents, increased thermogenesis by BAT is another mechanism by which leptin regulates body weight. Peripheral delivery of leptin leads to increased energy expenditure as measured by oxygen consumption and UCP-1 mRNA expression in BAT (Scarpace et al., 1997). The UCP-1 responses to chronic leptin enhancement were described in Chapter 5, here we sought to establish a dose response to rAAV-leptin treatment. In contrast to UCP-3 regulation in skeletal muscle by peripheral leptin, UCP-1 in BAT is regulated by the SNS via β3 receptors. We found a 2 fold induction in UCP-1 mRNA with either dose of rAAV-leptin \textit{icv}. Surprisingly the induction was not greater with the higher dose of rAAV-leptin treatment, leading us to speculate that there is a maximal response in UCP-1 induction post leptin treatment that does not increase with further elevations in leptin. Also we observed a significant decrease in BAT UCP-1 in the pair fed controls which was in line with the need for energy conservation induced by reduced caloric intake.

We further examined the expression of a few key neuropeptides involved in the regulation of appetite. NPY is a powerful stimulator of feeding (Clark et al., 1984). A sub-population of NPY producing neurons in the ARC express the long form of the leptin receptor and these cells are responsive to changes in circulating leptin levels (Basin et al., 1999). There is firm evidence that leptin inhibits NPY mRNA expression and decreases NPY mRNA levels in the ARC (Stephens et al., 1995). The NPY neurons
are, therefore, targets of leptin in the hypothalamus and the ability of leptin to induce hypophagia and weight loss is partly mediated by inhibition of NPY synthesis and secretion. In agreement with these observations, our studies demonstrate decreased NPY mRNA expression in the ARC with the high dose of rAAV-leptin in association with reduced FI. In the low dose treated rats there was no change in NPY gene expression in the hypothalamus. This may account for the unaltered food consumption in that group.

Another system involved in the mediation of leptin's effects on appetite regulation is the melanocortin system. POMC is produced by neurons in the ARC and is the precursor of αMSH, a hypothalamic peptide that inhibits feeding. Leptin enhances POMC mRNA expression in wild type rodents (Cheung et al., 1997) and in ob/ob mice (Mizuno et al., 1998). In an earlier study we have reported upregulation of POMC gene expression in ob/ob mice treated with rAAV-leptin (Dhillon et al., 2000). In the present study we observed an upregulation in POMC mRNA with the high dose of rAAV-leptin while the low dose did not affect POMC expression in correlation with the effect of these treatments on food intake. Expression of AGRP, the orexigenic peptide, was not affected by either dose of rAAV-leptin. It can be speculated that the increase in αMSH levels following the upregulation of POMC mRNA serves as the ligand for the melanocortin receptors to inhibit appetite. AGRP, the melanocortin receptor antagonist, apparently does not participate in the weight regulatory equation in this situation.

That these changes in NPY and POMC expression are the direct effect of leptin and not secondary to changes in food intake is supported by the results from the pair fed control group. These rats were given food matched to the amount consumed by the high dose rAAV-leptin group. Neither NPY nor POMC mRNA expression in the ARC was
affected by food restriction. Serum leptin levels in this group were not different from those observed in untreated controls. Thus in the absence of central elevation of leptin, moderate food restriction does not affect the expression appetite regulating neuropeptides.

We did not observe any symptoms of malaise associated with either dose of rAAV-leptin or the control vector. AAV is known to be non-immunogenic and non-pathogenic and not associated with the etiology of any disease (Berns and Bohenzky, 1987). Although we did not anticipate any inflammatory response we monitored age and weight matched, un-operated, untreated animals in the experiment as an additional control group. There were no differences in the BW, FI or hormonal profiles of rAAV-UF5 injected rats vs. these untreated rats. In summary, our dose response data demonstrate a dichotomy in the response to rAAV-leptin icv. The low dose selectively enhances energy expenditure without affecting FI whereas the high dose affects both energy expenditure as well as reduces FI. Food intake was suppressed in the latter group as a result of changes in the neuropeptide levels which were seen only in this group. There was no evidence of the development of leptin resistance in either dose group. This lack of any adverse effects of icv rAAV-injections strengthens the safety of using AAV therapy.
CHAPTER 7
GENERAL DISCUSSION

