

LEPTIN ANTAGONIST AND SOLUBLE LEPTIN RECEPTOR BLOCK LEPTIN ACTION
IN VIVO: EVIDENCE FOR AN ESSENTIAL ROLE OF LEPTIN
IN HOMEOSTATIC ENERGY REGULATION

By

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To my husband, Jack Shao, for his company and support through happiness and hardship
during my graduate study

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One-third of the US adults are obese, contributing to serious health issues. Leptin is an adipocyte-derived hormone that acts on the satiety center in the hypothalamus to suppress food intake and promote energy expenditure. The dissertation employed a leptin antagonist and soluble leptin receptor to block leptin action, *in vivo*, in order to dissect the role of leptin in energy regulation.

First, we verified that the leptin antagonist was able to block leptin-induced signaling and anorexic responses, *in vivo*, whereas central infusion of leptin antagonist alone increased food intake and body weight. When leptin antagonist was infused simultaneously with high-fat (HF) feeding, the normally observed caloric normalization following HF-induced hyperphagia was completely prevented, indicating this process is leptin dependent. Additionally, serum leptin level was significantly elevated and maximal leptin signaling was blunted after 2 days of HF-feeding compared with chow-fed controls.

Next, we demonstrated that a soluble leptin receptor (SLR) was able to fully neutralize central leptin signaling, *in vivo*. Central infusion of SLR alone increased food intake and body weight, suggesting it can neutralize endogenous leptin in the brain. Peripheral leptin infusion

induced significant anorexic responses, which were partially prevented by two doses of central SLR infusion. Interestingly, the leptin-induced UCP1 increase in BAT and leptin expression decrease in EWAT were completely inhibited by the higher dose.

The long-term effects of SLR central delivery were examined by recombinant adeno-associated viral (rAAV) gene therapy in leptin-responsive rats. The rAAV-mediated SLR expression, secretion and leptin binding was first verified, *in vitro*. However, no physiological responses were observed for 65 days after rAAV administration despite persistent transgene expression as detected by RT-PCR. It is likely the SLR produced by rAAV was not sufficient to neutralize significant amounts of CSF leptin. Further studies are necessary to investigate the long-term effects of SLR by optimized gene therapy or prolonged pharmacological method.

In summary, this dissertation utilized a leptin antagonist and soluble leptin receptor to block leptin action *in vivo*, and demonstrated an essential role for leptin in homeostatic energy regulation, in particular the counter-regulatory role of leptin in deterring high-fat feeding.

CHAPTER 1 BACKGROUND AND SIGNIFICANCE

The Obesity Epidemic

The obesity epidemic has increased dramatically in the U.S. over the past 20 years. More than one out of three U.S. adults—over 72 million people—are obese, defined as having a body mass index (BMI) of 30 or higher. The prevalence of obesity in children is also on the rise, with 16.3% of children and adolescents aged 2–19 years being obese over the period between 2003 and 2008 (Ogden *et al.*, 2008). It is estimated that obesity results in 300,000 deaths per year in the United States (Kopelman, 2000). Over 9% of total annual U.S. medical expenditures are accounted for by costs attributed to both overweight and obesity (Finkelstein *et al.*, 2003).

The Health Consequences of Obesity

Obesity is the excessive accumulation of adipose tissue to an extent that health is impaired (Aronne, 2002). Obesity generally results from an imbalance of food intake over energy expenditure (Cooney and Storlien, 1994). The cause of obesity is probably multi-factorial, often involving diet, genes and psychological factors (Proietto and Thorburn, 1994). Being overweight can reduce the quality of life, by triggering osteoarthritis, gout and sleep apnea. More importantly, obesity increases the risk of diseases and health conditions, including type 2 diabetes, hypertension, cardiovascular disease, stroke and certain types of cancer (Khaodhjar *et al.*, 1999).

Whole Body Energy Balance

The development of obesity is characterized by an imbalance between energy intake and energy expenditure. An increased energy intake, when not accompanied by increased energy expenditure, will result in a positive energy balance and an increase in body weight. On the other

hand, decreased energy expenditure, with aging for example, if not accompanied by decreased energy intake, will also lead to a positive energy balance.

High-Fat Diets

The increasing prevalence of obesity is often ascribed to the changes of life style in Western societies, among which is the consumption of a high-fat (HF) diet. High-fat diets are usually characterized by a high energy density and high palatability and promote hyperphagia and weight gain (Lissner *et al.*, 1987; Blundell *et al.*, 1993; Lissner and Heitmann, 1995; Westerterp *et al.*, 1996). Chronically HF-fed rats become obese and leptin resistant (Wilsey and Scarpace, 2004). Furthermore, it is demonstrated that HF-fed animals, independent of whether they get obese or not, have metabolic and hormonal disorders (Woods *et al.*, 2004).

Decreased Energy Expenditure

As the energy equation is composed of both energy intake and energy output, a decrease in physical activity has also been proposed to be responsible for the increasing prevalence of obesity in westernized societies. Increased automation in work and living lowers energy expenditure, and if not compensated for by elevated physical exercise, results in a positive energy balance even when caloric intake remains unchanged.

Complex Mechanisms of Energy Homeostasis

As the prevalence of obesity increases at an epidemic rate, a significant amount of research has been directed to understanding the physiology and underlying molecular mechanisms regulating body weight. The regulation of energy homeostasis is highly integrated, involving the interaction between central nervous system (CNS) and peripheral organ systems, such as white adipose tissue (WAT), gastrointestinal tract, thyroid, muscle, and reproductive organs (Foster-Schubert and Cummings, 2006; Lopez *et al.*, 2007). Circulating factors including nutrients, hormones and cytokines as well as afferent neural signals inform the brain of the nutritional

storage and metabolic status of the body. For example, insulin secretion by the pancreas increases rapidly after a meal, exerting an anorexic effect via the CNS (Schwartz *et al.*, 2000). By contrast, plasma levels of ghrelin, an orexigenic hormone secreted by the stomach, go up shortly before meals, signaling the brain to prepare for meal initiation (Cummings *et al.*, 2001). White adipose tissue (WAT), once considered simply for fat storage, is now recognized as an important endocrine organ. White adipose tissue secretes a family of adipokines that are actively involved in energy regulation, such as leptin, adiponectin and interleukin-6. In rodents, adiponectin levels increase after food restriction and peripheral administration reduces body weight by increasing oxygen consumption without affecting food intake (Berg *et al.*, 2001; Berg *et al.*, 2002). Interleukin-6 is a multifunctional immune-modulating cytokine and central administration was shown to reduce food intake (Wallenius *et al.*, 2002). However, among all adipose hormones, one of the most studied is leptin, which is a key regulator of energy balance.

Leptin

Leptin in Energy Regulation

Leptin, the *obese (ob)* gene product, is a 16 kD peptide hormone primarily produced in the white adipose tissues (WAT). Other sources of expression include brown adipose tissue (BAT) (Maffei *et al.*, 1995a), skeletal muscle (Wang *et al.*, 1998), stomach and placenta (Masuzaki *et al.*, 1997). Leptin is secreted into the circulation and transported across the blood brain barrier (BBB) via a saturable transport system in order to reach target sites in the brain (Banks *et al.*, 1996). Leptin acts within several sites in the brain, including the satiety center in the hypothalamus, leading to reduced appetite and increased energy expenditure (Friedman and Halaas, 1998). Leptin levels in the circulation are generally in proportion to whole body fat mass, therefore leptin serves as one afferent signal of nutritional status to the brain (Maffei *et al.*, 1995b; Considine *et al.*, 1996). While obesity, insulin, tumor necrosis factor-alpha and

glucocorticoids induce leptin expression, fasting and sympathetic stimulation reduce its synthesis (Sweeney, 2002).

Congenital Leptin Deficiency

Mice carrying homozygous recessive mutations of the *ob* gene, known as *ob/ob* mice, exhibit a profound obesity resulting from defects in energy expenditure, elevated food intake and altered nutrient partitioning. They also display several metabolic and neuroendocrine abnormalities (Bray and York, 1979; Tartaglia *et al.*, 1995). Congenital leptin deficiency in humans is extremely rare, but several cases have been reported. Two severely obese cousins of Pakistani origin were found to be homozygous for a frame-shift mutation in the *ob* gene (Δ G133), which results in a truncated protein that is not secreted. As a result, serum leptin levels were undetectable in both children (Montague *et al.*, 1997; Rau *et al.*, 1999). Another homozygous missense mutation was found in a large Turkish family, which are characterized by severe, early-onset obesity and intense hyperphagia (Farooqi *et al.*, 1999). Dramatic and beneficial effects have been observed for leptin treatment in these obese leptin-deficient children, in which daily leptin injection significantly reduced body weight and fat mass and effectively normalized hyperphagia (Farooqi *et al.*, 2002).

Other Physiological Functions of Leptin

In addition to its role in energy balance, leptin has diverse physiological functions, including metabolism, reproduction, immunity, angiogenesis, blood pressure regulation and bone growth (Fruhbeck, 2001). Long-term injections correct infertility in *ob/ob* mice (Chehab *et al.*, 1996; Mounzih *et al.*, 1997) and leptin also accelerates the onset of puberty in normal mice (Chehab *et al.*, 1997). Leptin enhances the production of cytokines by macrophages (Gainsford *et al.*, 1996) and also modulates T-cell proliferation (Lord *et al.*, 1998). Leptin promotes blood vessel formation (Sierra-Honigmann *et al.*, 1998) and accelerates wound healing (Ring *et al.*,

2000). Leptin increases sympathetic nerve activity into heart, kidney and adrenal (Haynes *et al.*, 1998) and hyperleptinemia has been linked to endothelial dysfunction (Knudson *et al.*, 2008).

Free and Bound Leptin in Circulation

Leptin circulates both in protein bound and free forms in blood (Sinha *et al.*, 1996), whereas only free leptin is detected in CSF, suggesting that free leptin is the biologically active form (Landt *et al.*, 2000). In lean subjects, the majority of leptin circulates in the bound form. Conversely, in obese subjects, the majority of leptin circulates as free leptin (Sinha *et al.*, 1996), which is consistent with the notion that leptin binding capacity in blood is saturated due to the elevated leptin levels associated with obesity. Therefore, leptin binding proteins may modulate bioavailability and bioactivity of leptin in circulation. Studies have demonstrated putative leptin binding proteins with various molecular weights, and one of these, the soluble leptin receptor is determined to be the major leptin binding protein in human circulation (Lammert *et al.*, 2001). Details of the soluble leptin receptor are discussed later in this chapter.

Leptin Receptor

Isoforms

First cloned in 1995, the leptin receptor is a single membrane-spanning receptor of the class I cytokine receptor family (Tartaglia *et al.*, 1995). There are multiple isoforms of leptin receptors (Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re and Ob-Rf) due to alternative RNA splicing, which occurs at the most C-terminal coding exon (Lee *et al.*, 1996; Wang *et al.*, 1996). Therefore, leptin receptor isoforms are identical throughout the entire length of the extracellular domain, with their intracellular domains differing in length and sequence composition (Lee *et al.*, 1996; Tartaglia, 1997). The long form leptin receptor, Ob-Rb, has a long intracellular domain with full signaling capacity and is expressed predominantly in the hypothalamus (Friedman and Halaas, 1998), although limited expression has been detected in the adrenal gland (Takekoshi *et*

al., 1999), the intestine (Morton *et al.*, 1998) and both brown and white adipose tissue (Siegrist-Kaiser *et al.*, 1997). Ob-Ra, the short form leptin receptor, has a much shorter intracellular domain devoid of signaling capacity. Ob-Ra is widely expressed in peripheral tissues as well as in the choroid plexus (Tartaglia *et al.*, 1995) and the cerebral microvessels (Bjorbaek *et al.*, 1998) and this form of the receptor is proposed to serve as a leptin transporter across the BBB (Hileman *et al.*, 2002). A soluble form of the leptin receptor, Ob-Re, which lacks the transmembrane domain, will be discussed in detail later in this chapter.

Differential Expression in the Brain

Leptin receptors are widely expressed in the brain, and sites of dense mRNA expression include hypothalamus, thalamus, choroids plexus and cerebellum. However, there is great isoform specificity to the expression pattern. *In situ* hybridization identified the highest density of Ob-Rb in the hypothalamus and cerebellum in humans (Burguera *et al.*, 2000) and rodents (Bjorbaek *et al.*, 1998; Elmquist *et al.*, 1998a). Within the hypothalamus, Ob-Rb is predominantly expressed in the neuronal populations, with high density in the arcuate nucleus (ARC), the ventromedial hypothalamic nucleus (VMH) and the dorsomedial hypothalamic nucleus (DMH) and with moderate density in the lateral hypothalamic area (LH) (Elmquist *et al.*, 1998a). All these regions are known to regulate feeding behavior, adding further evidence in support of a hypothalamic site of leptin action in energy regulation. However, relatively little hybridization was detected in the paraventricular nucleus of the hypothalamic (PVN), despite its influences in feeding and pituitary gland function (Elmquist *et al.*, 1998a). It is apparent that leptin activation of PVN is due to both direct receptor action and innervation from Ob-Rb dense regions, including ARC, VMH and DMH (Thompson *et al.*, 1996). Hybridization of Ob-Rb is also detected in brain regions outside the hypothalamus, with highest density in the cerebellum and moderate density in substantia nigra and several thalamic nuclei (Elmquist *et al.*, 1998a).

The presence of leptin receptor in these regions indicates the possible involvement of leptin in sensory, motor and association functions, which may modulate feeding behaviors in a reward-related paradigm. Short-form leptin receptors, Ob-Ra in particular, are detected at highest concentration in choroid plexus, microvessels and meninges (Bjorbaek *et al.*, 1998; Elmquist *et al.*, 1998a). High levels of short-form leptin receptors at barriers of the brain support the possible role of Ob-Ra in mediating leptin transport into or out of the CNS.

Leptin–Leptin Receptor Binding

All isoforms of the leptin receptor share the identical extracellular domain. Analysis of the amino acid sequence revealed that the extracellular domain is divided into several subdomains: 1) The signal peptide, amino acid 1–21; 2) N-terminal cytokine receptor homologous domain 1 (CRH1), amino acid 22–328; 3) an immunoglobulin-like domain (IGD), amino acid 329–427; 4) a second CRH domain, CRH2, amino acid 428–637; and 5) two consecutive F3 domains, amino acid 636–841. Further mutation studies have identified CRH2 domain as the major leptin-binding domain (Fong *et al.*, 1998). However, the mechanisms of leptin–leptin receptor clustering and receptor activation are largely unknown. Studies using resonance energy transfer in living cells suggested that the leptin receptor exists as preformed dimers (Couturier and Jockers, 2003) and leptin stimulation leads to *de novo* association of pre-dimerized receptors in addition to a conformational change (Biener *et al.*, 2005). Although previous studies have supported the 1:1 stoichiometry for leptin–leptin receptor binding (thus 2:2 in the activated complex) (Devos *et al.*, 1997), Tavernier and his group proposed a 2:4 model in which one leptin is bound to two leptin receptors and two such complexes then form a hexamer (Zabeau *et al.*, 2004). They further demonstrated that mutations in the IGD domain almost completely abolished leptin signaling, suggesting that in addition to the CRH2 domain, the IGD domain in the leptin

receptor also binds to leptin, mediating the formation of hexameric complex (Peelman *et al.*, 2006).

Intracellular Domain of Leptin Receptor

All isoforms of leptin receptor can be divided into three groups, based on the size of their intracellular domain: long (Ob-Rb), short (Ob-Ra, Ob-Rc, Ob-Rd and Ob-Rf) and soluble (Ob-Re). The intracellular domain of Ob-Rb contains approximately 306 amino acids, including a Box 1 consensus sequence involved in the interaction with Janus tyrosine kinases (JAKs) and a Box 2 consensus sequence, which is required for the binding of signal transducers and activators of transcription (STATs). Both Box 1 and Box 2 are conserved in the class I family of cytokine receptors, and are necessary for signal transduction. By contrast, the intracellular domain of all short form leptin receptors ranges from 30-40 amino acids, which only contains the membrane-proximal Box 1 sequence. Because the truncated intracellular domain lacks the STAT binding motif, short form leptin receptors are considered incapable of mediating signal transduction (Tartaglia *et al.*, 1995; Lee *et al.*, 1996).

Leptin Receptor Deficiency

Mutations in leptin receptor result in an obese phenotype very similar to that of leptin deficiency. In C57BL/Ks db/db mice, the mutation creates a new splice donor site (AGGTAAA) that inserts a premature stop codon into the cytoplasmic region, resulting in the replacement of the Ob-Rb isoform by Ob-Ra (Lee *et al.*, 1996). In Zucker fatty (*fa/fa*) rats, a missense mutation was found in the extracellular domain of all the leptin receptor isoforms, which resulted in an amino acid change from Glutamine to Proline at amino acid position 269 (Iida *et al.*, 1996; Phillips *et al.*, 1996). The *fa* leptin receptor displays reduced leptin binding affinity and signal transduction capacity. There has been only one reported case of leptin receptor mutation in humans (Clement *et al.*, 1998). Affected subjects were from the same consanguineous family and

all three individuals were found homozygous for a mutation that truncates the receptor before the transmembrane domain. Such mutation suggests that these subjects are deficient in all cell surface leptin receptors. The phenotype has similarities to leptin deficiency, but some unique endocrine defects suggest the loss of leptin receptor results in a more-severe neuroendocrine phenotype than loss of leptin itself.

Leptin Receptor Signal Transduction

Like other class I cytokine receptors, leptin receptor does not have intrinsic kinase activity and signal transduction is dependent on Janus tyrosine kinase 2 (JAK2) that associates with the receptor. Upon ligand binding to the receptor, JAK2 autophosphorylates and then activates the leptin receptor by phosphorylating three tyrosine residues (Tyr 985, 1077 and 1138) on the intracellular domain. There are three distinct signaling pathways associated with the phosphorylated receptor.

JAK2-STAT3 Pathway

The class I cytokine receptors are known to signal via the JAK-STAT pathway. Shortly after the cloning of leptin receptor, JAK2-STAT3 was established to be the major pathway of leptin signaling in the hypothalamus (Vaisse *et al.*, 1996). When leptin binds to leptin receptor, JAK2 autophosphorylates and it in turn phosphorylates tyrosine residues on the leptin receptor, including Tyr1138. Tyr1138 is critical for the recruitment and phosphorylation of STAT3 as mutations of Tyr1138 completely abolish STAT3 signaling. Once STAT3 is recruited to Tyr1138 via its Src homology 2 (SH2) domain, it is phosphorylated by JAK2. Activated STAT3 dissociates from leptin receptor, dimerizes and subsequently translocates into the nucleus to regulate gene transcription (Myers, 2004). Homozygous mutation of Ob-R in which Tyr1138 is replaced by Serine generated mice (*s/s*) that are hyperphagic with reduced energy expenditure, resulting in massive, early-onset obesity. Similarities in phenotype between *s/s* and *db/db* mice

underline the importance of JAK2-STAT3 pathway in body weight regulation. However, unlike *db/db* mice that are infertile and display retarded linear growth, *s/s* mice are fertile and demonstrate increased linear growth, suggesting reproduction and linear growth is regulated by STAT3 independent signaling (Bates *et al.*, 2003).