Obesity is a global epidemic and is a risk factor for medical conditions such as diabetes, hypertension, stroke and increased incidence of morbidity. Clinically, weight reduction and maintenance of reduced weight are socially desirable in the general population, and is recommended for obese patients for improvement of obesity-associated medical problems. Obesity occurs as a result of dysfunction in energy homeostasis. Successful maintenance of energy homeostasis depends on the brain being able to read, interpret and integrate a wide range of signals that describe the animal's nutritional state and its immediate environment and to make appropriate adjustments in food intake, energy expenditure and metabolism as a result of the information received. The brain tightly and accurately regulates BW and fat mass to within 0.5-1% under steady state conditions. Leptin a product of the obese gene produced primarily in adipose tissue is a major player in signaling the brain for the regulation of BW and energy expenditure (Ahima et al., 2000). Genetically obese ob/ob mice that lack functional leptin, are hyperphagic, hyperinsulinemic, and manifest a syndrome resembling non-insulin dependent diabetes mellitus (NIDDM, Zhang et al., 1994). Recombinant leptin treatment reverses the obese phenotype in ob/ob mice, along with normalization of the hyperinsulinemia, hyperglycemia and low basal metabolic rate characteristic of this model (Pelleymounter et al., 1995; Halaas et al., 1995; Campfield et al., 1995).

Whereas, there are several rodent models of genetic obesity as exemplified by the ob/ob mice that lack leptin, this is rare in obese humans. Morbid obesity associated with
leptin deficiency has been identified in very few humans (Montague et al., 1997). Most human obesity is not genetic in origin; on the contrary, it is associated with markedly elevated serum leptin levels that correlate positively with body mass (Considine et al., 1996). These observations have led to the speculation that obesity may be a consequence of resistance to the weight reducing actions of leptin. However, the site and mechanism of leptin resistance are not known. One likely possibility to account for the lack of leptin action despite the presence of high circulating levels is inadequate availability of leptin at its site of action in the brain. This concept is supported by the observation of decreased cerebrospinal fluid to serum leptin level ratio in obese humans implying decreased availability of leptin in the CNS of these patients (Caro et al., 1996).

Diet induced obesity in rodents also is linked to a decrease in the amount of leptin transported into the CSF (Bruguera et al., 2000). Evidence from rodent studies that diet induced obesity associated with peripheral hyperleptinemia can be attenuated with central but not peripheral leptin treatment (Halaas et al., 1997) and that centrally administered leptin has a greater potency than either intravenous infusion or intramuscular injection (Halaas et al., 1997; Campfield et al., 1995) clearly suggest that insufficient availability of leptin at its central sit of action may be a major cause of the obesity. This lone of reasoning raises the question of whether continuous elevation of leptin centrally would result in leptin resistance as seen with peripheral hyperleptinemia.

In view of the absence of defects in either leptin, leptin receptors, leptin receptor signaling, or transport in the majority of the obese population, the main hypothesis tested in this dissertation was that leptin resistance is not due to exposure of the hypothalamus to elevated levels of leptin but is a consequence of insufficient availability of leptin at its
site of action. The goals of this dissertation were, (1) to test the efficacy of peripheral rAAV-leptin gene therapy in a rodent model of obesity, the ob/ob mouse (2) to test the efficacy of centrally administered in a targeted manner to its site of action in the hypothalamus (3) to determine the effect of sustained centrally elevated leptin levels on BW, FI and development of leptin resistance, and (4) to elucidate the mechanism of action of leptin in the CNS on body weight regulation.

The data presented in Chapter 3 demonstrate the efficient transfer of leptin using a recombinant AAV vector. The results confirm earlier reports of the use of rAAV for gene transfer with long lasting effects (Murphy et al., 1997; Mandel et al., 1998; Samulski, et al., 1999). Three different doses of rAAV-leptin were injected via the tail vein to leptin deficient ob/ob mice. Serum leptin levels were elevated to the range seen in normal wt mice (2-3 ng/ml) with the high dose of rAAV-leptin, while the two lower doses had almost undetectable serum leptin levels. There was a dose dependent decrease in BW in the leptin deficient ob/ob mice with rAAV-leptin injection. The highest dose of rAAV-leptin (6x10^{11} particles) resulted in a significant decrease in BW vs. the rAAV-UF5 injected controls starting at 10 days post injection and was maintained for the duration of the study. Mice given the second highest dose of rAAV-leptin (6x10^{10} particles) maintained their initial body weights and failed to display the gain in BW characteristic of ob/ob mice, suggesting that the small amount of leptin contributed to the circulation by this dose was adequate to prevent BW gain. There was no effect on BW with the lowest dose of rAAV-leptin (6x10^{9} particles). Acute administration of the leptin protein to ob/ob mice leads to decreased body weight through reduction in caloric consumption accompanied with increase in energy expenditure (Zhang et al., 1994;
Halaas et al., 1995; 1997; Pelleymounter et al., 1995; Harris RB et al., 1998). In my study, only the highest dose of the vector elicited a decrease in food intake. Thus the weight loss in this group may be attributed to a decrease in energy intake, however, the observation that the middle dose blunted BW gain without reducing FI is the most interesting. Although this study did not measure parameters of energy expenditure, it is well documented in the literature that leptin enhances energy expenditure in \textit{ob/ob} mice (Halaas et al., 1995, 1997; Pelleymounter et al., 1995). This brings forward a dichotomy in the action of leptin based on the dose administered. Whereas the higher dose affects both the energy expenditure and energy intake aspects of the energy homeostasis equation, the lower dose retards the rate of BW gain presumably by affecting energy expenditure. Another interesting observation of this study was that reinstatement of leptin via rAAV-leptin injection in the \textit{ob/ob} mice was not associated with development of leptin resistance. Resistance to leptin action occurs generally in the presence of higher than normal levels of leptin. In the experiments detailed in Chapter 3, serum leptin levels, even in the highest dose group, did not exceed normal wild type levels and do not constitute hyperleptinemia as seen in obese rodents. This may account for the lack resistance to leptin in these animals. It is also likely that since the \textit{ob/ob} mice are supersensitive to leptin administration much higher peripheral leptin levels may be required to induce resistance.

In order to test the hypothesis that leptin resistance is a consequence of insufficient availability of leptin at the site of action, the objective of the study in Chapter 4 was to induce leptin production locally in the hypothalamus and to determine the efficacy of sustained leptin elevation within the CNS on BW and FI in adult lean \textit{wt}
Sprague-Dawley male and female rats. rAAV vector encoding leptin or GFP was delivered directly into the CSF of these rats. We observed effective transduction of neurons in the hypothalamus with GFP indicating successful transgene delivery at six weeks post-injection. The spread of transduced cells around the third ventricle was in areas associated with the regulation of appetite such as the ARC, mediobasal hypothalamus and the posterior hypothalamus. A significant elevation in leptin mRNA in the medial basal hypothalami of rAAV-leptin treated rats confirmed the successful use of rAAV-vector to induce local production in the hypothalamus. This centrally produced leptin was effective in maintaining body weight in Sprague-Dawley rats for extended periods. We observed a significant decrease in BW with leptin treatment in both male and in female rats as compared with the control rats. This was due to attenuation of the rate of weight gain achieved by a single icv injection of rAAV-leptin. Contrary to reports in the literature, in our study the retardation in BW was not accompanied by a decrease in caloric consumption since FI in both the male and female rats was not reduced. These results raised the question as to how the weight loss was effected by leptin in these animals and argued against the hypothesis that leptin is predominantly an appetite suppressing hormone. The mechanism underlying BW regulation without associated changes in FI post icv rAAV-leptin injection was determined in experiments in Chapter 5.

With advancing age there is an increase in the propensity towards adiposity in humans as well as in rodents (Wolden-Hanson et al., 1999). This increase in adipose mass brings about increases in peripheral leptin levels which in turn leads to the development of leptin resistance followed often by development of obesity (Friedman and Halaas, 1998). Another important finding was that we did not observe the onset of
leptin resistance in either males or females at the end of the 12-week experiment. None
of the rAAV-leptin injected animals displayed any inclination towards developing
obesity. This strongly supports our hypothesis that leptin resistance is not a result of
sustained elevations in leptin or saturation of leptin receptors in the CNS, but due to
insufficiency of leptin at its target sites. Cumulatively, these results document the
efficacy of centrally administered rAAV-leptin gene therapy and reveal a dichotomy in
BW regulatory mechanisms by leptin that is independent of caloric consumption.