ERK Pathway

The phosphorylation of Tyr985 recruits the SH2-containing tyrosine phosphatase SHP-2, which is then phosphorylated and leads to the activation of extracellular signal-regulated kinase (ERK) signaling pathway in cultured cells. ERK is a serine/threonine kinase that belongs to the mitogen-activated protein kinase (MAPK) family and the physiological significance for its activation regarding energy regulation is still unclear. In addition to SHP-2, Tyr985 also interacts with suppressor of cytokine signal 3 (SOCS3), which mediates feed-back inhibition on STAT3 signaling (Bjorbak *et al.*, 2000). Mice homozygous for Tyr985 mutations are lean and demonstrate increased sensitivity to leptin, which is consistent with a role for the interaction between SOCS3 and Tyr985 in attenuating STAT3 signaling (Bjornholm *et al.*, 2007).

Phosphatidylinositol-3 Kinase (PI3K)-cAMP Pathway

In addition to the signaling pathways mediated by phosphorylation of tyrosine residues on the leptin receptor, leptin induces an insulin-like signaling pathway via JAK2. Activated JAK2 phosphorylates insulin receptor substrate (IRS) protein, which subsequently recruits and activates the PI3K (Niswender *et al.*, 2001; Zhao *et al.*, 2002). PI3K leads to the activation of phosphodiesterase 3B (PDE3B) and subsequent reduction in cyclic adenosine monophosphate (cAMP) levels in the hypothalamus. Central administration of PI3K inhibitors reversed the anorexic action of leptin (Niswender *et al.*, 2001), and administration of a PDE3B inhibitor also reversed the effect of leptin on food intake and body weight (Zhao *et al.*, 2002). These results

indicate that the PI3K-PDE3B-cAMP pathway is an important component of leptin signaling in the hypothalamus.

On top of these three pathways, the role of Tyr1077 is less characterized. Myers and his group have demonstrated that phosphorylation of Tyr1077 during receptor activation mediates the recruitment and activation of STAT5, and proposed an alternate leptin receptor signaling pathway in cultured cells (Gong *et al.*, 2007).

Negative Regulators

There are several negative regulators in the leptin signaling pathways. The most studied inhibitory signal is suppressor of cytokine signal 3 (SOCS3), which is induced by prolonged leptin receptor activation and mediates feedback inhibition. SOCS3 is induced by pSTAT3 signaling, and SOCS3, in turn, blocks leptin receptor–STAT3 signaling by binding to Tyr 985 via the SH2 domain (Bjorbaek *et al.*, 2000). Neuron-specific SOCS3 conditional knockout mice showed enhanced leptin-induced hypothalamic STAT3 phosphorylation and a suppression of food intake leading to greater body weight loss. Moreover, the SOCS3-deficient mice were resistant to high fat diet-induced weight gain and hyperleptinemia, and insulin-sensitivity was retained (Mori *et al.*, 2004). Protein tyrosine phosphatase 1B (PTP1B) inhibits leptin receptor signaling by preferentially dephosphorylating JAK2 (Myers *et al.*, 2001). Moreover, PTP1B is also shown to inhibit insulin signaling, by inactivating the insulin receptor (Bandyopadhyay *et al.*, 1997) and by dephosphorylating important substrates of insulin receptors (Goldstein *et al.*, 2000). PTP1B knockout mice are resistant to weight gain when fed a high-fat diet and display increased sensitivity to insulin (Zabolotny *et al.*, 2002).

Neuropeptides Regulated by Leptin

In the hypothalamus, the leptin receptor is found in at least two distinct populations of neurons. One population expresses neuropeptide Y (NPY) and agouti-related protein (AgRP) and

the other expresses pro-opiomelanocortin (POMC) (Elmqvist *et al.*, 1999; Schwartz *et al.*, 2000). In POMC neurons, leptin receptor signaling stimulates the synthesis of POMC and promotes firing of the neurons. POMC is processed to produce alpha-melanocyte-stimulating hormone (α -MSH), which decreases food intake by activating downstream melanocortin-3 and -4 receptors. In NPY/AgRP neurons, leptin receptor inhibits neuron firing and suppresses synthesis of these neuropeptides. NPY is an orexigenic hormone and AgRP is an inverse agonist of melanocortin-3 and -4 receptors. Therefore, leptin receptor signaling stimulates anorexigenic neurons and inhibits the orexigenic neurons.

Leptin Antagonist

The novel leptin antagonist that is employed in this dissertation is a triple mutant (L39A/D40A/F41A) of rat leptin (Protein Laboratory Rehovot, Israel). This antagonist maintains typical cytokine secondary structure and binds to the leptin receptor with a similar affinity as wild-type leptin. *In vitro* assays demonstrated that the antagonist inhibited leptin-induced phosphorylation of MAPK and STAT3 and also attenuated leptin-induced transactivation of luciferase reporter gene. In addition, it was able to inhibit leptin-induced proliferation of BAF/3 cells stably transfected with long-form leptin receptor. These results indicate that it is a true antagonist without agonist activity and specifically inhibits leptin action, *in vitro* (Niv-Spector *et al.*, 2005; Solomon *et al.*, 2006).

Soluble Leptin Receptor

Soluble Receptors in General

Leptin receptor is a member of the class I family of cytokine receptors. Several member of this receptor family have soluble isoforms of the receptor that are truncated before the transmembrane domain, and are therefore secreted into the circulation. Among them, one of the best characterized is the growth hormone binding protein (GHBP). GHBP is a soluble, truncated

isoform of the growth hormone receptor (GHR) that encompasses its extracellular domain, and it binds growth hormone (GH) with approximately the same affinity as the full-length receptor. GHBP is generated by proteolytic cleavage of the membrane-bound GHR or by alternative mRNA splicing. The GHBP modulates GH action through both inhibitory and enhancing mechanisms. By complexing with growth hormone, the GHBP buffers GH level in the circulation and delays its clearance. The GHBP also inhibits GH binding to membrane-anchored receptors by competing for ligand. Serum GHBP level is in parallel regulation with GHR under several physiological conditions and it is positively correlated with GH responsiveness. Because of its complex *in vivo* effects, the biological role of the GHBP is still not completely understood. The main clinical application of GHBP measurements is in the diagnosis of genetic growth hormone insensitivity (Laron syndrome) (Baumann, 1995; Baumann, 2001).

Soluble Leptin Receptor (SLR)

One isoform of leptin receptor, Ob-Re, is devoid of transmembrane domain, and therefore is termed the soluble leptin receptor. Similar to GHBP, it is generated by ectodomain shedding of membrane-anchored leptin receptor, but it can also be generated by alternative mRNA splicing, as observed in rodents (Li *et al.*, 1998; Maamra *et al.*, 2001; Ge *et al.*, 2002). SLR binds leptin with a similar affinity as the long form leptin receptor, and regulates the bioavailability of leptin in the circulation (Liu *et al.*, 1997). To this date, SLR has not been found in the brain, suggesting circulating SLR in the blood cannot pass the blood brain barrier. However, one study reported that mRNA for SLR was detected in the hypothalamus and in the cerebral microvessels that compose the blood brain barrier, suggesting SLR may be produced in the brain by alternative mRNA splicing (Tu *et al.*, 2008). Whether SLR is synthesized in the brain needs further investigation.

Physiological Relevance of SLR

In humans, obesity is associated with decreased levels of circulating soluble leptin receptors, whereas weight loss increases SLR levels (Laimer *et al.*, 2002; Ogier *et al.*, 2002). In other studies, however, obese and normal individuals were found to have similar levels of SLR in the circulation, but in the obese individuals, most of the SLR is populated with bound leptin, whereas in lean subjects most SLR binding sites are unoccupied, with only a small fraction of SLR associated with bound leptin (Wu *et al.*, 2002). Thus, the inability to upregulate SLR in parallel with leptin is a factor contributing to the elevated levels of free leptin with obesity, and could potentially be a factor contributing to the development of obesity. In addition, opposite modulation of circulating levels of leptin and SLR is observed by fasting and circadian rhythms. Fasting reduces leptin levels while increasing SLR levels. SLR follows a circadian rhythm inverse to that of leptin, with peak levels in the early to mid-afternoon, whereas leptin levels peak in the late evening. SLR levels are also regulated by gender, adiposity, hormones and leptin administration (Chan *et al.*, 2002).

SLR Modulation of Leptin

A number of studies have been focused on how SLR modulates leptin action. Gavrilova *et al.* reported that in mice, circulating leptin levels increase by almost 40-fold during late stages of pregnancy with no detectable increase in leptin production and such increase is associated with an increase in SLR level in the circulation (Gavrilova *et al.*, 1997). Similarly, overexpression of SLR in Zucker (*fa/fa*) rats leads to a parallel rise in circulating leptin level without an increase in leptin expression (Huang *et al.*, 2001). Both studies indicate that SLR prolongs the half-life of leptin in the circulation, presumably by binding leptin and protecting it from clearance. Moreover, mice studies using ¹²⁵I-leptin demonstrated leptin transport across the BBB is inhibited by SLR (Tu *et al.*, 2008). Collectively, these data suggest upregulation of SLR leads to

increased circulation of total leptin, but decreased availability of leptin in the brain. Cell culture studies indicate that SLR dose-dependently prevents leptin binding to its membrane receptor, with a corresponding decrease in leptin-mediated signaling (Yang *et al.*, 2004).

Brown Adipose Tissue (BAT) and Uncoupling Protein 1 (UCP1)

Leptin positively regulates energy expenditure, and one mechanism underlying the increased energy expenditure involves increased thermogenesis in brown adipose tissue. Brown adipose tissue serves as a thermogenic organ to defend body temperature in a cold environment. The thermogenic function is characterized by dense sympathetic innervation and vascularization, high lipolytic capacity and mitochondrial density as well as the unique expression of uncoupling protein 1 (UCP1). A mitochondrial carrier protein, UCP1 is inserted into the inner mitochondrial membrane and channels protons from mitochondrial intermembrane space back into the matrix. UCP1 uncouples ATP synthesis from mitochondrial respiration and dissipates the proton motive force as heat (Schrauwen *et al.*, 1999). Studies have demonstrated that UCP1 is essential for nonshivering thermogenesis in brown adipose tissue and leptin induction of UCP1 gene expression is dependent on sympathetic innervation (Scarpace and Matheny, 1998).

Leptin Resistance

Leptin Treatment in Human Obesity

When leptin was first cloned in 1994, there was great enthusiasm about its potential as a powerful therapeutic agent to treat human obesity. Humans that are deficient in leptin as a result of congenital mutations exhibit severe obesity, and administration of exogenous leptin induced significant weight loss and dramatic normalization of hyperphagia (Farooqi and O'Rahilly, 2005). However, serum leptin concentrations are already elevated in obese subjects with normal genetic background and exogenous leptin treatment demonstrated little effects (Heymsfield *et*

al., 1999). Except for rare genetic defects, human obesity is associated with hyperleptinemia and apparent leptin resistance (Heymsfield *et al.*, 1999).

Mechanisms

Similar to human obesity, although leptin administration causes weight loss in leptin deficient *ob/ob* mice and normal lean rodents, studies in obese rodents evoked minimal responses (Levin and Dunn-Meynell, 2002). Many rodent models of obesity have elevated circulating leptin as a consequence of their large fat mass, but they do not adequately respond to these increased leptin levels nor to exogenously administered leptin. This inability to respond to leptin is termed leptin resistance. The mechanism underlying leptin resistance remains a matter of debate, but most likely it is multi-factorial. The two hypotheses that have received the most attention are 1) failure of circulating leptin to reach its targets in the brain due to a limitation of leptin transport across the blood-brain barrier and 2) impaired central leptin signal transduction. Evidence supporting these mechanisms are the observations with both diet-induced obesity and aged-related obesity that, leptin responses were blunted with either peripheral or central administration of leptin, but to a greater extent following peripheral leptin administration (El-Haschimi *et al.*, 2000; Scarpace *et al.*, 2000; Scarpace *et al.*, 2001). Thus, leptin resistance may have both a peripheral and central component. The Scarpace lab has also demonstrated that leptin receptors are diminished with age (Scarpace *et al.*, 2001) and diet-induced obesity (Wilsey and Scarpace, 2004). Reduced leptin receptors may be contributing to the reduced maximal level of STAT3 phosphorylation in obese animals. In addition, the expression level of SOCS-3, an inhibitor of STAT3 phosphorylation, is elevated with age (Wang *et al.*, 2001) and with diet-induced obesity as well (Munzberg *et al.*, 2004). Thus, impaired STAT3 phosphorylation may be one factor in leptin resistance as well.

Role of Elevated Leptin Level in Leptin Resistance

Both serum and cerebrospinal fluid (CSF) leptin concentrations increase with body fat (Schwartz *et al.*, 1996). Growing evidence suggest that a prolonged exposure to exogenously administered leptin may result in the development of leptin resistance. Indeed, chronic subcutaneous infusion of leptin for 21 days in male Long-Evans rats desensitized the subsequent anorexic response to a peripheral leptin challenge (Martin *et al.*, 2000). Similarly, chronic central infusion in chow-fed male Sprague-Dawley rats results in attenuation of the leptin-induced anorexia (Sahu, 2002), indicating a leptin-induced hypothalamic leptin resistance. Both diet-induced and age-related leptin resistance are associated with elevated leptin levels, and the above findings suggest that the elevated leptin, in and of itself, may be the causative factor in the development of the leptin resistance. Further supporting this hypothesis are the results from several studies of the Scarpace lab using leptin gene delivery into the brain that generated sustained elevation of central leptin in the absence of either obesity or elevated serum leptin (Scarpace *et al.*, 2002a; Scarpace *et al.*, 2002b). In these studies, not only did the anorexic response to leptin completely attenuate, but also the energy expenditure response waned over time following chronic leptin delivery in both young and older rats. Furthermore, the leptin-induced leptin-resistant rats were completely unresponsive when challenged by intracerebroventricular injection of a supra-pharmacological dose of leptin, thus confirming their full hypothalamic leptin resistant state (Scarpace *et al.*, 2002a; Scarpace *et al.*, 2003). These findings suggest that prolonged leptin exposure may be one cause for the development of leptin resistance.

Caloric Restriction Reverses Leptin Resistance

In obese rats with high serum leptin, basal leptin signaling level is elevated by over two-fold whereas maximal signaling capacity is significantly reduced compared with normal rats

(Scarpace *et al.*, 2001; Wilsey and Scarpace, 2004). The change in leptin signaling is accompanied by a parallel decrease in the expression level of the leptin receptor in the hypothalamus (Wilsey and Scarpace, 2004), which may be one of the factors contributing to leptin resistance. Caloric restriction restores leptin receptor expression and central leptin signaling capacity in both aged-obese and young diet-induced obese rats (Fernandez-Galaz *et al.*, 2002; Wilsey and Scarpace, 2004). Interestingly, caloric restriction not only reduces adiposity levels, but also dramatically reduces serum leptin, usually to a much greater extent than predicted from the loss of adiposity (Shimokawa and Higami, 2001; Miyawaki *et al.*, 2002). However, it is unclear whether it is the reduction in serum leptin level or the decrease in adiposity that is the causative factor in restoring leptin responsiveness. To date, there is no study that has reduced leptin level independent of obesity and examined the effects on leptin responsiveness.

Recombinant Adeno-Associated Viral Gene Therapy

Wild Type Adeno-Associated Virus

Adeno-associated virus (AAV) is a small (25-nm), nonenveloped virus that packages a 4.7-kb linear single-stranded DNA genome. It belongs to the parvovirus family and is dependent on helper virus, either adenovirus or herpesvirus, for productive infection. In the absence of helper virus, AAV can establish latent infection by integrating into a 4-kb region on chromosome 19 (Kotin *et al.*, 1990). The AAV genome is characterized by two open reading frames (ORF) flanked by inverted terminal repeats (ITR) (Daya and Berns, 2008). The ITR forms a characteristic T-shaped hairpin structure, which is essential for AAV replication and genome packaging. The left ORF encodes the Rep proteins, which are important regulatory proteins in AAV replication and gene expression. The right ORF encodes the Cap genes, which produces three capsid proteins to form an icosahedral viral particle.

Recombinant AAV

Recombinant AAV vectors (rAAV) are engineered from wild-type AAV but with all viral coding sequences removed. The ITRs are maintained, as they are required for vector packaging. As viral sequence required for site-specific integration is also removed, rAAV vectors persist primarily as extrachromosomal elements. Increasing interest in rAAV as a delivery vehicle for gene therapy is justified by several advantages, including an apparent lack of pathogenicity of the virus, persistence of gene expression, and many available serotypes targeting a broad host and cell type. However, there are still a few limitations in using AAV vectors. 1) The size of the gene expression cassette is limited by the small genome size of AAV. A novel technique of *trans*-splicing AAV vectors has been used to increase vector capacity (Yan *et al.*, 2000), despite a reduced efficiency. 2) Gene expression has a slow onset, as second-strand synthesis is a rate-limiting step before the initiation of gene expression. The invention of self-complementary AAV (scAAV) vectors has bypassed the limitation, but maximal size of transgene is reduced by 50% (McCarty *et al.*, 2001). 3) Although random integration of rAAV has been observed at a very low frequency, possibility for insertional mutagenesis still remains.

Examples of Application

Gene delivery mediated by rAAV has become increasingly common in preclinical studies as well as in human clinical trials. Over forty clinical trials have been approved for a variety of diseases, including cystic fibrosis, hemophilia B, rheumatoid arthritis, Parkinson's diseases and others (Mueller and Flotte, 2008). In the Scarpace lab, rAAV vectors have been successfully applied to mediate central overexpression of leptin and POMC in rats (Scarpace *et al.*, 2002a; Li *et al.*, 2003; Li *et al.*, 2005). Vector delivery induced significant responses in food intake and body weight, and transgene expression was verified for up to 300 days post administration (Scarpace *et al.*, 2003). Thus, rAAV-based gene therapy is particularly useful for conducting

chronic pre-clinical animal studies to evaluate the long-term efficacy of an agent that is otherwise impractical to deliver by pharmacological means.

Central Hypothesis

In this dissertation, the major objective was to further understand the role of leptin in energy regulation. The availability of a leptin antagonist and soluble leptin receptor are powerful tools to study the role of leptin in physiological processes. Specifically, we were interested in using a leptin antagonist and soluble leptin receptor to block leptin action, *in vivo*, and examine the consequences in leptin-responsive rats. Therefore we put forward two major hypotheses. First, leptin is necessary for the normalization of high-fat feeding induced hyperphagia and this counter-regulatory response will be blocked by central delivery of a leptin antagonist. Second, soluble leptin receptor neutralizes leptin, *in vivo*, thus increasing food intake and body weight in leptin-responsive rats. The goals of this dissertation are, first, to characterize the leptin antagonist, *in vivo*; second, to examine the effects of leptin antagonist on caloric normalization following HF feeding; third, to characterize soluble leptin receptor, *in vivo*; and finally, to examine the long-term effects of leptin neutralization by soluble leptin receptor on energy regulation in leptin-responsive rats.