The retardation in age related weight gain via rAAV-leptin delivery into the CNS,
without development of leptin resistance in either male or female rats was also observed
when the experiment was extended to 24 weeks. Data in Chapter 5 demonstrate
suppression of normal weight gain for 24 weeks after a single icv rAAV-leptin injection.
Untreated as well as rAAV-UF5 treated control rats displayed the normal weight gain
pattern during the 6 month study, with 25% gain of BW over the course of the study. In
female rats this age related BW gain was abolished with rAAV-leptin treatment whereas,
in male rats the rate of gain of BW was retarded. This is similar to the short-term data
presented in Chapter 4 demonstrating maintenance of BW in females at their initial
values, while males continue to grow, albeit at a slower rate than the control rAAV-UF5
treated males. This sex-specific divergence in response to rAAV-leptin may be explained
by the "fat melting" action of leptin (Chen et al., 1996). Leptin acts to reduce BW by
specifically targeting the adipose mass in the body, without affecting the lean mass (Chen
G et al., 1996; data in Chapter 4). As females have a higher fat mass compared with
males this may account for part of the difference in response to rAAV-leptin. Another
possible explanation could be the effective dose of rAAV-leptin in the different sexes.
Since both male as well as female rats received the same actual dose, the males with a 30% higher initial BW received a 30% lower relative dose. It is possible that increasing the relative dose in male rats would produce the same changes in BW as displayed by the female rats.

Central rAAV-leptin resulted in a drastic suppression of peripheral leptin levels. Blood leptin levels were significantly attenuated in rAAV-leptin treated male and female rats versus their controls at all time points examined. Interestingly, we observed that while in control rats leptin levels continued to increase in correlation with advancing age and adiposity, the rAAV-leptin treated rats of both sexes did not show this age related increase suggesting a protective effect of rAAV-leptin on the aging process. The idea that icv leptin may protect against age related changes is further supported by our observation of a significant decrease in serum insulin levels at 6 months post rAAV-leptin injection. Serum insulin levels are higher with advancing age (Wolden-Hanson et al., 1999) and are associated with insulin resistance and impaired glucose tolerance which contribute to the development of NIDDM with age (Porte Jr. et al., 2000). Rising insulin levels in the periphery correlate with rising adiposity and leptin levels in the periphery. Leptin has been suggested to inhibit insulin release by a direct action on the pancreatic β cells. However, in our study peripheral levels were markedly reduced yet no "breakthrough" increase in insulin was noted. This suggests that the insulin regulating effect of leptin is manifested centrally and may be transmitted via the SNS to the pancreas.

The reduced blood insulin levels in our model associated with normoglycemia in both sexes point towards an increase in insulin sensitivity. The insulin sensitizing actions of leptin have been previously reported (Chen et al., 1997; Kahn and Flier 2000).
however, it is not known whether this is due to leptin action in the CNS or if leptin directly acts on target organs such as the pancreas in the periphery to affect insulin sensitivity. In pancreatic β cells and in muscle leptin increases lipid oxidation and inhibits lipid synthesis, factors known to promote insulin sensitivity. Although leptin could affect insulin sensitivity via its effects on decreased appetite and reduced nutrient flux, this is unlikely to be the case in our studies in the absence of any effects on food intake. Alternatively leptin may enhance insulin sensitivity by modulation of neuroendocrine effectors such as glucocorticoids, which are increased by icv leptin and are known to enhance insulin sensitivity. In the presence of reduced peripheral leptin, our data clearly add support to the idea that the site of action of leptin to regulate insulin sensitivity resides within the hypothalamus since in our model enhanced CNS leptin levels resulted in enhanced peripheral insulin sensitivity. The molecular basis for the insulin sensitizing effect of leptin remains to be explored further.