CHAPTER 2 GENERAL METHODS AND MATERIALS

Generation of Soluble Leptin Receptor Transgene

Rat Ob-Re is a splice variant from full-length leptin receptor, Ob-Rb. It is identical to Ob-Rb from amino acid (aa) 1–796, plus a distinct 10-aa sequence at the C-terminal (Takaya *et al.*, 1996). Thus, we first used rat hypothalamic total cDNA as a template to clone the 1–2388 bp (aa 1–796) sequence of Ob-Rb. The PCR primers used were: forward 5'- ATG GGT GTC TAT CTC TGA AGT AAG -3' and reverse 5'- CAT GGA TAT AAT ACT TGT TAA CAT T -3'. A second round of PCR added *NotI* site at the N-terminal and c-myc tag sequence (EQKLISEEDL), stop codon and *NotI* site at the C-terminal. The primer sequences were: forward 5'- CAT AGC GGC CGC ATG ACG TGT CAG AAA TTC TAT G and reverse 5'- GCG GCC GCC TAC AGA TCT TCT TCA GAA ATA AGT TTT TGT TCA TGG ATA TAA TAC TTG TTA AC. PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) was used for PCR amplification. The PCR products were cloned into pCR-BluntII-TOPO vectors (Invitrogen, Carlsbad, CA). The plasmids were verified by restriction enzyme digestion and DNA sequencing.

Construction of rAAV Vector Plasmid

SLR transgene was subcloned into pTR backbone by ligation at the *XhoI* and *HindIII* restriction enzyme sites. Flanked by the TRs, the expression cassette of pTR-SLR includes the following components, in 5' to 3' order: 1) the hybrid cytomegalovirus immediate early (CMV ie) enhancer, chicken beta-actin (CBA) promoter, exon 1 and chimeric intron (Dhillon *et al.*, 2001); 2) SLR transgene, containing c-myc tag at the C-terminal; 3) The woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) is placed downstream of the SLR transgene to enhance its expression (Loeb *et al.*, 1999); 4) and the poly A tail from bovine growth hormone.

The control vector, termed pTR-GFP, encodes the humanized version of green fluorescent protein (hGFP) (Owen *et al.*, 2002).

***In Vitro* Analysis of rAAV-SLR Plasmid**

The pTR-SLR construct was tested for *in vitro* expression of SLR transgene in mammalian cells. Human embryonic kidney 293T cells (HEK 293T) were transfected using Lipofectamine 2000 transfection reagent (Invitrogen), according to the protocol provided by the manufacturer. Six hours after transfection, media were replaced by 4% reduced serum media. Two day after transfection, media were collected and replaced with serum-free Opti-MEM I media (Invitrogen). One day after (3 days after transfection), the media were collected and cells harvested in RIPA buffer (Pierce, Rockford, IL). All media samples were concentrated 50-fold in Centricon-50 units (Millipore, Bedford, MA). The reduced-serum media samples were then separated on a 7.5% Tris-HCl polyacrylamide gel (Bio-Rad, Hercules, CA) and electrotransferred to nitrocellulose membrane. The blot was probed for SLR using a rabbit anti-c-myc tag polyclonal antibody (Cell Signaling, Danvers, MA) at a dilution of 1:1000. Cells were sonicated in RIPA buffer and centrifuged at 14,000 x g for 15 minutes. Cell lysate was examined for SLR by the same Western methods. Media and lysate of GFP plasmid transfected cells were processed in an identical fashion and used as a negative control.

Leptin Binding Activity *In Vitro*

Serum-free media from pTR-SLR or GFP transfected cells was concentrated by 50 fold and was either incubated in 65°C or kept on ice for 30 minutes. On day 1, 2 μL , 10 μL and 20 μL of the media was mixed with 50 μL ^{125}I -leptin and 50 μL rat leptin antibody (Rat leptin RIA kit, Linco Research, St. Charles, MO), adjusted to a total volume of 200 μL and incubated at room temperature for 24 h. On day 2, 0.5 mL of precipitating reagent containing secondary antibody was added to the mixture and incubated for another one hour. The samples were centrifuged at

2,500x g for 40 minutes at 4°C. Supernatant was aspirated and radioactivity in the precipitant was counted in a gamma counter. The assay was performed in duplicate and total binding (without media) and non-specific binding (NSB) were both included.

Packaging of rAAV Vector

To produce rAAV, rAAV plasmid was cotransfected with the helper plasmid pDG carrying the AAV rep and cap genes, as well as adenovirus helper genes required for rAAV replication and packaging (Grimm *et al.*, 1998). The vectors were purified by iodixanol density gradient followed by HiTrap Q HP (GE healthcare, Piscataway, NJ) chromatography and concentrated by centrifugation through Apollo 150 MWCO filter (Zolotukhin *et al.*, 1999). Titration of rAAV vector was carried out following a previously reported dot-blot protocol (Grimm *et al.*, 1998). The titer of rAAV-SLR was 1.85×10^{13} viral genome/mL. The control virus pTR-GFP, titered at 1.8×10^{12} viral genome/mL, was prepared by the vector core at the University of Florida.

Experimental Animals

Young adult (age 3–4 months) male Fischer 344 x Brown Norway rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN) for all experiments described in this dissertation. Upon arrival, rats were examined and remained quarantined for one week. Animals were individually caged with a 12:12 hour light:dark cycle (lights on from 07:00 to 19:00). Animals were cared for in accordance with the principles of the NIH *Guide to the Care and Use of Experimental Animals*. Protocols were approved by the University of Florida Institutional Animal Care and Use Committee.

Intracerebroventricular (i.c.v.) Injections

Rats were anesthetized with ketamine (75 mg/kg) and xylazine (7 mg/kg). The animal's head was prepared for surgery, and the animal placed in a stereotaxic frame. A small incision (1.5-cm) was made over the midline of the skull to expose the landmarks of the cranium (bregma

and lamda). The following coordinates were used for injection into 3rd cerebral ventricle: 1.3 mm anterior to bregma and 9.4 mm ventral from the skull surface on the midline (medial fissure), with the nose bar set at 3.3 mm below the ear bars (below zero) and the cannula set at 20° anterior to posterior. The following coordinates were used for injection into the left lateral ventricle: 1.3 mm posterior to bregma, 1.9 mm lateral to the midsaggital suture, and 3.5 mm ventral from the surface of the skull, with no angle. A small hole was drilled through the skull and a 23-gauge stainless steel guide cannula was inserted followed by an injection cannula. Using 10- μ L syringe, a 4- or 5- μ L volume was delivered over a 5-minute period.

Intracerebroventricular Infusions

Rats were anesthetized with 5% isoflurane by inhalation and maintained on 2.5% isoflurane when surgical platform was reached. The animal's head was prepared for surgery, and the animal placed in a stereotaxic frame. A small incision (1.5 cm) was made over the midline of the skull to expose the landmarks of the cranium (bregma and lamda). A brain infusion cannula (Durect Corporation, Cupertino, CA) was placed into the left lateral ventricle using the following coordinates: 1.3 mm posterior to bregma, 1.9 mm lateral to the midsaggital suture, and to a depth of 3.5 mm. The cannula was anchored to the skull using acrylic dental cement. A subcutaneous pocket on the dorsal surface was created using blunt dissection and an osmotic mini pump (Durect Corporation) was inserted. A catheter was employed to connect the cannula to the mini-osmotic pump flow moderator. The pumps (model 2001, Alzet, Durect Corporation, Cupertino, CA) infuse 1 μ L of fluid per hour for a minimum of 7 days and have a total capacity of 200 μ L. All prefilled minipumps were incubated in sterile saline at 37°C for 24 h before implantation. A catheter tube was employed to connect the cannula to the osmotic minipump flow moderator. The incision for the minipump was then closed with sutures. Rats were kept warm during the manipulation and until fully recovered.

Subcutaneous Infusions

Rats were anesthetized with 5% isoflurane by inhalation and maintained on 2.5% isoflurane when surgical platform was reached. The animal's back was prepared for surgery and a small incision was made over the midline on the dorsal surface. A subcutaneous pocket was created using blunt dissection and an osmotic minipump (Durect Corporation) was inserted. The incision was then closed with sutures. Rats were kept warm during the manipulation and until fully recovered.

Body Composition Measurement

Body composition was determined by time domain-nuclear magnetic resonance (TD-NMR) analyzer (Minispec, Bruker Optics, The Woodlands, TX). The MiniSpec measures whole body fat, lean and fluid mass on restrained but conscious animals in approximately 2 minutes. Validation of TD-NMR methodology has been provided (Tinsley *et al.*, 2004).

Tissue Harvesting and Preparation

Rats were euthanized by thoracotomy under anesthesia (5% isoflurane inhalation or pentobarbital injection 150 mg/kg). Blood samples were collected by heart puncture, and serum was harvested by a 10-min centrifugation in serum separator tubes. The circulatory system was perfused with 30 mL of cold saline. Perirenal (PWAT), retroperitoneal (RTWAT), and epididymal white adipose tissues (EWAT), interscapular brown adipose tissue (BAT), and the hypothalamus were excised. For removal of hypothalamus, an incision was made medial to the piriform lobes, caudal to the optic chiasm and anterior to the cerebral crus to a depth of 2-3 mm. The hypothalamus was sonicated in 10 mM Tris-HCl (pH 6.8), 2% SDS, and 0.08 µg/mL okadaic acid plus protease inhibitors. Protein concentrations were determined using the DC Bradford assay kit (Bio-Rad, Hercules, CA). BAT samples prepared similarly were filtered

through a 0.45- μ m syringe filter (Whatman, Clifton, NJ) to remove lipid particles prior to protein measurement.

Serum Leptin

Serum leptin was measured using a rat leptin radioimmunoassay kit (Linco Research, St. Charles, MO).

Western Analysis

Protein homogenate (10–80 μ g) was boiled for 5 minutes and separated on a Tris-HCl polyacrylamide gel (7.5–12.5 %, BioRad) and electrotransferred to nitrocellulose membranes (BioRad). Immunoreactivity was assessed with an antibody specific to Tyr 705-phosphorylated STAT3, total STAT3, c-myc tag (Cell Signaling), UCP1 (Linco Research) or GFP (Molecular Probes, Eugene, OR). Immunoreactivity was visualized by the ECL Plus detection system (GE Healthcare, Piscataway, NJ) and quantified by ImageQuant TL (GE Healthcare).

RNA Isolation and Reverse Transcription

Total cellular RNA was extracted using TRI reagent (Sigma-Aldrich, St. Louis, MO), based on a modification of the method of Chomczynski (Chomczynski and Sacchi, 1987). The integrity of the isolated RNA was verified using agarose gels (1%) stained with ethidium bromide. The RNA was quantified by spectrophotometric absorption at 260 nm using multiple dilutions of each sample. Total RNA (3 μ g) was treated with a DNA-free kit (Ambion, Austin, TX), and first-strand cDNA synthesis generated from 1 μ g RNA in a 20- μ L volume using random primers (Invitrogen) containing 200 units of M-MLV reverse transcriptase (Invitrogen).

Relative-Quantitative PCR

Relative quantitative PCR was performed using QuantumRNA 18S Internal Standards kit (Ambion). PCR was performed by multiplexing target gene primers and 18s primers and coamplifying for a number of cycles in the linear range of the target. For example, the primer

sequences for leptin are forward 5'- TGA CAC CAA AAC CCT CAT CA -3', reverse 5'- TGA GCT ATC TGC AGC ACG TT -3'. Linearity for the leptin amplicon was determined to be 25 to 30 cycles. The optimum ratio of 18s primer to competitor is 1:9. PCR was performed at 94°C denaturation for 120 sec, 59°C annealing temperature for 60 sec, and 72°C elongation temperature for 120 sec for 27 cycles. The PCR conditions used for other gene targets are listed as follows. Primer sequences are POMC forward 5'- GCT TGC AAA CTC GAC CTC TC -3', reverse 5'- CTT GAT GAT GGC GTT CTT GA -3'; NPY sense 5'- ATG GGG CTG TGT GGA CTG ACC -3', reverse 5'- GTC AGG AGA GCA AGT TTC ATT T -3'; AgRP forward 5'- AGG GCA TCA GAA GGC CTG ACC A -3', reverse 5'- CTT GAA GAA GCG GCA GTA GCA C -3'. The optimum ratio of 18S primer to competitor was 1:7 for leptin, 1:9 for POMC, 1:6 for NPY and 1:7 for AgRP. PCR were performed for 23 cycles for leptin, 28 cycles for POMC, 22 cycles for NPY or 24 cycles for AgRP. The PCR product was electrophoresed on a 5% Tris-Borate EDTA acrylamide gel (BioRad) and stained with SYBR green (Molecular Probes). Gels were scanned using a STORM fluorescent scanner (GE Healthcare) and quantified using ImageQuant (GE Healthcare).

Detection of SLR Transgene by PCR

Two sets of PCR primers were designed to detect SLR transgene and distinguish its expression from the endogenous leptin receptor. Primer set 1 was targeted toward the exon 1 of the pTR vector and the N-terminal of SLR (forward: 5'- GGC TCT GAC TGA CCG CGT TA - 3'; reverse: 5'- TCA TTC CCA AAG CAA CAG TG -3'). Primer set 2 was targeted toward the C-terminal of SLR and the c-myc tag (forward: 5'- TCA GTG CTT ATC CCC TGA GC -3'; reverse: 5'- GGC CGC CTA CAG ATC TTC TT -3'). For comparison, GFP expression levels were examined by RT-PCR using GFP primers (forward: 5'- GGC GTG GTC CCA ATT CTC GTG GAA -3'; reverse: 5'- GCG GTC ACA AAC TCC AGC AGG AC CA -3'). PCR was

performed at 94°C denaturation for 45 sec, 56°C (SLR) or 59°C (GFP) annealing for 45 sec, and 72°C elongation for 60 sec for 28 cycles (SLR) or 25 cycles (GFP). Alternatively, SLR expression was measured by relative quantitative PCR using QuantumRNA 18S Internal Standards kit (Ambion). The optimum ratio of 18S primers to competitors was 1:9 for SLR.

Statistical Analysis

All data are expressed as mean \pm standard error of mean (SE). Data were analyzed by one-way ANOVA, two-way ANOVA, or Student's *t*-test, as appropriate, with α level set at 0.05 for all analyses. For ANOVA, when the main effect was significant, a Tukey post-hoc test was applied to determine individual differences between means. GraphPad Prism software version 3.0 (San Diego, CA) was used for all statistical analysis and preparation of figures.

CHAPTER 3
LEPTIN ANTAGONIST REVEALS THAT THE NORMALIZATION OF CALORIC INTAKE
AND THE THERMIC EFFECT OF FOOD AFTER HIGH-FAT FEEDING ARE LEPTIN
DEPENDENT

Introduction

The consumption of a high-fat diet leads to obesity and severe metabolic consequences (Rothwell and Stock, 1985; Levin, 2005). When rodents are provided with high-fat diet, *ad libitum*, the immediate response is an increase in caloric intake and a corresponding increase in energy expenditure. In most circumstances, the increase in caloric intake is transitory, with caloric intake returning to control or nearly control levels within several weeks (Ramirez, 1990; Wilsey *et al.*, 2003). The exact mechanism underlying this homeostatic normalization of caloric intake is unknown, but presumably, it involves leptin. Leptin is one factor that mitigates food consumption while promoting energy expenditure (Campfield *et al.*, 1995; Halaas *et al.*, 1995; Elmquist *et al.*, 1998b; Friedman and Halaas, 1998; Schwartz *et al.*, 2000). Because leptin level rises following food consumption (Dallongeville *et al.*, 1998), it is reasonable to conjecture that this hormone mediates the normalization of the caloric intake following high-fat feeding. This is supported by the recent study by the Scarpace group at the University of Florida. When lean leptin resistant rats are provided with a high-fat diet, they respond with unabated increased caloric intake, indicating that leptin resistant rats are unable to homeostatically downregulate caloric intake following high-fat feeding (Scarpace *et al.*, 2005).

In addition to decreasing food intake, leptin enhances energy expenditure, and this hormone may mediate the well-described thermic effect of food consumption, which in rodents is manifested by elevated thermogenesis in brown adipose tissue (BAT) (Scarpace *et al.*, 1997; Scarpace and Matheny, 1998). In contrast to the transient nature of the increased caloric intake,

the temporal relationship of the elevated energy expenditure with high-fat feeding is less well documented, but likely dissipates as obesity and leptin resistance emerge.

Although circumstantial evidence indicates a role for leptin in both the normalization of the caloric intake and the enhanced energy expenditure following high-fat feeding, direct evidence is lacking. In this chapter, we used a leptin antagonist (a triple mutant of rat leptin resulting in a L39A/D40A/F41A) mutant to examine the role of leptin in these biological processes. To this end, we first established the effectiveness of the leptin antagonist in blocking centrally introduced leptin-mediated STAT3 phosphorylation in the hypothalamus and the anorexic response to leptin, as well as the consequences of a short infusion of the antagonist on food consumption and body weight. We then fed rats chow or a high-fat diet, and simultaneously infused the leptin antagonist or vehicle while assessing caloric intake, hypothalamic leptin signaling, and UCP1 levels in BAT.

Experimental Design

This chapter consisted of four experiments.

Experiment 1

Murine leptin (100 ng), leptin (100 ng) + rat leptin antagonist (0.3, 1, 3.5, 10, or 20 µg; Protein Laboratories Rehovot), or vehicle was administered by i.c.v. injection into the third ventricle. Rats were euthanized 1 hour later, and hypothalamic leptin signaling was assessed by STAT3 phosphorylation levels. In a separate group, after central administration of 20 µg of rat leptin antagonist, leptin signaling was compared with that in vehicle-injected controls.

Experiment 2

Rats fed standard chow were infused with leptin antagonist (25 µg/day into the lateral ventricle) or vehicle (control) by minipump for 7 days. A separate group of rats was fed standard chow and infused with leptin (1 µg/day into the lateral ventricle), leptin plus leptin antagonist

(200 µg/day), or vehicle (control) by minipump for 7 days. Food intake and body weight were recorded daily, and animals were euthanized on day 7 for tissue analysis.

Experiment 3

Rats were fed a high-fat (HF) diet (60% fat, 5.2 kcal/g; diet D12492, Research Diets, New Brunswick, NJ) and simultaneously infused with leptin antagonist (25 µg/day into the lateral ventricle) or vehicle by minipump for 7 days (Control/HF and Antagonist/HF groups, respectively). Rats fed standard chow (4.4% fat, 3.1 kcal/g; diet 8604, Harlan Teklad, Madison, WI) were also infused with vehicle (Control/Chow group). Caloric intake and body weight were recorded daily. Animals were euthanized 7 days after infusion for tissue analysis.

Experiment 4

Rats were fed chow or a high-fat diet for 2 days and i.c.v. injected with 250 ng of leptin or vehicle as control into the third ventricle, thus yielding four groups (Chow-Control, Chow-Leptin, HF-Control and HF-Leptin groups, respectively). Rats were euthanized 1 hour later, and hypothalamic leptin signaling was assessed by STAT3 phosphorylation levels. Serum was collected from control animals for assessment of leptin levels.

Results

Leptin Signaling After Antagonist Blockade

Hypothalamic leptin signaling was measured as leptin-induced STAT3 phosphorylation using 100 ng of leptin, a dose previously determined to evoke maximal signaling (Scarpace *et al.*, 2001). This dose of leptin, in combination with increasing doses of antagonist (0.3, 1, 3.5, 10, or 20 µg), or vehicle was centrally administered, and hypothalamic leptin signaling was assessed 1 hour later. As expected, phosphorylated STAT3 was increased fourfold in leptin- compared with vehicle-injected rats. The leptin antagonist inhibited this leptin-mediated signaling in a dose-response manner (Fig. 3-1, A). The highest dose of the antagonist, which is 200-fold higher

in concentration than the administered leptin, mostly inhibited the leptin-mediated signaling (Fig. 3-1, A). Administration of higher doses of the leptin antagonist was limited by solubility of the compound. To verify that the compound does not have partial agonist characteristics, a separate group of rats was injected with the highest dose of antagonist (20 μg), and the STAT3 phosphorylation levels were compared with those of vehicle-injected rats (Fig. 3-1, B).

Leptin Antagonist Infusion

To characterize the physiological effects of the leptin antagonist, chow-fed rats were infused with antagonist (25 $\mu\text{g}/\text{day}$ into the lateral ventricle) or vehicle for 7 days and caloric intake and body weight were recorded. The caloric intake was moderately, but significantly, higher in rats infused with antagonist than in control rats throughout most of the infusion period, with a peak increase of 16.1 kcal/rat (equal to 5.2 g of chow diet) on day 2 (Fig. 3-2, A). However, over time, the increase waned to less than 20% and, by day 6, was no longer statistically significant. Over the 7-day period, the antagonist-infused rats not only consumed 32% more energy but also gained more weight than the control rats: 12.71 ± 10.36 g vs. 8.74 ± 2.40 g (Fig. 3-2, B). At death, leptin signaling was assessed by hypothalamic STAT3 phosphorylation, yet there were no significant differences between the groups (control vs. leptin antagonist, 100.00 ± 17.47 vs. 91.80 ± 17.62).

Leptin Antagonist Blocks Leptin-Induced Anorexic Effects

To further demonstrate the blockade of leptin by the antagonist, we infused chow-fed rats with leptin alone (1 $\mu\text{g}/\text{day}$ into the lateral ventricle), the same dose of leptin + leptin antagonist (200 $\mu\text{g}/\text{day}$), or vehicle for 7 days. In the leptin infused rats, daily food intake was significantly lower than in control rats starting at day 2, whereas body weight loss was significant from day 5 (Fig. 3-3). In contrast, food intake and body weight in rats infused with leptin + leptin antagonist were not different from controls. At death, leptin infusion resulted in a 70% increase in

hypothalamic STAT3 phosphorylation, which was completely blocked in the leptin + antagonist group (Fig. 3-4, A). Leptin infusion elevated UCP1 levels in the BAT nearly six fold. Antagonist infusion completely prevented the increase in UCP1 protein (Fig. 3-4, B). Additionally, leptin infusion reduced serum leptin, abdominal adiposity and BAT weight, and these were all prevented by the antagonist (Table 3-1).

High-Fat Feeding

Rats were fed a high-fat diet (60% kcal as fat) and simultaneously infused with antagonist (25 µg/day into the lateral ventricle) or vehicle by minipump for 7 days. Rats fed standard chow were also infused with vehicle. Daily caloric intake in Antagonist/HF and Control/HF groups peaked on day 2, with increases of 90.2% and 66.7%, respectively, over Control/Chow rats (Fig. 3-5, A). In Control/HF rats, the increased caloric intake rapidly declined, with a nearly complete attenuation by day 7, whereas that of Antagonist/HF rats remained elevated and was significantly higher throughout the remainder of the experiment. Over the 7-day period, the Control/HF group consumed 42% more energy than the Control/Chow group, whereas the Antagonist/HF group consumed 90% more than Control/Chow group and 33% more than the Control/HF group (Table 3-2). Moreover, the peak antagonist-induced increase in caloric intake in animals fed the high-fat diet was similar to the increase observed in chow-fed rats in Figure 3-2. For example, on day 2, the antagonist evoked a 15.2-kcal/day increase in rats fed the high-fat diet (Antagonist/HF vs. Control/HF group; Fig. 3-5) compared with a 16.2 kcal/day increase (calculated from an increase of 5.21 g/day) in chow-fed rats also at day 2 (Antagonist/Chow vs. Control/Chow group; Fig. 3-2).

Body weight in the Control/Chow group was relatively stable throughout the 7-day experiment, whereas body weight increased in both HF groups. Body weight increased more rapidly in the Antagonist/HF than in Control/HF group, and weight gains between the two

groups significantly diverged by day 3. By the end of the experiment, the control- HF group gained almost 6-fold more weight than the Control/Chow group, whereas the Antagonist/HF group gained 14-fold more weight than the Control/Chow group and 2.4-fold more than the Control/HF group (Fig. 3-5B).

Serum Leptin Levels

HF feeding significantly elevated serum leptin level by day 7 and such increase was further enhanced by antagonist infusion. Serum leptin level in the Antagonist/HF group was significantly higher than that in the Control/HF group (Table 3-2).

Adiposity Levels

The high-fat diet significantly elevated white adipose tissue and BAT weights in Antagonist/HF and Control/HF groups (Table 3-2), but the increase was not significantly greater in the Antagonist/HF than in the Control/HF group ($P > 0.05$; Table 3-2).

UCP1 Protein in BAT

Animals were killed 7 days after antagonist or vehicle infusion, and UCP1 protein levels in BAT were assessed. Consistent with our previous findings (Wilsey *et al.*, 2003), the high-fat diet increased UCP1 protein levels in BAT (Fig. 3-6). Antagonist infusion completely blocked the effect of the high-fat diet, inasmuch as UCP1 levels were nearly identical in the Antagonist/HF and Control/Chow groups (Fig. 3-6). When expressed as UCP1 per milligram of BAT protein, the high-fat diet induced a similar 2.6-fold elevation in UCP1 (258 ± 32.4 vs. 100.0 ± 11.9 , arbitrary units, in Control/HF and Control/Chow groups, respectively, $P < 0.01$) that was also blocked by the antagonist infusion (94.3 ± 16.7 , arbitrary units).

Leptin Signal Transduction in the Hypothalamus

Unexpectedly, STAT3 phosphorylation levels were not significantly different between any of the three groups. However, PTP1B expression levels were found to be elevated only in the Antagonist/HF group (Table 3-2).

Leptin Challenge Following High-Fat Feeding

The lack of an increase in phosphorylated STAT3 levels 7 days after HF feeding suggests that leptin signaling may already have returned to baseline level in parallel with the normalization of caloric intake. For this reason, we examined leptin signaling at the peak of HF-induced caloric intake. At this time, 2 days after initiation of HF feeding, serum leptin levels were elevated 3-fold in the HF-fed compared with the chow-fed rats (Fig. 3-7, A). However, despite this elevated serum leptin, basal levels of STAT3 phosphorylation were similar in the HF- and chow-fed rats (Fig. 3-7, B, Chow-Control vs. HF-Control). We then examined maximal leptin signaling by i.c.v. administration of leptin to chow- and HF-fed rats. Leptin elevated STAT3 phosphorylation more than threefold in the chow-fed rat but just over twofold in the HF-fed rats, with the maximal level of leptin signaling significantly reduced in HF-fed compared with the chow-fed rats (Fig. 3-7, B).

Discussion

The importance of leptin in long-term homeostatic body weight regulation is well established (Friedman and Halaas, 1998; Schwartz *et al.*, 1999), but its exact role in the short-term regulation of food intake and energy expenditure is less certain. In the studies under Chapter 3, we employed a leptin antagonist to ascertain the role of leptin in the normalization of caloric intake after HF feeding and in the thermic effect of increased caloric intake. We first characterized the antagonist properties of this compound. This antagonist fully blocked, in a dose-response manner, the leptin-mediated signaling after an acute dose of centrally

administered leptin. A one-week central infusion of the antagonist resulted in the predicted increase in food consumption and body weight gain. Moreover, when infused for one week simultaneously with leptin, the antagonist prevented the anorexic and weight-reducing responses to leptin as well as the increase in leptin signaling. Additionally, the action of this agent appears to be that of a pure antagonist: the compound neither stimulated STAT3 phosphorylation nor reduced basal levels of phosphorylated STAT3. Finally, the antagonist blocked the leptin-mediated elevation of UCP1 protein in BAT. These data indicate that this compound is a pharmacologically and physiologically active antagonist of rat leptin receptor.

When administered simultaneously with the initiation of high-fat feeding, the antagonist prevented the normalization of caloric intake that ordinarily occurs, demonstrating the importance of leptin in this process. The inability to restore normal caloric intake resulted in an exaggerated weight gain during high-fat feeding, signifying the importance of endogenous leptin in everyday weight control. Moreover, these results support the previous findings by the Scarpace lab. In rats that were made leptin resistant by chronic central overexpression of leptin, they displayed the same response to high-fat feeding as did the leptin antagonist-treated rats in the present study; i.e., they failed to downregulate the high-fat induced increase in caloric intake, thus resulting in an exacerbated weight gain (Wilsey *et al.*, 2003; Scarpace *et al.*, 2005). These data further support our contention that leptin resistance is not only a consequence of obesity but also one cause of obesity.

Increased caloric intake, whether by high-fat feeding or otherwise, often results in elevated energy expenditure (Arnold and Richard, 1987). High-fat feeding stimulates the sympathetic outflow to BAT and the subsequent activation of thermogenesis, the putative thermic effect of food (Rothwell and Stock, 1985; Rothwell *et al.*, 1985; Stock and Rothwell, 1985). Although

thermogenesis was not assessed in this chapter, UCP1 protein, a reasonable marker for BAT thermogenesis, was elevated by nearly 2-fold after high-fat feeding. Moreover, this increase in UCP1 protein levels was completely prevented by the antagonist infusion, indicating that the BAT thermogenic effect of food in response to high-fat feeding is leptin dependent.

Despite the overt detrimental outcomes of the leptin antagonist treatment on body weight, we were unable to relate the failure of caloric restoration to a specific inhibition of leptin signaling. This was mainly because the HF-fed rats did not express elevated hypothalamic leptin signaling on day 7 after high-fat feeding or on day 2, during the peak increase in caloric intake. This lack of an endogenous increase in leptin signaling occurred, despite a 3-fold elevation in serum leptin at the peak of caloric intake. These data suggest that any leptin mediated signaling event triggered by increased caloric intake is below detection by our method of examining whole hypothalamic STAT3 phosphorylation, that leptin signaling has already returned to basal levels by day 2, or that this physiological response of leptin is mediated by another signaling pathway, such as the phosphatidylinositol 3-kinase pathway (Zhao *et al.*, 2002; Sahu and Metlakunta, 2005). Nevertheless, it is apparent that the normalization of caloric intake after high-fat feeding is a leptin-mediated event.

Even though we were unable to detect an increase in leptin signaling associated with high-fat feeding, when exogenous leptin was centrally administered on day 2, at the peak caloric intake, there was a robust increase in hypothalamic leptin signaling, confirming pharmacological responsiveness to leptin. However, maximal leptin signaling during this period of peak caloric intake was diminished, suggesting that desensitization may have occurred, potentially in response to the high-fat diet-induced elevated leptin. The Scarpace group has previously demonstrated that high-fat feeding reduces the maximal signaling capacity and that this is

associated with a similar reduction in leptin receptor expression. In that study, however, the high-fat feeding was prolonged (115 days) and the result was leptin-resistant animals (Wilsey and Scarpace, 2004). The present study suggests that this putative desensitization occurs rapidly and is likely not related to the development of obesity.

In summary, the studies under Chapter 3 describe the physiological responses to the central infusion of a leptin antagonist in chow- and HF-fed rats. The antagonist was able to block hypothalamic leptin signaling in response to an acute exogenous central challenge with leptin or a one-week central infusion of leptin. Infusion of the antagonist resulted in the predicted increase in food consumption and weight gain, and the antagonist prevented the anorexic response to a leptin infusion. The homeostatic normalization of elevated caloric intake after high-fat feeding and the increase in UCP1 protein in BAT were prevented by the leptin antagonist, indicating that these processes are leptin dependent. These data demonstrate an important role for leptin in the homeostatic response to high-fat feeding.

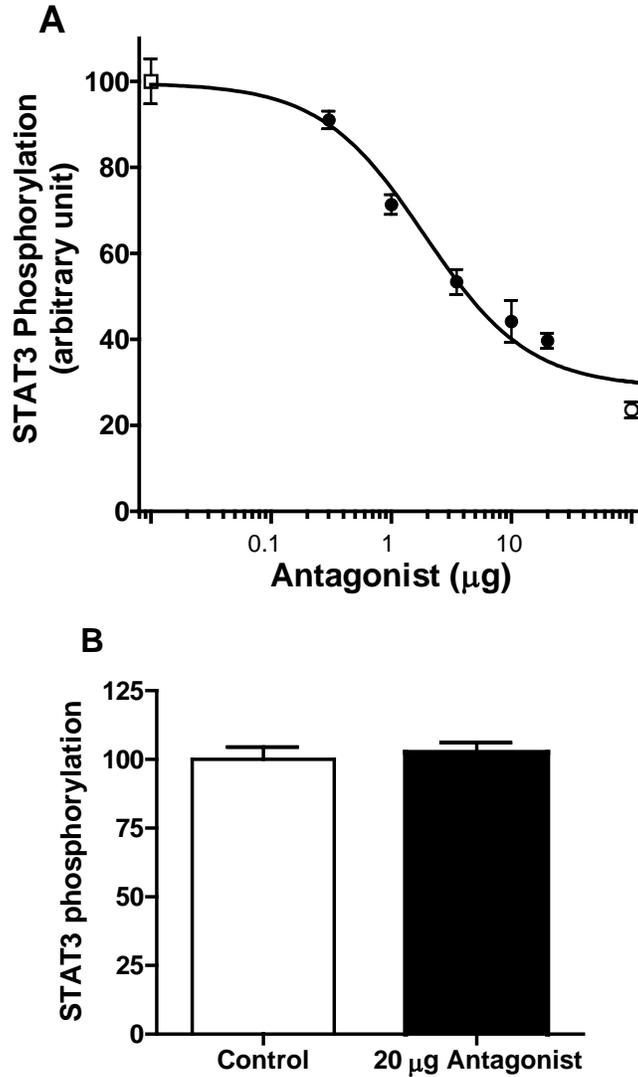


Figure 3-1. Dose-dependant inhibition of hypothalamic leptin signaling by leptin antagonist. A) STAT3 phosphorylation 1 h after i.c.v. administration of vehicle, leptin (100 ng) and leptin (100 ng) plus increasing doses of antagonist (0.3, 1, 3.5, 10, or 20 μg). Open square, leptin alone; open circle, basal activity in the absence of leptin or antagonist. Results are expressed in arbitrary units per microgram of hypothalamic protein. STAT3 phosphorylation was normalized to total STAT3, and levels of STAT3 phosphorylation in the leptin alone-injected rats were set to 100 with SE adjusted proportionally. Values represent the mean \pm SE of 5 rats per group. B) STAT3 phosphorylation with the highest dose of antagonist (20 μg) compared to vehicle-injected control rats. Levels of STAT3 phosphorylation in the control rats were set to 100 with SE adjusted proportionally.

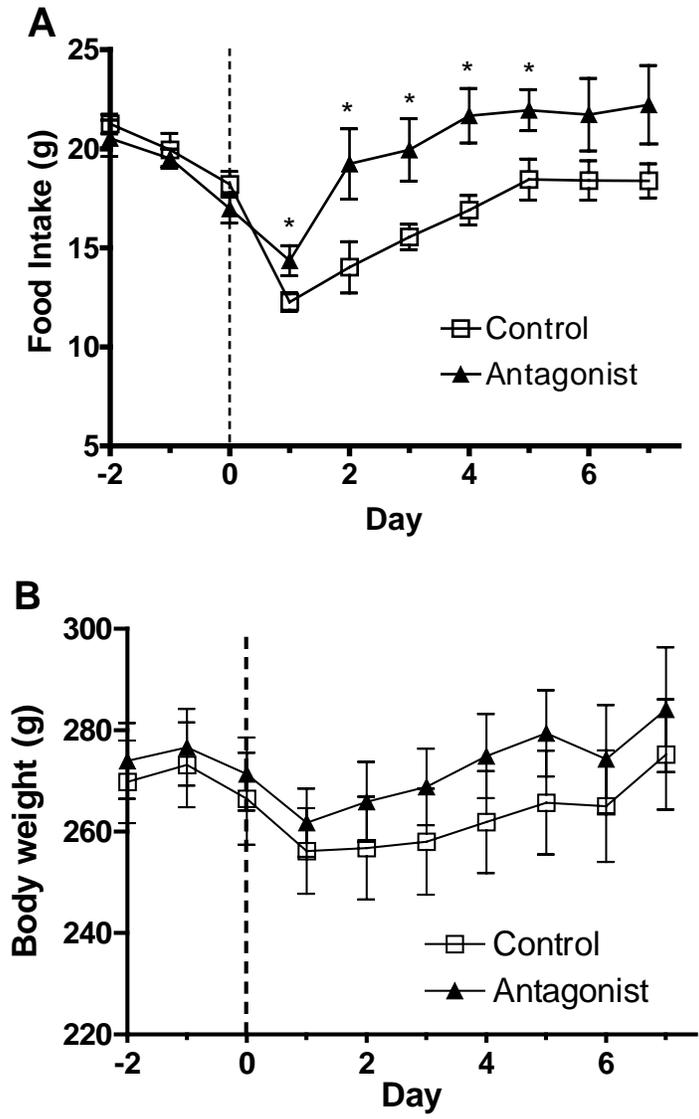


Figure 3-2. Daily food intake and body weight in chow-fed rats following a 7-day infusion of antagonist (25 µg/day) or vehicle. The antagonist or vehicle was infused from day 0. Values represent the mean ± SE of 6 control and 6 antagonist rats. A) Daily food intake. * $P < 0.05$ for difference compared with controls. B) Body weight in the antagonist infused rats was significantly different from controls ($P < 0.01$ by repeated measures).