BAT is the principal anatomic location of non-shivering thermogenesis (Himms-Hagen 1985). Although BAT constitutes only ~1% of the total body mass in the adult rodent, this tissue contributes as much as one third to thermogenesis during cold exposure (Foster and Frydman 1979). Leptin increases sympathetic outflow to BAT in ob/ob mice suggesting that the mechanism of increased energy expenditure may involve increased thermogenesis in this tissue (Collins et al., 1996). In order to elucidate the basis for the weight loss in the absence of an obvious decrease in FI we examined UCP-1 expression in BAT. We detected a 2 fold up-regulation in UCP-1 mRNA levels at 24 weeks post rAAV-leptin injection in male and female rats. The magnitude of UCP-1 induction did not vary in a sex specific manner nor was it dose dependent (Chapter 6). These results
confirm the effects of leptin administration or central leptin infusion on enhancing UCP-1 expression in BAT (Scarpace et al., 1997, Cusin et al., 1998). Our results demonstrated that the prolonged BW maintenance action of icv rAAV-leptin in male and female rats in the absence of reduced energy intake may be due to increased energy expenditure in the form of non-shivering thermogenesis implied by the dramatic increase in UCP-1 expression in BAT.

In Chapter 6 we examined the dose dependent effects of icv rAAV-leptin and investigated the effects of rAAV-leptin on hypothalamic expression of NPY, POMC and AGRP, neuropeptides involved in the regulation of appetite. Two doses of rAAV-leptin were delivered icv in lean female Sprague-Dawley rats and the effects on BW, FI and potential development of resistance to leptin action were studied. The lower dose was the same dose tested in Chapters 4 and 5. In the short-term six week experiment in Chapter 6, we confirmed our earlier observation of maintenance in BW in the initial range with a single injection of rAAV-leptin. However, a 2-fold increase in rAAV-leptin dose caused a significant decline in BW; the final BW with the high dose of rAAV-leptin being 14% below initial BW while the controls gained 13%. As anticipated, there was a decline in BW in the pairfed controls; a group fed a restricted diet to match the caloric intake by the high dose rAAV-leptin treated rats. Interestingly although the PFC rats lost weight initially, at week 4 post injection in spite of the still reduced caloric intake these animals seemed to be rebounding to their initial weight, in fact the weight of the PFC rats was 3% higher than their starting weight. This "rebounding" in BW can perhaps be explained by the compensatory mechanisms that exist in the body to maintain the BW at a certain "set point." A dose dependent effect on FI was also noted. There was a significant 15%
decrease in FI with the increased dose of rAAV-leptin. This decreased FI possibly accounts for the increased weight loss in this group.

Serum leptin levels were also significantly decreased with the lower dose of rAAV-leptin, as seen in the earlier experiments. The depletion of serum leptin was greater in the higher dose. Negative feedback effects of elevated leptin levels in the CNS combined with the greater fat loss may account for the almost total absence of peripheral leptin in the high dose group. In obese individuals a decreased CSF: serum leptin ratio is observed implying decreased availability of leptin in the CNS (Considine et al 1995). Contrary to expectation, despite the severely depleted serum leptin levels in rAAV-leptin treated rats the CSF leptin levels were not diminished. Secretion of leptin by the cells transduced with rAAV-leptin in the hypothalamus may account for maintaining CSF leptin levels. With both doses we observed a significant elevation in the CSF: serum leptin ratio, opposite to that seen in the leptin resistant state. We can thus state that chronic central leptin elevation does not lead to the development of leptin resistance.

Serum insulin levels were unchanged with the single injection of rAAV-leptin at six weeks post injection; however, with the higher dose of rAAV-leptin, serum insulin levels were significantly diminished, along with a significant reduction in serum glucose levels. As discussed above, this may be due to the insulin sensitizing effects of leptin in the CNS.

To facilitate our understanding of the difference in the mechanism of action of the two doses we studied the effect of icv rAAV-leptin on serum thyroid hormones. The thyroid hormones regulate the resting metabolic rate in rodents as well as in humans (Jekabsons et al 1999). Leptin has been reported to enhance metabolism by increasing
the turnover of the less potent thyroid hormone T4 to the active form T3 (Cusin et al., 2000). There was no effect of a single injection of rAAV-leptin on circulating T3 or T4 at week 6 post-injection, however, there was a significant decrease in serum T4 levels with the higher dose rAAV-leptin suggesting increased conversion of T3 to T4. Thus, increased turnover of T4 to T3 may contribute to the leptin induced increase in energy expenditure. However, to unequivocally establish the effects of leptin on thyroid hormones it may be necessary to examine the hypothalamic-pituitary-gonadal axis including the analysis of hypothalamic thyroid releasing hormone mRNA levels and plasma TSH levels in response to leptin. Also, the activity and expression of hepatic 5'-monodeiodinase (Type I), the main enzyme responsible for converting T4 to T3 might shed light on the nature of modulation of thyroid hormones by leptin. Interestingly, in experiments in Chapter 5 circulating T3 and T4 levels at 16 weeks post injection were not affected by rAAV-leptin treatment. Taken together, these results suggest that in the short-term, leptin does augment thyroid hormones, however, by 16 weeks post-injection compensatory changes to rAAV-leptin may have normalized the thyroid hormone responses.

The UCP-1 responses to chronic leptin enhancement were described in Chapter 5, in Chapter 6 we sought to establish a dose dependent response to icv rAAV-leptin treatment. We found a 2 fold induction in UCP-1 mRNA with both doses of rAAV-leptin, but surprisingly the induction was not greater in the higher dose of rAAV-leptin treated rats. This leads us to speculate that the lowered dose elicited the maximal response in UCP-1 induction. We did not observe any changes in skeletal muscle UCP-3
expression in any of our experiments; thus it appears that only the thermogenic activity of BAT UCP-1 is involved in leptin's effects on increasing energy expenditure.

We further examined the expression of a few neuropeptides known to regulate appetite. NPY is a powerful stimulator of feeding (Clark et al., 1984). A sub-population of ARC NPY neurons express the long form of the leptin receptor and these cells respond specifically under conditions when circulating leptin levels are altered (Basin et al., 1999). There is firm evidence that leptin inhibits NPY mRNA expression and decreases NPY expression in the ARC (Stephens et al., 1995). The NPY neurons are, therefore, potential targets of leptin in the hypothalamus and the ability of leptin to induce hypophagia and weight loss is partly due to leptin induced inhibition of NPY synthesis and secretion. In our studies we demonstrate decreased NPY mRNA expression in the ARC only with the high dose of rAAV-leptin without any changes in the low dose. We did not quantitate the effects of rAAV-leptin on NPY mRNA in other areas of the hypothalamus. Since the DMN NPY producing neurons are also involved in regulation of appetite, it is possible that there were changes associated with rAAV-leptin treatment in these areas as well.

Another system involved in the mediation of leptin's effects on appetite regulation is the melanocortin system. POMC is produced by neurons in the ARC and is the precursor of αMSH, a hypothalamic peptide that inhibits feeding. Leptin induces POMC mRNA expression and thus enhances αMSH levels, which inhibit feeding (Cheung et al., 1997). POMC mRNA is up-regulated by leptin treatment in ob/ob mice (Mizuno et al., 1998). We observed a significant up-regulation of POMC gene expression with the high dose of rAAV-leptin while the low dose did not affect POMC expression. Expression of
AGRP the orexigenic peptide, was not affected by either dose of rAAV-leptin. It can be speculated that the increase in αMSH levels following the up-regulation of POMC mRNA serves as the ligand for the melanocortin receptors to inhibit appetite. AGRP, the melanocortin receptor antagonist, apparently does not participate in the regulation of BW in this model.

Interestingly, the PFC rats included in the study did not show any differences in either NPY or POMC mRNA in the ARC. Since the serum leptin level in this group is not different from that observed in controls it is not surprising that there are no differences in the expression of appetite regulating neuropeptides. Although these animals were on a restricted diet to match that of the rAAV-leptin treated group, we observed a compensatory rebounding in the BW, hence, it is entirely possible that at the termination of the experiment, the neuropeptide changes associated with caloric restriction were also normalized.