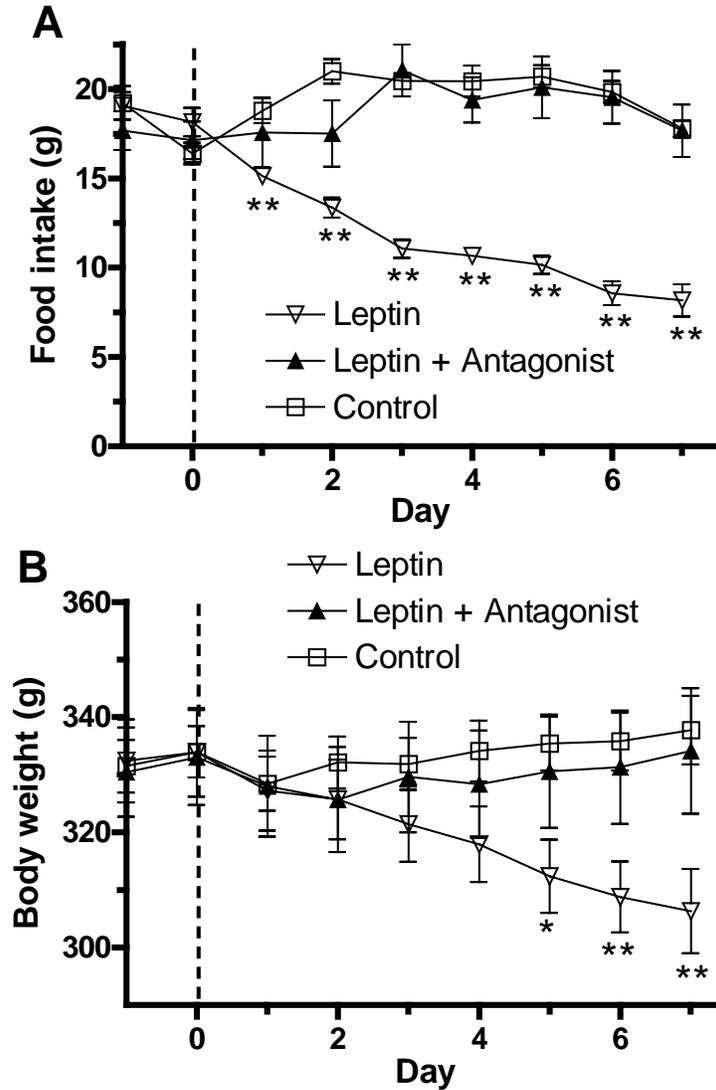


Figure 3-3. Daily food intake and bodyweight in chow-fed rats following a 7-day infusion of leptin (1 $\mu\text{g}/\text{day}$), leptin (1 $\mu\text{g}/\text{day}$) plus antagonist (200 $\mu\text{g}/\text{day}$), or vehicle. The infusions commenced at day 0. Values represent the mean \pm SE of 6 rats in each group. A) Daily food intake. B) Body weight. Neither food intake nor bodyweight in the Leptin + Antagonist group was different from control group during the infusion. * $P < 0.05$, ** $P < 0.01$ compared with controls.

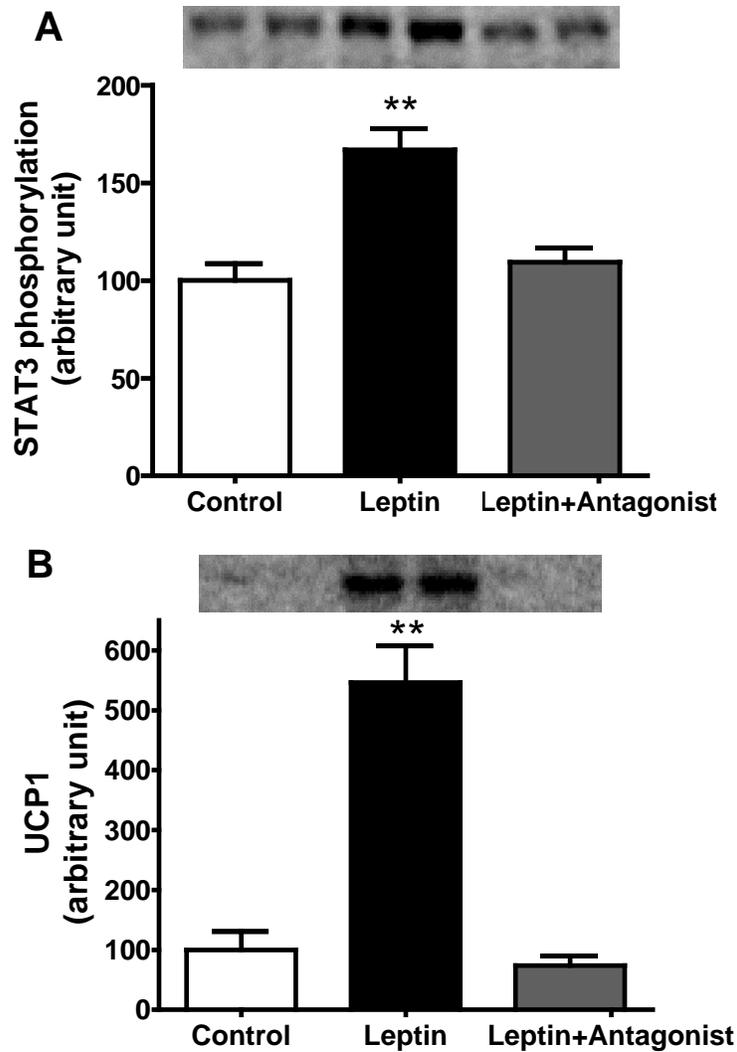


Figure 3-4. Hypothalamic STAT3 phosphorylation and UCP1 protein levels in BAT after a 7-day infusion of leptin (1 $\mu\text{g}/\text{day}$), leptin (1 $\mu\text{g}/\text{day}$) plus antagonist (200 $\mu\text{g}/\text{day}$), or vehicle. Top panel, representative bands of each group from Western analysis. Bottom panel, quantified results of Western analysis. Values represent the mean \pm SE of 6 rats in each group. A) STAT3 phosphorylation was normalized to total STAT3 and levels in the control rats were set to 100 with the SE adjusted proportionally. B) UCP1 levels in the control rats were set to 100 with the SE adjusted proportionally. ** $P < 0.001$ for difference over control and Leptin + Antagonist group.

Table 3-1. Energy balance-related parameters on day 7 following central infusion of leptin plus leptin antagonist

Parameter	Control	Leptin	Leptin + Antagonist
Serum leptin level, ng/mL	3.94 \pm 0.21	0.64 \pm 0.10**	4.62 \pm 0.50
Visceral adiposity, g	8.11 \pm 0.29	5.13 \pm 0.37**	8.35 \pm 0.85
BAT weight, mg	407.8 \pm 22.3	217.6 \pm 5.2**	412.3 \pm 21.7

Data represent the mean \pm SE of 6 animals in each group. ** $P < 0.01$ vs. Control.

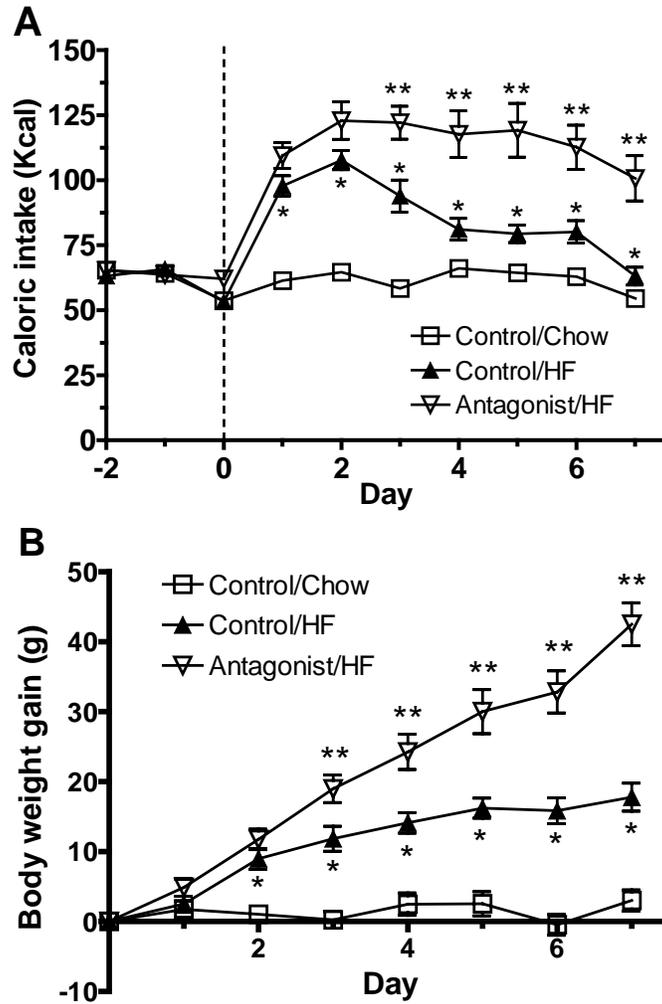


Figure 3-5. Daily caloric intake and body weight gain in HF-fed rats following a 7-day infusion of antagonist (25 $\mu\text{g}/\text{day}$) or vehicle and in chow-fed rats following vehicle infusion. The antagonist or vehicle infusion and HF feeding started at day 0. Values represent the mean \pm SE of 9 Antagonist/HF, 8 Control/HF and 8 Control/Chow rats. A) Caloric intake per day was based on 3.10 kcal/g of chow and 5.24 kcal/g of HF diet. * $P < 0.01$ or $P < 0.05$ on day 7, compared with Control/Chow group. ** $P < 0.01$ for difference from Control/HF group. B) Body weight gain in the two HF groups significantly diverged from Control/Chow by day 2 (* $P < 0.001$), and significantly diverged between the two HF groups by day 3 (* $P < 0.01$).

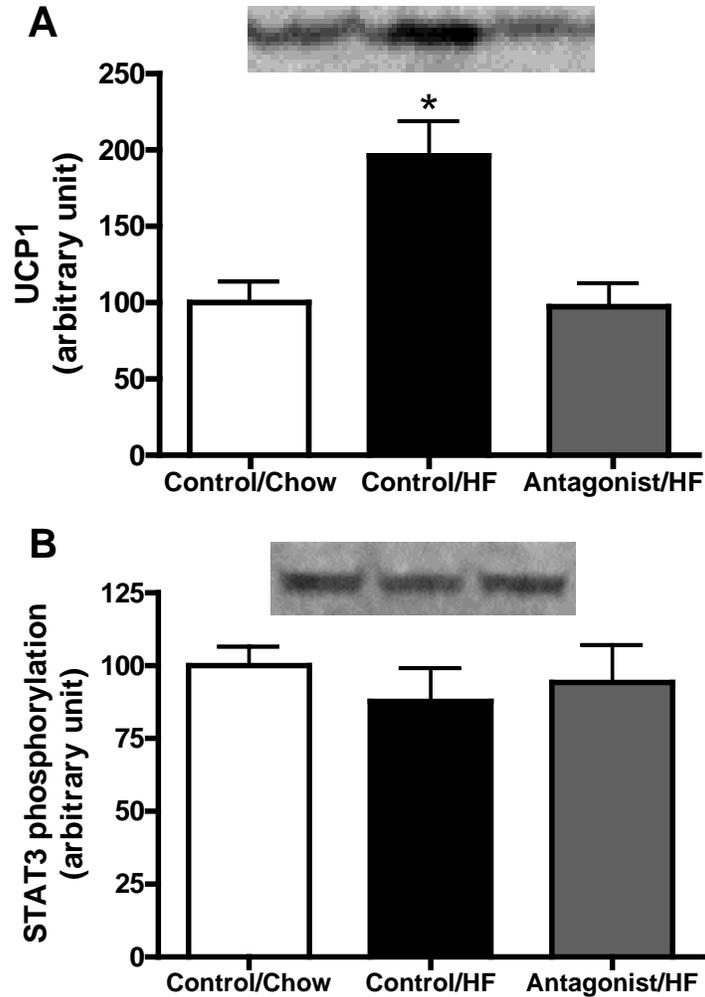


Figure 3-6. UCP1 protein levels in BAT and hypothalamic STAT3 phosphorylation in HF-fed rats after a 7-day infusion of antagonist (25 $\mu\text{g}/\text{day}$) or vehicle and in chow-fed rats following vehicle infusion. Top panel, representative bands of each group from Western analysis. Bottom panel, quantified results of the Western analysis. Results are expressed in arbitrary units per microgram of protein. Values represent the mean \pm SE of 8 rats in each group. A) UCP1 levels in the Control/Chow group were set to 100 and with the SE adjusted proportionally. * $P < 0.01$ for difference compared with Control/Chow or Antagonist/HF. B) STAT3 phosphorylation was adjusted to total STAT3 and levels in the Control/Chow group were set to 100 with the SE adjusted proportionally.

Table 3-2. Energy balance-related parameters on day 7 following high-fat feeding with or without simultaneous leptin antagonist infusion

Parameter	Control/Chow	Control/HF	Antagonist/HF
Cumulative caloric intake, kcal	424.0 ± 10.5	603.1 ± 21.1**	804.9 ± 45.3†
Serum leptin level, ng/mL	2.70 ± 0.22	7.97 ± 0.99**	15.40 ± 1.50†
Visceral adiposity, g	6.48 ± 0.57	10.35 ± 0.84*	13.11 ± 1.15‡
BAT weight, mg	403.3 ± 26.0	574.8 ± 39.1**	656.6 ± 29.9‡
PTP1B level, arbitrary units	100.0 ± 5.91	102.63 ± 7.14	136.79 ± 12.65*

Data represent the mean ± SE of 7 to 9 animals in each group. * $P < 0.05$ vs. Control/Chow. ** $P < 0.01$ vs. Control/Chow. † $P < 0.001$ vs. Control/Chow and Control/HF. ‡ $P < 0.001$ vs. Control/Chow. $P > 0.05$ vs. Control/HF.

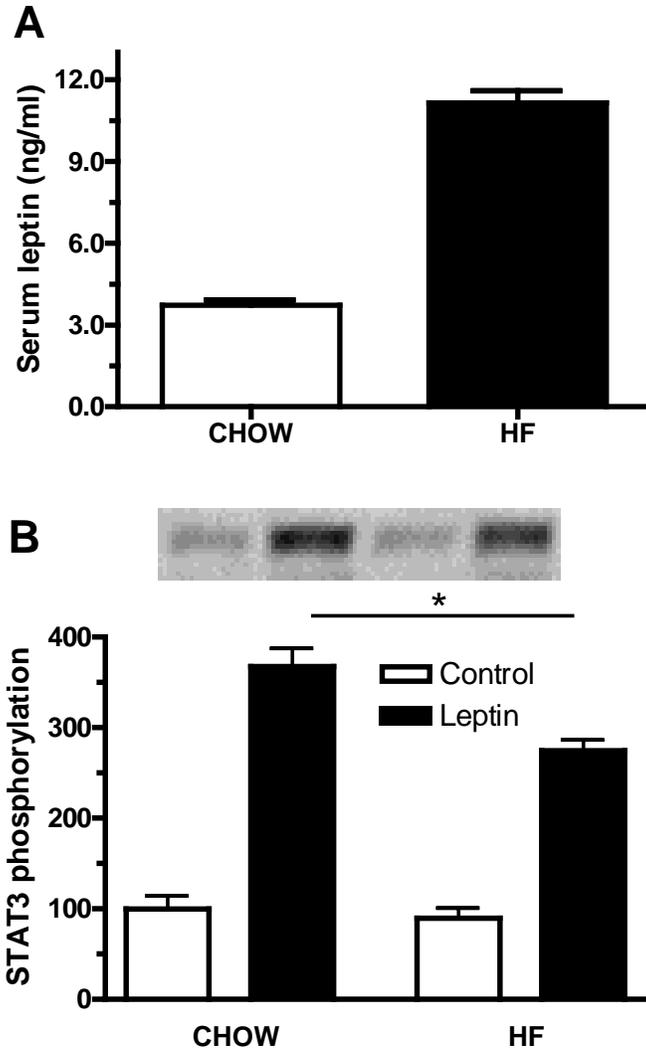


Figure 3-7. Serum leptin levels and STAT3 phosphorylation following central leptin administration after 2 days of HF feeding. Values represent mean \pm SE of 5 to 6 rats in each group. A) Serum leptin levels in control-injected rats (Chow-Control and HF-Control). B) STAT3 phosphorylation levels. Top panel, representative Western bands for each group. Bottom panel, quantified results of the Western analysis. STAT3 phosphorylation was normalized to total STAT3 and levels in the Chow-Control rats were set to 100 with the SE adjusted proportionally. * $P < 0.01$ between HF-Leptin and Chow-Leptin groups. $P < 0.001$ between HF-Leptin and both control groups.

CHAPTER 4
SOLUBLE LEPTIN RECEPTOR NEUTRALIZES LEPTIN MEDIATED STAT3 SIGNALING
AND ANOREXIC RESPONSES, *IN VIVO*

Introduction

Leptin is a key peptide hormone in energy homeostasis. Produced in white adipose tissue, leptin is secreted into the circulation and transported across the blood brain barrier (BBB) via a saturable transport system (Banks *et al.*, 1996). Leptin acts within several sites in the brain, including the satiety center in the hypothalamus, leading to reduced appetite and increased energy expenditure (Friedman and Halaas, 1998). Leptin levels in the circulation are generally in proportion to whole body fat mass (Maffei *et al.*, 1995b; Considine *et al.*, 1996).

There are multiple isoforms of leptin receptor as a result of alternative splicing (Lee *et al.*, 1996). The full-length leptin receptor, Ob-Rb, consists of an extracellular domain, a transmembrane domain and a cytoplasmic tail with full signaling capacity. Ob-Rb is most abundantly expressed in the hypothalamus. Ob-Ra, also called the short form leptin receptor, shares the same sequence with Ob-Rb, except for a truncated cytoplasmic tail, and thus is devoid of signaling capacity (Friedman and Halaas, 1998). Ob-Ra is widely expressed in various tissues, including the choroid plexus and the cerebral microvessels, which compose the blood brain barrier (BBB), and this form of the receptor is proposed to serve as a leptin transporter across the BBB (Hileman *et al.*, 2002). A third isoform, Ob-Re, or the soluble leptin receptor (SLR), is the major leptin binding protein in the circulation (Gavrilova *et al.*, 1997). Consisting of only the extracellular domain of full-length receptor, it is generated by ectodomain shedding of membrane-anchored leptin receptors, and in rodents, it can also be generated by alternative splicing (Li *et al.*, 1998; Maamra *et al.*, 2001; Ge *et al.*, 2002). The SLR binds leptin with a similar affinity as the full-length Ob-Rb, and thus regulates the bioavailability of leptin (Liu *et al.*, 1997). Studies in Zucker rats indicate that SLR prolongs half-life of leptin in the circulation,

presumably by protecting it from clearance (Huang *et al.*, 2001). Mice studies using ¹²⁵I-leptin demonstrated leptin transport across the BBB was inhibited by SLR (Tu *et al.*, 2008). Cell culture studies indicate that SLR prevents leptin binding to its membrane receptor effectively inhibiting leptin-mediated signaling (Yang *et al.*, 2004).

Most soluble forms of receptors act as antagonists by binding the hormone, thus preventing interaction with the native receptor. Leptin is a member of the class I family of cytokine receptors, several of which have soluble forms of their receptors that inhibit ligand action. These include the soluble forms of the growth hormone receptor and various interleukin receptors (Fisker, 2006).

Collectively, the previous studies with SLR and known actions of other soluble forms of receptors predict that the SLR will also act as a negative regulator of leptin's physiological function, although direct evidence of this function by SLR is lacking, *in vivo*. Therefore, in Chapter 4, we examined whether SLR is able to neutralize leptin-mediated STAT3 signaling and leptin-induced anorexic responses and elevated energy expenditure in normal leptin responsive rats.