In summary, AAV is able to effectively introduce leptin into leptin deficient ob/ob mice. This reinstatement of leptin has a dose dependent effect on decreasing body mass along with reduction in energy intake and enhancement of energy expenditure without induction of leptin resistance. We have confirmed the observation that the potency of leptin action is greater when delivered icv as opposed to peripheral iv delivery. Obese ob/ob mice that are supersensitive to the effects of leptin were given a greater dose peripherally than that given to wt rats centrally with similar outcomes in BW reduction. In experiments in Chapters 4, 5 and 6 we have demonstrated the successful transduction of cells with rAAV within the hypothalamus and expression of the leptin transgene delivered via rAAV-leptin in the hypothalamus at the primary site of leptin action. With
the discovery of leptin mRNA and protein in the hypothalamus (Morash et al., 1999) it is now likely that locally produced leptin may have an important role in the regulation of BW associated with increased leptin production in the hypothalamus. In our experiments, we have demonstrated phenotypic effects on BW in 6, 12 and 24 week studies without the development of leptin resistance. We have demonstrated that 24 weeks of sustained rAAV-leptin mediated local leptin production does not lead to a loss in the ability of the brain to respond to leptin. Even with a higher dose that induced a decrease in FI we observed the weight reducing response to rAAV-leptin. Thus leptin resistance is likely due to insufficient availability within sub-hypothalamic sites integral to the regulation of BW. Our data show that leptin levels in the CNS do not have to be supraphysiological to achieve BW reduction and fat depletion, yet at the same time we demonstrate the importance in delivering leptin to the appetite regulating sites in the hypothalamus. It is thus possible that local leptin production within the hypothalamus regulates BW and appetite. Thus leptin resistance could be a consequence of disruption of the local leptin production within the hypothalamus. One possible signal controlling CNS production of leptin could be the blood leptin levels. An increase in peripheral leptin level beyond a set threshold could then conceivably initiate a chain of events that render the brain unresponsive to leptin with disruption of the local leptin production. This in turn may lead to insufficiency at certain target sites critical in BW regulation by leptin and eventually obesity.

We have effectively demonstrated a dichotomy in the response to rAAV-leptin. A high dose icv as well as iv elicits weight loss through reduction in FI. In Sprague-Dawley rats, the lower dose produced significant increases in energy expenditure in form
of activation of UCP-1 mRNA in the thermogenic organ BAT only. The higher dose caused changes in appetite regulating neuropeptides AGRP, NPY and POMC and this resulted in decreased FI along with UCP-1 mRNA expression. These dose response data suggest that leptin maintains BW by primarily increasing energy expenditure; changes in appetite may be secondary effects of leptin seen only when the dose is sufficiently high.

A major outcome of our studies is that by circumventing the peripheral effects of hyperleptinemia and leptin-resistance imposed by the blood barrier in \textit{wt} lean adult Sprague-Dawley rats, a new strategy has been identified that is effective, long lasting, non-pathogenic and hence therapeutically relevant for the control of weight gain in the normal population and for reducing and maintaining weight in patients genetically prone to obesity. A single central injection of rAAV-leptin may, on a long-term basis, provide an alternative to pharmacological approaches once the clinical feasibility and safety of this strategy is established. This therapy however, needs further testing in large animals such as non-human primates before it can be applied towards the treatment of human obesity.
Central leptin gene therapy

Dose dependent changes in BW and FI

Low Dose
Maintain BW ↔ FI

High Dose
↓BW  ↓FI

Depletion of WAT
Increased energy expenditure (UCP-1 ↑)

Fig 7-1. Summary Diagram
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BIOGRAPHICAL SKETCH

Harveen Dhillon was born in Saharanpur, UP, India, on the 20th of September, 1971, to Abnash and Jagdish Dhillon. She spent her childhood moving every two years as demanded by her father's military career. Harveen and her two younger siblings were adept at making friends in every new town in India that they made their home in. She had studied in 11 different schools by the time she graduated high school. Harveen attended Miranda House College at the University of Delhi, Delhi, India, where she received her BSc (Hons) degree in zoology in 1992. Harveen went on to graduate school at the University of Arizona at Tucson, AZ, where she graduated with an MS in animal sciences in 1996, following which she joined the University of Florida's Interdisciplinary Program in Biomedical Sciences in August 1996, where she pursued doctoral studies under the guidance of Dr Pushpa Kalra. Harveen found a home away from home in Gainesville in the Kalra lab and in the departments of Physiology and Neuroscience where she made enduring friendships. Harveen married Parakh Hoon in December 1997 in India, with whom she has spent three happy years. She hopes to travel with him to Africa during the course of his doctoral studies in political science in the coming year, and anywhere his journeys might take him later in life. In the future, she is looking forward with renewed excitement to pursuing a full time career in science, and aspires to sharpen the skills acquired during the course of her graduate studies.