To this end, we used a recombinant murine soluble leptin receptor–Fc chimera to verify if SLR can block leptin-mediated energy regulation. First we established the effectiveness of SLR in blocking central leptin-mediated STAT3 signaling in the hypothalamus. Then, we used two doses SLR to counter a peripheral leptin infusion and examined the consequent anorexic responses, weight reduction and BAT thermogenesis.

Experimental Design

This chapter consists of four experiments. The soluble leptin receptor used in these experiments is a recombinant chimera of 1-839 aa of murine leptin receptor fused to Fc fragment of human IgG (R&D systems Minneapolis, MN).

Experiment 1

Three-month-old male F344 x BN rats were administered murine leptin (90 ng), leptin (90 ng) plus soluble leptin receptor (13.5 μ g), soluble leptin receptor alone or artificial cerebral spinal fluid (ACSF) by intracerebroventricular (i.c.v.) injection into the third ventricle. Rats were sacrificed 1 hour later and hypothalamic leptin signaling assessed by STAT3 phosphorylation levels.

Experiment 2

Rats were infused with leptin (0.1 mg/day, subcutaneous minipump) for 7 days and were simultaneously infused with soluble leptin receptor (4.3 μ g/day, lateral ventricle) or ACSF as vehicle. Rats infused with saline (subcutaneous) and ACSF (lateral ventricle) were included as controls. Food intake and body weight were recorded daily and animals were euthanized at day 7 for tissue analysis.

Experiment 3

Rats were infused with leptin at three different doses (0.07, 0.05 and 0.03 mg/day) by subcutaneous minipump for 7 days. Rats infused with saline were included as controls. A separate group of rats were centrally infused with leptin into the lateral ventricle at three different doses (0.15, 0.25 and 0.35 μ g/day). Rats centrally infused with ACSF were included as controls. Food intake and body weight were recorded daily.

Experiment 4

Rats were infused with leptin (0.05 mg/day) or saline peripherally for 7 days and simultaneously infused with soluble leptin receptor (5.5 μ g/day, lateral ventricle) or ACSF. This yielded four groups: Saline-ACSF, Saline-SLR, Leptin-ACSF and Leptin-SLR. Food intake and body weight were recorded daily and animals were euthanized at day 7 for tissue analysis.

Results

Experiment 1

SLR neutralizes leptin signaling, *in vivo*

Hypothalamic leptin signaling was assessed as leptin-induced STAT3 phosphorylation (p-STAT3) in response to 90 ng of leptin, a dose previously determined to evoke sub-maximal signaling (Scarpace *et al.*, 2001). This dose of leptin alone, in combination with soluble leptin receptor (13.5 μ g), soluble leptin receptor alone (13.5 μ g) or ACSF control were centrally administered and hypothalamic leptin signaling assessed 1 hour later. As expected, leptin increased p-STAT3 by 3-fold over control rats. This increase in p-STAT3 was fully blocked by co-administration of the soluble leptin receptor. Injection of SLR alone did not alter p-STAT3 levels when compared with the controls (Fig. 4-1).

Experiment 2

SLR partially blocks leptin-induced anorexic effects

Rats were infused peripherally with leptin (0.1 mg/day) via subcutaneous minipumps for 7 days and simultaneously infused centrally with soluble leptin receptor (4.3 μ g/day, lateral ventricle) or ACSF. This yielded two groups: Leptin and Leptin+SLR. A third group, infused with saline (subcutaneously) and ACSF (lateral ventricle) served as the control. Food intake was significantly reduced in the Leptin group from day 1, immediately after infusion. In the Leptin + SLR group, the initial drop in food intake was similar to the Leptin group, a response likely due to a surgical effect. Subsequently, the anorexia was generally attenuated throughout the remaining infusion period (Fig. 4-2, A).

Moreover, the Leptin group consumed 27.4 ± 2.7 g less food in total from day 2 to day 7 when compared to its own pre-infusion baseline. In comparison, the Leptin + SLR group only consumed 17.5 ± 3.3 g less than its pre-infusion baseline, and this is significantly less than the

Leptin group ($P < 0.05$). In contrast, the control group consumed nearly the same amount of food (-0.2 ± 2.9 g less than pre-infusion consumption).

Body weight reduction was significant in both Leptin and Leptin + SLR groups starting from day 2. The slope of body weight reduction in Leptin +SLR group was not as steep as the Leptin group (Leptin, -1.85 ± 0.11 ; Leptin + SLR, -1.01 ± 0.10 , $P < 0.001$) and decrease in body weight significantly diverged beginning at day 5 continuing through day 7, in the Leptin + SLR compared with the Leptin group (Fig. 4-2, B)

Whole body fat mass and visceral adiposity

Body composition was measured and the changes in whole body fat mass were compared after the 7-day leptin, leptin plus SLR, or control infusions. The control group had a slight increase, while the leptin infusion reduced fat mass by nearly 10 g. Infusion with leptin plus SLR partially blocked this decrease, with the Leptin + SLR group experiencing a decrease of 5.1 g (Table 4-1). At tissue harvest, EWAT, PWAT and RTWAT were collected and visceral adiposity was defined as the sum of these three tissues. However, visceral adiposity levels were found to be similar among the three groups (Table 4-1).

Leptin induced BAT UCP1 is partially blocked by SLR

At death, UCP1 protein levels in BAT were assessed. Consistent with our previous finding (Scarpace et al., 1997), leptin infusion elevated UCP1 level in BAT by almost 40%, whereas simultaneous central SLR infusion partially prevented the increase in UCP1 protein (Fig. 4-3, A).

Serum leptin levels

Serum leptin was determined in 7 randomly selected rats prior to any infusion, and the average serum leptin level was found to be 3.38 ± 0.18 ng/mL. At day 7, blood was collected from all the rats at death. Serum leptin in control rats was unchanged at day 7, whereas following

leptin infusion, serum leptin was elevated by 4-fold. Similarly, in rats infused with leptin plus SLR, there was a comparable increase in serum leptin (Table 4-1).

Leptin signaling markers at death

At the end of the 7-day infusion of leptin plus SLR, hypothalamic leptin signaling markers, including p-STAT3 and expression levels of POMC, NPY and AgRP were measured. Despite the significant physiological responses to the leptin infusion and partial blockade of these responses by SLR, no difference in STAT3 phosphorylation was detected among the three groups (Fig. 4-3, B). Similarly, expression levels of hypothalamic neuropeptides downstream of the leptin receptor–STAT3 signaling pathway were also unchanged (Table 4-1).

Experiment 3

Dose-dependent response curve to peripheral leptin infusion

In order to lower the dose for peripheral leptin infusion, anorexia and weight reduction were compared in response to three leptin doses, 0.03, 0.05 and 0.07 mg/day. All three doses induced significant reduction in food intake and body weight in a dose-dependent manner (Fig. 4-4). Interestingly, the group received the highest dose, 0.07 mg /day, exhibited a big variance in the anorexic and weight-reducing responses to leptin.

Dose-dependent response curve to central leptin infusion

For comparison with the peripheral dose-response study, rats were infused with leptin into the lateral ventricle by minipump at a rate of 0.15, 0.25 or 0.35 $\mu\text{g}/\text{day}$ for 7 days. The higher two doses significantly reduced body weight in a dose-dependent manner (Fig. 4-5, A). In contrast, the lowest dose, 0.15 $\mu\text{g}/\text{day}$, only slightly increased body weight over the controls, which was not statistically significant. When food intake was compared with the controls, both doses of 0.25 and 0.35 $\mu\text{g}/\text{day}$ decreased food intake to a similar extent, while the lowest dose, 0.15 $\mu\text{g}/\text{day}$, did not have an effect (Fig. 4-5, B).

Experiment 4

Higher dose of SLR infusion

In order to raise the ratio of SLR to exogenously infused leptin, we increased the dose of central SLR infusion to 5.5 $\mu\text{g}/\text{day}$ and coupled this with a lower dose of peripheral leptin (0.05 mg/day) infusion. This leptin dose was determined based on the dose-response study described in Experiment 3, in which this dose induced significant anorexia and weight reduction. In addition, in this experiment, an additional group was included, treatment with SLR alone.

When the SLR was infused centrally by itself, both food intake and especially, body weight were increased over the controls (Fig. 4-6), suggesting the SLR blocked the action of endogenous leptin in the brain. Leptin infusion, at a dose lower than that used in Experiment 2, significantly reduced food intake and body weight as expected (Fig. 4-6). However, despite the higher SLR dose employed, SLR infusion still only partially blocked these leptin-induced responses (Fig. 4-6).

Whole body fat mass and visceral adiposity

Parallel to the body weight results, rats centrally infused with SLR demonstrated a slight but not significant increase in whole body fat mass over the controls ($P > 0.05$). The leptin peripheral infusion significantly reduced fat mass in the leptin group, while this decrease was completely prevented by SLR infusion in the Leptin + SLR group ($P < 0.05$ vs. Leptin, Table 4-2). Consistent with the body composition results, visceral adiposity at death (sum of EWAT, PWAT and RT-WAT) was only slightly increased in rats centrally infused with SLR, but significantly reduced by leptin peripheral infusion. However, concurrent SLR infusion only slightly inhibited the decrease, which was not significant from the Leptin group (Table 4-2).

UCP1 elevation in BAT is fully blocked by SLR infusion

Despite the changes in body weight and food intake, the SLR central infusion alone did not change the basal UCP1 levels in the BAT. Peripheral leptin infusion increased UCP1 by almost 20%, which was completely prevented by the addition of SLR infusion ($P < 0.05$ vs. Leptin, $P > 0.05$ vs. Control. Fig. 4-7, A).

Leptin expression levels in EWAT

Leptin expression levels in EWAT were measured at the end of the infusion. The SLR infusion alone did not change the leptin expression compared with the controls. Consistent with previous data (Scarpace et al., 1998), leptin infusion inhibited leptin expression in EWAT by 25%. Moreover, this inhibition was completely prevented by the simultaneous infusion with leptin plus SLR (Fig. 4-7, B).

Serum leptin levels

Serum levels were sampled from 7 randomly selected rats from all animals before the infusion and the average was 3.23 ± 0.30 ng/mL. At day 7, leptin levels in SLR infused rats were almost identical to the controls, whereas in leptin infused rats, serum leptin was almost two-fold greater than control level ($P < 0.01$ vs. Control, Table 4-2). Interestingly, the Leptin + SLR group had the highest serum leptin, 40% higher than the leptin group ($P < 0.05$ vs. Leptin).

Leptin signaling markers at death

Similar to the infusion results in Experiment 2, there were no significant changes in STAT3 phosphorylation among the groups (Fig. 4-8).

Discussion

Soluble receptors in various ligand-receptor systems have an important role in the regulation of ligand availability and subsequent receptor-mediated physiology (Heaney and Golde, 1993; Baumann, 2002; Rose-John *et al.*, 2006). The soluble leptin receptor (SLR) is the

major leptin binding protein in the circulation. It was previously demonstrated that the SLR inhibits leptin-induced signaling, *in vitro* (Yang *et al.*, 2004). In addition to this indirect antagonism of the leptin receptor, other studies have demonstrated potential roles for the SLR in leptin pharmacokinetics. The SLR prolongs the half-life of leptin in blood in Zucker rats, presumably by binding and thus protecting leptin from clearance (Huang *et al.*, 2001). In addition, a study in mice demonstrated SLR inhibits leptin transport across the blood brain barrier, thus limiting the availability of leptin to access receptors in the brain (Tu *et al.*, 2008).

Leptin is an important peptide hormone in energy regulation. This is readily apparent in rodents with genetic mutations lacking leptin or with defective leptin receptors (Halaas *et al.*, 1995). It stands to reason, that factors, such as the SLR, that have the potential to limit the access of leptin to its receptor may play an important role in energy homeostasis. However, the direct demonstration of the impact of SLR on energy balance (food intake or energy expenditure) in normal animals is lacking. In this chapter, we used an available SLR, a chimera of murine SLR and Fc of human IgG, to examine the impact of SLR on leptin physiology in F344xBN rats.

First, we demonstrated that SLR could neutralize leptin signaling, *in vivo*, by demonstrating that SLR inhibits leptin-mediated STAT3 signaling in the hypothalamus. Leptin-induced STAT3 phosphorylation was completely prevented by the simultaneous administration of SLR without pre-incubation, verifying the antagonistic features of SLR, *in vivo*. The rapid nature of this neutralization is consistent with the known high affinity binding between SLR and leptin (Liu *et al.*, 1997).

Moreover, we demonstrated that the SLR is able to counteract leptin's regulation on energy balance by co-infusion of leptin and SLR. Due to their high binding affinity, SLR and leptin were infused by separate routes, avoiding formation of any leptin–SLR complexes prior to

the individual compounds reaching the physiologically relevant regions of the brain. Leptin was infused peripherally, to mimic the elevation of serum leptin in physiological conditions, whereas the SLR was infused centrally in order to most efficiently block leptin action. At a dose of 4.3 $\mu\text{g}/\text{day}$, SLR infusion partially blocked leptin-induced anorexia and weight reduction. Although whole body energy expenditure was not measured, UCP1 protein level, a reasonable marker for BAT thermogenesis, was elevated by leptin infusion. This increase was also partially inhibited by the SLR co-infusion. Collectively, these data indicate that SLR is able to prevent leptin, at least partially, from acting in the CNS to regulate energy balance. The nature of the partial inhibition may simply be a matter of competition, i.e, there was insufficient SLR to fully bind the available leptin.

In an attempt to achieve a full blockade of leptin action, we increased the SLR infusion dose to 5.5 $\mu\text{g}/\text{day}$ while decreasing the dose of the peripherally infused leptin to 0.05 mg/day. In addition, we included a group that received only SLR central infusion. Interestingly, SLR infusion alone increased food consumption and body weight significantly over the control, presumably by neutralizing endogenous CSF leptin. Moreover, the increase in body weight with this amount of SLR infusion paralleled the increase in body weight following full leptin receptor blockade with a leptin receptor antagonist. In our previous study, we determined the dose of a leptin receptor antagonist that achieved full leptin receptor blockade, and found that this dose increased body weight by 12.71 ± 10.36 g over a 7-day treatment period (Zhang *et al.*, 2007). This increase in body weight is almost identical to that observed with the SLR in the present study and both studies used rats of same strain, age, and body weight. Collectively, these data indicate the SLR effectively neutralizes endogenous central leptin-mediated receptor activation.

However, when infusion of this higher SLR dose was coupled with the lower dose of leptin, this combination still resulted in only a partially blockade of leptin-induced anorexia and weight reduction. In contrast, the leptin-induced BAT UCP1 increased was completely prevented by SLR. Similarly, the infusion of central SLR fully reversed the leptin-inhibition of leptin expression in the EWAT. This leptin inhibition of adipose leptin expression is consistent with our previous finding that leptin inhibits leptin expression in white adipose tissue through a central mechanism (Scarpace *et al.*, 1998; Wilsey *et al.*, 2006). Complete prevention of these two markers of leptin action indicates that our higher dose of SLR coupled with the lower dose of leptin was able to achieve a more complete neutralization of the leptin, although some anorexia and weight reduction remained.

There are several potential explanations for the incomplete blockade of exogenous leptin-mediated responses. First and most likely is that neither dose of SLR was sufficient to fully bind all the available leptin. Use of higher doses was impractical due to cost. Second, the separate routes of delivery may have allowed a temporal dissociation between when the leptin and SLR mixed within the brain. The leptin-containing, subcutaneously implanted minipumps infused leptin directly into the subcutaneous tissues, where it was available for rapid uptake into the brain. In contrast, each SLR-containing, subcutaneously implanted minipump was connected to a brain cannula via a 7.5-cm tubing. We calculated that that the SLR may require 28 hours to transverse this tubing before reaching the lateral ventricle. It is likely that the leptin reached the brain and activated leptin receptors before the first of the SLR made an appearance. Thus, there was an initial burst of leptin receptor activity that was unchallenged, and thus accounting for the partial blockade of leptin action. Third and most intriguing is the possibility that the response due to central leptin action was fully blocked and that the residual activity is due to peripheral leptin

action. This suggests a role for peripheral leptin action in appetite and weight regulation, although most studies, so far, have failed to tease out a peripheral component of leptin action separate from central-mediated leptin responses. Further experiments would be necessary to resolve this issue.

Despite the distinct physiological responses between the Leptin and Leptin + SLR groups, we were unable to relate these differential responses to an inhibition of leptin signaling at the end of the infusions. This was mainly because the peripheral leptin infusion failed to elevate hypothalamic leptin signaling on day 7 despite a significant reduction in food consumption and high serum leptin levels. In contrast, when a pharmacological dose of leptin was injected centrally, leptin signaling was significantly increased by 1 h, and was fully inhibited by co-administration of SLR. Similarly, when leptin is administered peripherally by intravenous injection, the increase in STAT3 phosphorylation is observed within 30 minutes, is maximal by 1 h and returns to basal level by 14 h post injection (Scarpace *et al.*, 2000). These data suggest that either timing or dosing is responsible for the lack of an increase in STAT3 phosphorylation with a peripheral leptin infusion. For instance, the amount of leptin that reaches the brain by peripheral infusion may not be sufficient to activate leptin signaling, at least to a level detectable by our method of examining whole hypothalamic STAT3 phosphorylation, or the signaling is transient and has already returned to basal level by day 7. Nevertheless, it is apparent that SLR can prevent leptin signaling due to an acute injection and infused leptin from exerting its full effect on food intake, body weight, and BAT thermogenesis.

Obese animals and humans have elevated leptin whereas circulating SLR levels remain comparable to their lean counterparts (Wu *et al.*, 2002). The inability to upregulate SLR with the development of obesity results in an excessively high level of free leptin in the circulation.

Whether the elevated leptin is simply secondary to the obesity or a causative factor in pathogenesis of obesity is controversial (Scarpace and Zhang, 2007). In dietary obese rats, leptin treatment worsens rather than deters further high-fat induced obesity (Scarpace *et al.*, 2005). Elevated leptin in humans, independent of obesity, is a predictor of metabolic syndrome at 5 and 10 years (Franks *et al.*, 2005). There is no direct evidence that normalization of the elevated leptin associated with obesity is desirable, but neutralization of leptin is potentially beneficial. The study in Chapter 4 indicates neutralization of leptin prevents receptor activation and the subsequent physiological responses, thus demonstrating the feasibility of leptin normalization. The SLR, in addition to other agents, such as leptin synthesis blockers or leptin receptor antagonists are potential approaches to reduce the consequences, if any, of hyperleptinemia.

In summary, the study in Chapter 4 describes the physiological responses to the central infusion of SLR. This isoform of the leptin receptor was able to block hypothalamic leptin signaling induced by an acute central challenge of leptin. In addition, central infusion of SLR significantly increased food intake and body weight, effectively neutralizing endogenous central leptin. When infused centrally in conjunction with a peripheral leptin infusion, SLR partially blocked leptin-induced anorexic responses and body weight reduction, and fully prevented the elevation in BAT UCP1 levels as well as the inhibition on leptin expression in EWAT. These data imply that SLR is able to neutralize leptin action, *in vivo*, suggesting the potential of a regulatory role in energy homeostasis.

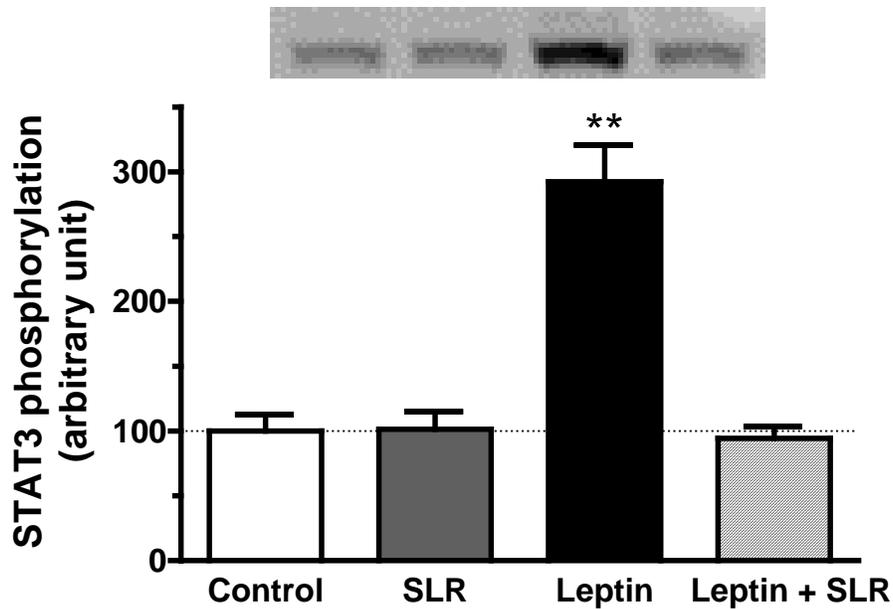


Figure 4-1. SLR inhibition of leptin-mediated STAT3 phosphorylation in the hypothalamus 1 h after i.c.v. administration of control, SLR (13.5 μ g), leptin (90 ng) or leptin (90 ng) + SLR (13.5 μ g). Top panel, representative Western bands for each group. Bottom panel, quantified results of the Western analysis. Results are expressed in arbitrary units per microgram of hypothalamic protein. STAT3 phosphorylation was normalized to total STAT3 and levels in control rats were set to 100, with SE adjusted proportionally. Values are means \pm SE of 6 animals per group. ** $P < 0.01$ vs. ACSF control.

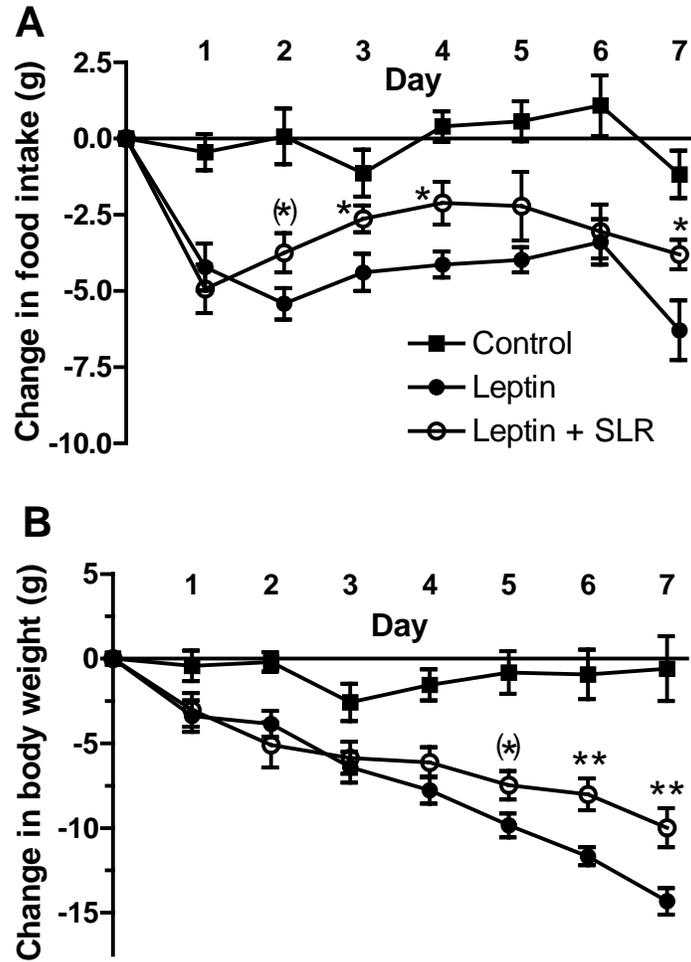


Figure 4-2. Change in daily food intake and body weight in rats following 7-day infusion of vehicle, leptin (0.1 mg/day, subcutaneous) or leptin (0.1 mg/day, subcutaneous) + SLR (4.3 μ g/day, lateral ventricle). Values are mean \pm SE of 7 animals per group. A) Change in daily food intake was calculated from the average food intake before treatment (Control, 16.7 ± 0.8 g; Leptin, 17.7 ± 0.5 g; Leptin + SLR, 17.1 ± 0.5 g) B) Change in body weight was calculated from body weight at day 0 before treatment (Control, 309.2 ± 10.8 g; Leptin, 312.0 ± 8.5 g; Leptin + SLR, 308.3 ± 6.2 g). (*) $P < 0.05$ vs. leptin by one-tailed t -test; * $P < 0.05$, ** $P < 0.01$ vs. leptin by two-tailed t -test.

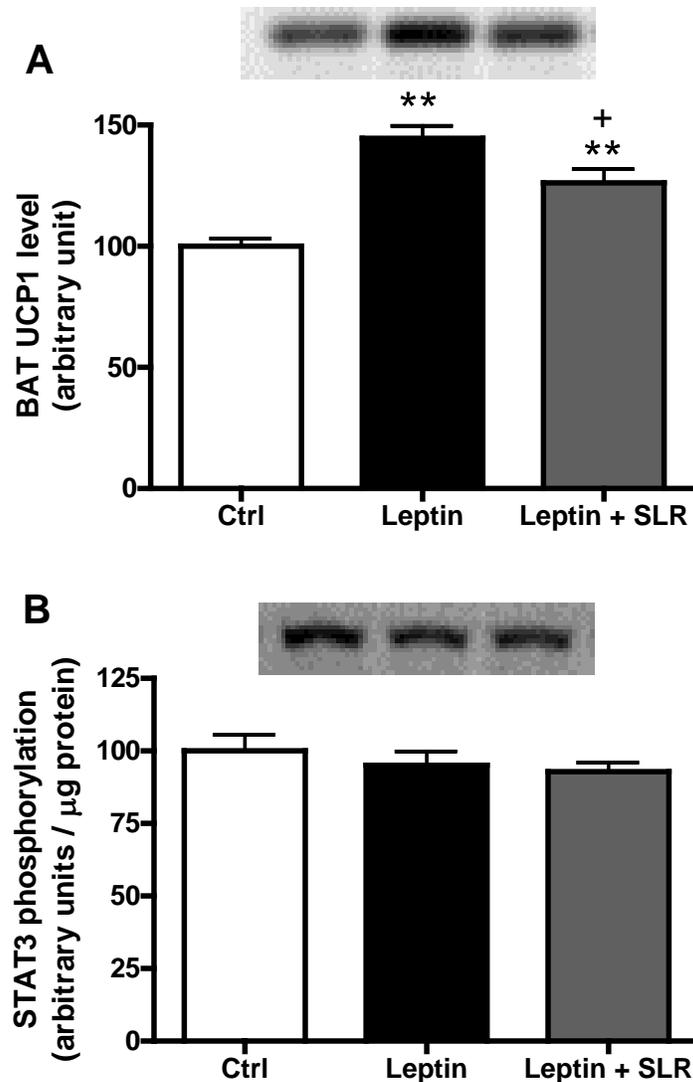


Figure 4-3. UCP1 protein levels in BAT and hypothalamic STAT3 phosphorylation following 7-day infusion of vehicle, leptin (0.1 mg/day, subcutaneous) or leptin (0.1 mg/day, subcutaneous) + SLR (4.3 μ g/day, lateral ventricle). Top panel, representative Western bands for each group. Bottom panel, quantified results of the Western analysis. Results are expressed in arbitrary units per microgram of protein. Values are means \pm SE of 6 or 7 animals per group. A) UCP1 levels in control rats were set to 100, with SE adjusted proportionally. ** $P < 0.01$ vs. Control; † $P < 0.05$ vs. Leptin. B) STAT3 phosphorylation was normalized to total STAT3 and levels in control rats were set to 100, with SE adjusted proportionally. There was no statistically significant difference among the groups.

Table 4-1. Energy homeostasis-related parameters following low-dose SLR central infusion (4.3 $\mu\text{g}/\text{day}$)

Parameter	Control	Leptin	Leptin + SLR
Change in whole body fat mass (g)	2.8 \pm 1.3	-9.4 \pm 0.7**	-5.1 \pm 1.6** †
Visceral adiposity, day 7 (g)	6.57 \pm 0.51	5.95 \pm 0.41	5.37 \pm 0.42
Serum leptin, day 7 (ng/mL)	3.73 \pm 0.23	13.49 \pm 0.90**	11.84 \pm 1.42**
POMC expression (AU)	100.0 \pm 7.4	91.6 \pm 6.2	99.13 \pm 5.6
NPY expression (AU)	100.0 \pm 2.5	97.0 \pm 2.5	101.6 \pm 3.9
AgRP expression (AU)	100.0 \pm 2.6	100.0 \pm 2.7	99.0 \pm 3.1

Values are means \pm SE of 6 or 7 animals in each group. * P < 0.05, ** P < 0.01 vs. Control; † P < 0.05 vs. Leptin. AU, arbitrary unit.

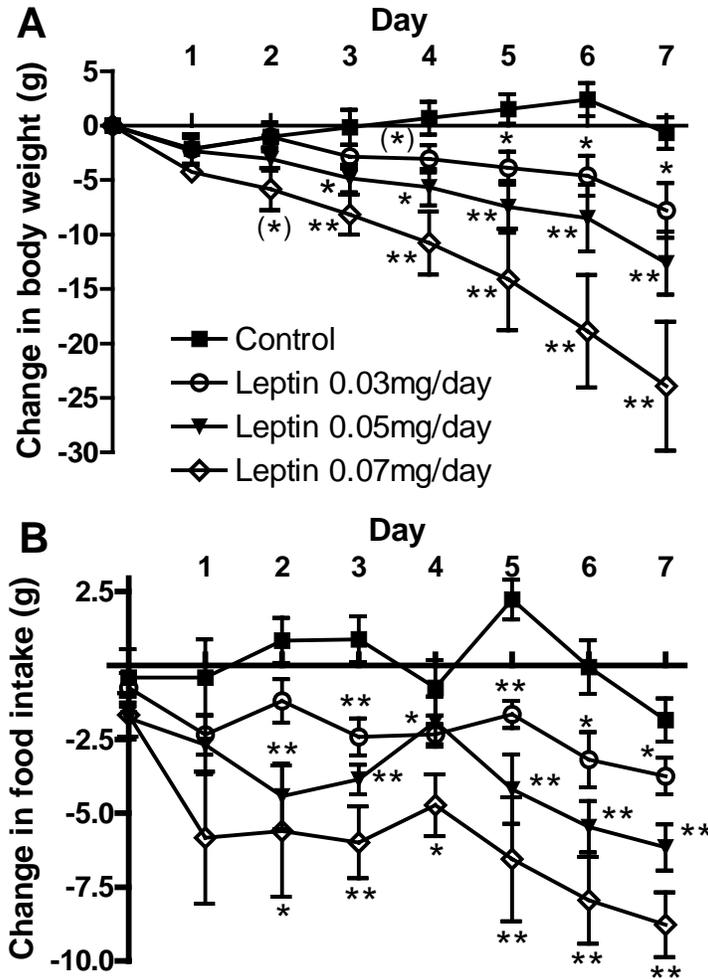


Figure 4-4. Dose-dependent response curve to peripheral leptin infusion following 7-day subcutaneous infusion of saline or leptin (0.03, 0.05 or 0.07 mg/day). A) Change in body weight. B) Change in food intake. Values are mean \pm SE of 6 or 7 animals per group. (*) $P < 0.05$ vs. Control by one-tailed t -test; * $P < 0.05$, ** $P < 0.01$ vs. Control by two-tailed t -test.

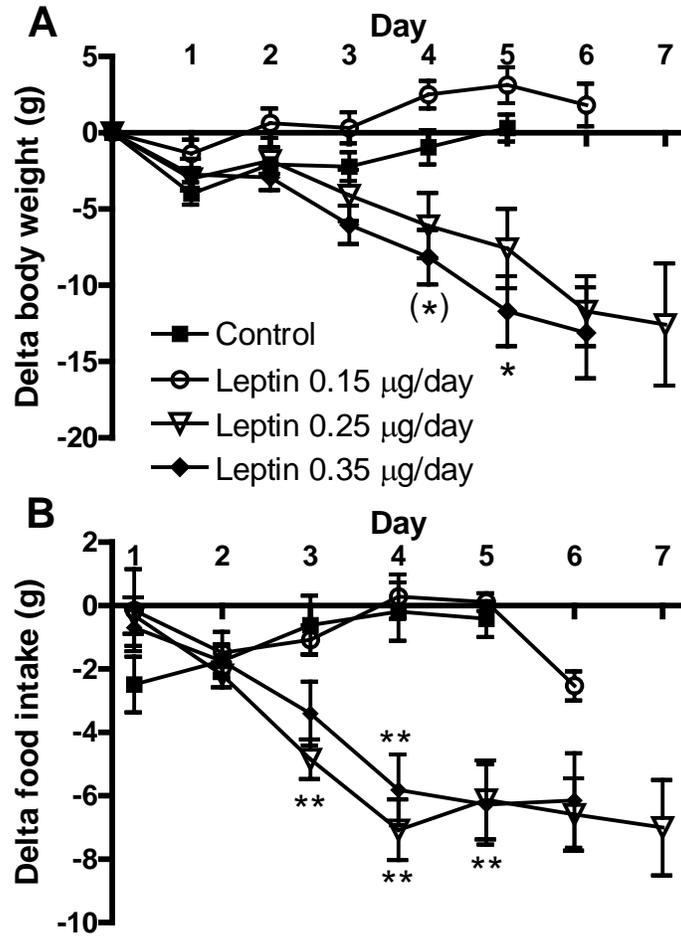


Figure 4-5. Dose-dependent response curve to central leptin infusion following 7-day infusion of ACSF or leptin (0.15, 0.25 or 0.35 $\mu\text{g}/\text{day}$) into the lateral ventricle. A) Change in body weight. B) Change in food intake. Values are mean \pm SE of 6 or 7 animals per group. (*) $P < 0.05$ vs. Control by one-tailed t -test; * $P < 0.05$, ** $P < 0.01$ vs. Control by two-tailed t -test.

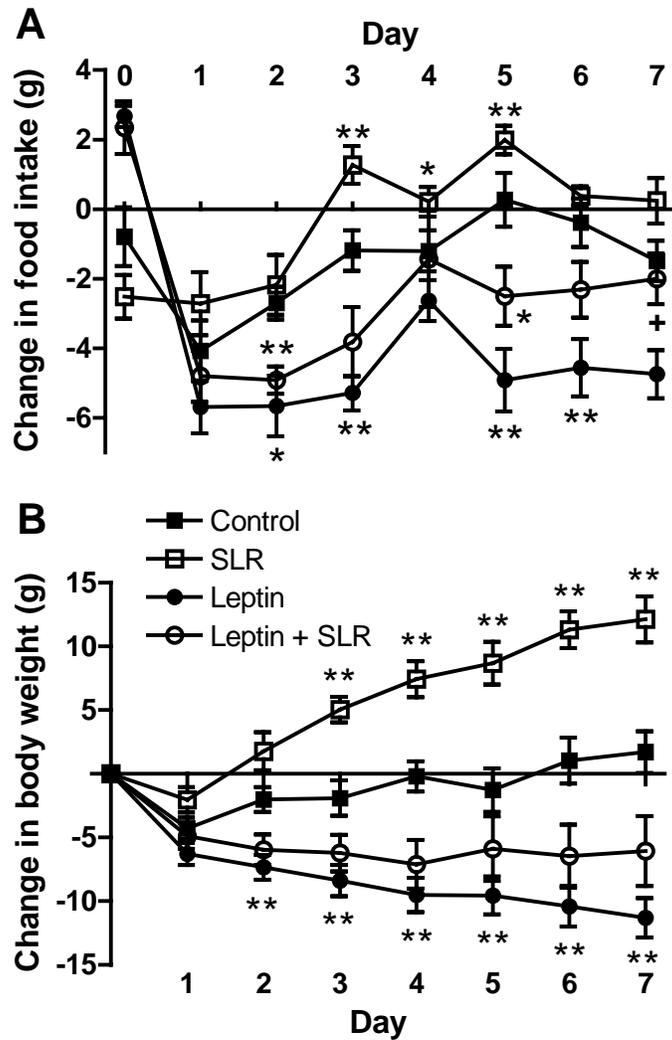


Figure 4-6. Change in daily food intake and body weight in rats following 7-day infusion of vehicle, leptin (0.05 mg/day, subcutaneous), SLR (5.5 μ g/day, lateral ventricle) or leptin (0.05 mg/day, subcutaneous) + SLR (5.5 μ g/day, lateral ventricle). A) Change in food intake was calculated from the average food intake before treatment (Control, 17.9 ± 0.5 g; SLR, 18.9 ± 0.7 g; Leptin, 18.2 ± 0.5 g; Leptin + SLR, 17.4 ± 0.3 g). B) Change in body weight was calculated from body weight at day 0 before treatment (Control, 306.3 ± 8.2 g; SLR, 299.3 ± 11.8 g; Leptin, 304.1 ± 6.9 g; Leptin + SLR, 302.2 ± 7.2 g). Values are mean \pm SE of 7 animals per group. * $P < 0.05$, ** $P < 0.01$ vs. Control; † $P < 0.05$ vs. Leptin.

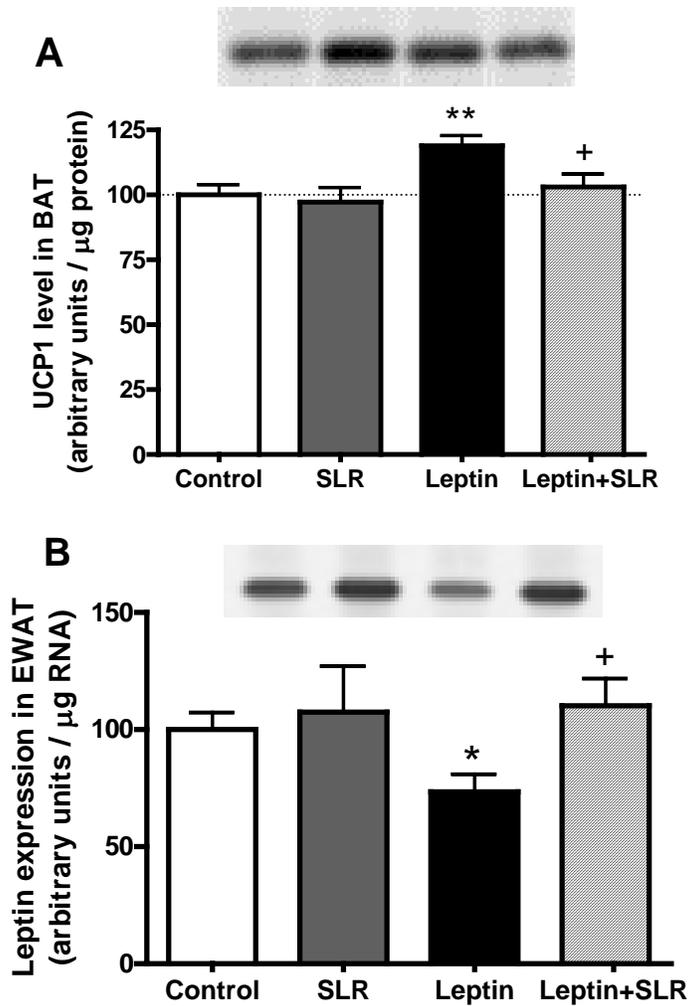


Figure 4-7. UCP1 protein levels in BAT and leptin expression levels in EWAT following 7-day infusion of vehicle, leptin (0.05 mg/day, subcutaneous), SLR (5.5 μg /day, lateral ventricle) or leptin (0.05 mg/day, subcutaneous) + SLR (5.5 μg /day, lateral ventricle). Values are means \pm SE of 6 or 7 animals per group. A) UCP1 protein levels in BAT. Top panel, representative Western bands for each group. Bottom panel, quantified results of the Western analysis. Results are expressed in arbitrary units per microgram of BAT protein and levels in control rats were set to 100, with SE adjusted proportionally. B) Leptin expression levels in EWAT. Top panel, representative RT-PCR product for each group. Bottom panel, quantified results of the RT-PCR analysis. Values are expressed in arbitrary units per microgram of total RNA and levels in control rats were set to 100, with SE adjusted proportionally. * $P < 0.05$, ** $P < 0.01$ vs. control; † $P < 0.05$ vs. leptin.

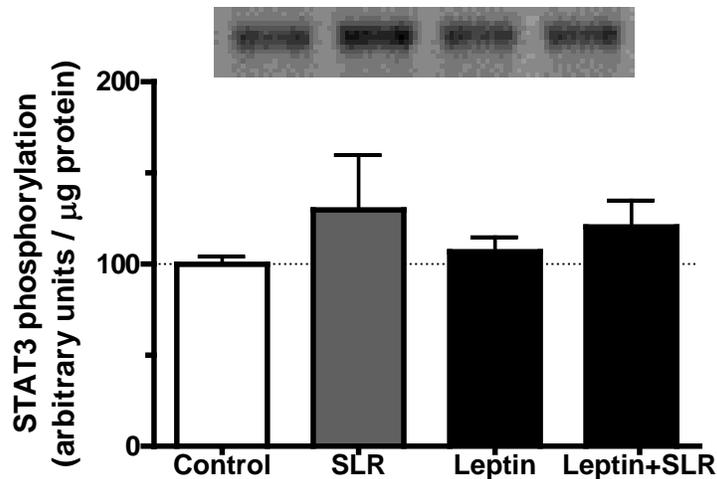


Figure 4-8. Hypothalamic STAT3 phosphorylation following 7-day infusion of vehicle, leptin (0.05 mg/day, subcutaneous), SLR (5.5 μg/day, lateral ventricle) or leptin (0.05 mg/day, subcutaneous) + SLR (5.5 μg/day, lateral ventricle). Top panel, representative Western bands for each group. Bottom panel, quantified results of the Western analysis. Results are expressed in arbitrary units per microgram of hypothalamic protein. STAT3 phosphorylation was normalized to total STAT3 and levels in control rats were set to 100, with SE adjusted proportionally. Values are means ± SE of 6 or 7 animals per group. There was no statistically significant difference among the groups.

Table 4-2 Energy homeostasis-related parameters following high-dose SLR central infusion (5.5 μg/day)

Parameter	Control	SLR	Leptin	Leptin + SLR
Change in whole body fat mass (g)	3.4 ± 2.0	7.0 ± 1.6	-2.6 ± 1.0*	2.7 ± 1.7†
Visceral adiposity (g)	6.49 ± 0.47	6.75 ± 0.64	4.31 ± 0.33**	5.01 ± 0.30*
Serum leptin, day 7 (ng/mL)	2.53 ± 0.32	2.66 ± 0.31	4.89 ± 0.44**	6.81 ± 0.73**†

Values are means ± SE of 6 or 7 animals in each group. * $P < 0.05$ ** $P < 0.01$ vs. Control; † $P < 0.05$ vs. Leptin.

CHAPTER 5
SOLUBLE LEPTIN RECEPTOR GENE THERAPY IN YOUNG F344XBN RATS

Introduction

Soluble leptin receptor is the major leptin binding protein in the circulation. It consists of the extracellular domain of the full-length leptin receptor and is generated by alternative splicing or ectodomain shedding of the leptin receptor. Previous studies have demonstrated that the SLR is able to block leptin action, by binding leptin and preventing its activation of leptin receptor signaling, *in vitro* (Yang *et al.*, 2004). Studies in Chapter 4 further verified the leptin-neutralizing characteristic of SLR by demonstrating that SLR can inhibit leptin-induced anorexic responses and weight reduction, *in vivo*. Moreover, a central infusion of SLR for 7 days significantly increased food intake and body weight.

In this chapter, we hypothesized that long-term SLR treatment into the brain will prevent leptin-mediated energy homeostasis, leading to enhanced food intake and accelerated body weight gain. To test the hypothesis, we utilized a gene deliver system to chronically overexpress SLR in the brain. This method of SLR long-term treatment was chosen because prolonged infusion of recombinant SLR was prohibitively expensive.

Recombinant adeno-associated virus was used as the gene delivery system for SLR transgene, due to its advantages over other methods: The rAAV vector lacks virally encoded genes, thus is non-pathogenic. More importantly, rAAV-mediated gene delivery is able to establish long-term gene expression in various tissues, including the brain (Peel and Klein, 2000). Previous studies from the Scarpace lab successfully achieved long-term gene expression in the hypothalamus using type 2 rAAV-leptin and rAAV-POMC vectors (Scarpace *et al.*, 2002b; Li *et al.*, 2003).

In the experiments of Chapter 5, we used a rAAV type 2 vector encoding rat soluble leptin receptor (rAAV-SLR) to assess the long-term consequences of central SLR gene delivery on energy balance in young leptin-responsive rats. To this end, we first examined synthesis, secretion and activity of the transgene product, *in vitro*, by transfection of SLR plasmid into HEK 293T cells. We then verified SLR transgene expression, *in vivo*, at day 10 and day 65 after rAAV-SLR or control vector administration. Food intake and body weight were examined for 65 days in rats fed a chow or high-fat diet.

Experimental Design

Experiment 1: Verification of SLR Synthesis, Secretion and Activity, *In Vitro*

The SLR transgene was cloned from the 1-2388 bp of the full-length leptin receptor, with a c-myc tag added at the C-terminal (Figure 5-1, A). SLR transgene was then subcloned into pTR backbone to construct the pTR-SLR plasmid (Figure 5-1, B) for rAAV-packaging. The pTR-SLR plasmid was characterized *in vitro* by transfecting into HEK 293T cells. Culture media (reduced-serum and serum-free) and cells were harvested at 72 hours post transfection. SLR protein levels were examined by Western analysis in both cell lysate and concentrated media, using an antibody targeted to c-myc tag. Leptin-binding activity in serum-free media was assessed as the interference of ¹²⁵I-leptin immunoprecipitation. Culture media and lysate from GFP plasmid transfected cells were processed identically and included in all analysis as controls. The rAAV-SLR was produced and titered at 1.85×10^{13} viral genome/mL. The control virus, pTR-GFP, was prepared by the vector core at the University of Florida and was titered at 1.8×10^{12} viral genome/mL.

Experiment 2: Verification of SLR Expression, *In Vivo*

At day 0, two rats were administered rAAV-SLR (9.25×10^{10} particles in 5 μ l). One rat was injected with the vector into the basal hypothalamus targeting the 3rd cerebroventricle and the

other into the lateral ventricle. Ten days after vector administration, the hypothalamus and tissues surrounding the left ventricle were collected, and SLR mRNA levels were measured by RT-PCR. SLR transgene expression was also measured at 65 days after vector delivery as part of Experiment 4 described below.

Experiment 3: Food Intake and Body Weight Following rAAV Administration

Rats were administered of rAAV-SLR (9.25×10^{10} particles in 5 μ l) or rAAV-UF11 (1.8×10^9 particles in 5 μ l) into the basal hypothalamus with coordinates targeting the 3rd cerebroventricle, thus yielding two groups: rAAV-GFP and rAAV-SLR. Food intake and body weight were monitored for 65 days.

Experiment 4: High-Fat Feeding

At day 40 after rAAV administration, a subset of rats from Experiment 2, including rats from both the rAAV-GFP and rAAV-SLR groups, were switched to a high-fat (HF) diet (60% fat, 5.2 kcal/g; diet D12492, Research Diets, New Brunswick, NJ). Food intake and body weight was recorded daily for 13 days. Body composition was measured by TD-NMR at day 0 and day 13. At day 15 of the HF feeding (day 65 after virus delivery), the hypothalamus was collected and SLR transgene expression was measured by RT-PCR.

Results

Verification of SLR Synthesis and Secretion, *In Vitro*

The expression of SLR protein was assessed *in vitro* by transient transfection of HEK 293T cells with pTR-SLR plasmid. The cells transfected with pTR-SLR released significant amount of SLR protein into the media, which was detected as a 140 kD band by Western analysis using an antibody targeted to the c-myc tag (Figure 5-2, A). However, in the cell lysate, a diffused band over a broad range of molecular weight was detected by c-myc tag antibody above 150 kD, while no sharp band was detected at the 140kD molecular weight that was

identified in cell media (Figure 5-2, B). No SLR was detected in either media or lysate of cells transfected with GFP plasmid (Figure 5-2).

Leptin Binding Activity of SLR in the Culture Media

Leptin binding activity in the culture media from pTR-SLR transfected HEK293T cells was assessed by the inhibition of ^{125}I -leptin immunoprecipitation (Figure 5-3). When increasing amount of culture media was incubated with a fixed amount of ^{125}I -leptin and leptin antibody, subsequent precipitation of ^{125}I -leptin was inhibited in a dose-dependent manner. Moreover, when the media was heat inactivated before incubation with ^{125}I -leptin, no interference of ^{125}I -leptin immunoprecipitation was observed. Similarly, when culture media from GFP transfected cells was used, immunoprecipitation of ^{125}I -leptin was also not affected, with or without prior heat-inactivation. These data suggest that factors that are unique to the culture media of pTR-SLR transfected cells have leptin binding activity and such factor is SLR, as confirmed by Western analysis of the media.

Verification of SLR Expression, *In Vivo*

As SLR shares majority of the sequence with full-length leptin receptor, two sets of PCR primers were designed to specifically amplify the transgene. Primer set 1 was targeted toward the exon 1 sequence unique to the pTR vector and the N-terminal sequence of SLR (Figure 5-4, A). As the target region includes a chimeric intron, the size of PCR product will be 611 bp with the intron or 457 bp if intron was removed by mRNA splicing. Primer set 2 was targeted toward the C-terminal of the SLR and the c-myc tag sequence, and thus, will amplify a DNA fragment of 203 bp (Figure 5-5, A).

At day 0, two rats were administered rAAV-SLR by i.c.v. injection, one was injected into the 3rd ventricle and the other into the lateral ventricle. Ten days later, SLR mRNA level was measured in the hypothalamus and also in tissues surrounding the lateral ventricle by RT-PCR.

In the rat that was administered rAAV-SLR into the 3rd ventricle, SLR mRNA expression was detected only in the hypothalamus but not in the tissues surrounding the lateral ventricle. By contrast, in the rat that was administered rAAV-SLR into the lateral ventricle, SLR transgene expression was only detected in lateral ventricle but not in the hypothalamus (Figure 5-4, B; Figure 5-5, B). RT-PCR results using primer set 1 generated fragments of two sizes, a 457-bp fragment, which corresponds to the spliced form of RNA without the intron, and a larger 611-bp fragment, which represents the unspliced RNA. The 457-bp band was much stronger than the 611-bp band, suggesting that rAAV-encoded transgene was actively transcribed and processed, *in vivo* (Figure 5-4, B). Hypothalamic cDNA from untreated rats and pTR-SLR vector were included in the PCR as negative and positive controls, respectively. RT-PCR results using primer set 2, which targeted toward the N-terminal of SLR, revealed the same results, in that SLR expression was only detected in tissues surrounding the injection site (Figure 5-5, B).

Food Intake and Body Weight after rAAV-SLR Delivery

In a separate group of rats, rAAV-SLR (9.25×10^{10} particles in 5 μ l) or rAAV-UF11 (1.8×10^9 particles in 5 μ l) was administered by 3rd ventricle injection. Animals were maintained on chow and their food intake and body weight monitored regularly for 65 days. With both treatments, food intake dropped significantly on day 1 due to surgical effect, but recovered quickly to pre-surgical level by day 4 and remained stable throughout the experiment (Figure 5-6, A). Similarly, there was an initial loss of body weight for two days after surgery, but from day 3 both groups started gaining weight steadily (Figure 5-6, B) However, there was no difference in food intake or body weight between the rAAV-SLR and rAAV-GFP rats (Figure 5-6).

High-Fat Feeding

At day 40 after vector delivery, a subset of rats from the rAAV-SLR and rAAV-GFP groups were switched to a 60% HF diet. Caloric intake of both groups increased significantly on

day one immediately after HF feeding, and gradually normalized to the basal level by day 9. However, neither group differed from the other in terms of peak caloric intake or days required for caloric normalization (Figure 5-7, A). Meanwhile, although both groups gained substantial weight on the HF diet, body weight remained the same between the two groups (Figure 5-7, B).

Change in Body Composition on HF Diet

Whole body fat, lean, and fluid mass were measured at day 0 and day 13 of the HF feeding. Although both groups had significant increase in fat and lean mass, no significant difference was observed between rAAV-SLR and rAAV-GFP rats in any parameter of body composition in response to HF feeding (Figure 5-8).

SLR Transgene Expression at Day 65 Post Vector Delivery

Because no physiological responses were observed after rAAV-SLR delivery, this raised the question whether SLR transgene was still being expressed *in vivo*. At day 65 post delivery, hypothalamic SLR mRNA expression was verified by RT-PCR in rAAV-SLR rats, whereas none was detected in rats given rAAV-GFP, indicating rAAV-mediated transgene expression is persistent for 65 days (Figure 5-9, A). Additionally, SLR expression levels on day 65 were compared with day 10 by relative-quantitative PCR, which co-amplified SLR transgene with primers specific for 18S rRNA (Figure 5-9, B). SLR expression level on day 65 was 100 ± 40.89 (arbitrary unit), as compared to 82.71 (from only one rat) on day 10. When normalized to 18S rRNA, SLR expression levels were also comparable between the two time points (day 65, 100.0 ± 5.27 vs. day 10, 79.82, arbitrary units). Day 10 hypothalamic cDNA from lateral ventricle injected rat was included as a negative control, and no SLR expression was detected in this hypothalamus.

SLR Protein Levels in the Hypothalamus Following Vector Delivery

At day 10 and 65 after rAAV vector delivery, SLR protein levels were measured in hypothalamic homogenate by Western blot using c-myc tag antibody. At day 10, a diffused band over a broad range of molecular weight was detected above 150 kD (Figure 5-10, A), which is similar to the pattern observed with Western analysis of cell lysate in the *in vitro* study (Figure 5-2, B). Hypothalamic homogenate from an untreated animal was included as a control, and as expected, no SLR was detected. However, at day 65, no SLR protein was detectable by Western in the hypothalamus of rAAV-SLR or rAAV-GFP injected rats (Figure 5-10, B).

Immunohistochemistry also revealed no apparent SLR expression in the hypothalamus.

GFP mRNA and Protein Levels, *In Vivo*

In order to confirm the successful delivery of virus by i.c.v. injection, hypothalamic GFP mRNA was assessed by RT-PCR at day 65 in rAAV-GFP rats. Using primers specific for GFP, A 650-bp band was detected, however, in only 4 out of the 6 animals (Figure 5-11, A, lane 1-6). Moreover, in those 4 rats, there was a big variance in the expression level of GFP. pTR-GFP plasmid and rAAV-GFP were included as positive controls, and PCR amplified a 650-bp fragment, consistent with the results in rAAV-GFP rats (Figure 5-11, A, lane 7, 8).

Hypothalamic cDNA from rAAV-SLR injected rats were pooled and used as a negative control. As expected, PCR did not detect any GFP expression (Figure 5-11, A, lane 7). For comparison, GFP protein levels at day 65 were also examined by Western using GFP antibody. However, even with 70 μ g hypothalamic proteins, there was no clearly detectable band near 27 kD, which is the molecular weight of GFP (Figure 5-11, B).

Discussion

Results from Chapter 4 demonstrated that central infusion of recombinant SLR protein is able to increase food intake and body weight in leptin responsive rats, presumably by

neutralizing free leptin in the CSF. As leptin is important for long-term homeostatic body weight regulation, we evaluated the effects of long-term leptin neutralization by SLR. In this chapter, we hypothesized that long term SLR gene delivery by rAAV will increase food intake and body weight for a prolonged period of time.

The SLR transgene sequence was engineered so that it is 99% identical to endogenous soluble leptin receptor (Ob-Re) in amino acid sequence (1-796), with the last 9 amino acid of Ob-Re (GHICYVLLLN) replaced by c-myc tag (EQKLISEEDL). Deletion and substitution studies of leptin receptor have localized the leptin-binding domain to aa 323-640 (Fong *et al.*, 1998), and this domain is retained in the SLR transgene. Another report studied the leptin binding action of Ob-R839, a leptin receptor construct truncated immediately before the transmembrane domain. It is identical to Ob-Re in aa 1-796, with additional 43 aa at C-terminal. Despite the sequence difference, Ob-R839 was found to be similar to Ob-Re with respect to the binding of leptin (Tu *et al.*, 2008). The above data predict that SLR transgene will have similar leptin binding characteristics to the endogenous Ob-Re.

In vitro analysis of the SLR transgene construct included transient transfection in HEK 293T cells and interference of a ¹²⁵I-leptin binding assay. SLR was readily detected in media of transfected cells, suggesting SLR was synthesized and secreted. When this media was incubated with ¹²⁵I-leptin in an immunoprecipitation assay, it greatly reduced the amount of ¹²⁵I-leptin precipitated by leptin antibody, suggesting the SLR in the media was able to bind leptin and prevent binding from leptin antibody. Collectively, *in vitro* analysis confirmed that the SLR transgene construct was able to express and secrete a functional SLR, *in vitro*.

However, a sharp band corresponding to SLR could not be detected in the cell lysate. Instead a diffused band over a broad range of molecular weight above 150 kD was observed in

the pTR-SLR transfected but not in the GFP transfected cells. Since the samples were separated under reducing conditions, this diffused band was not likely due to the dimerization of SLR, and more likely to represent intermediate products of glycosylation. Theoretically, up to 20 N-linked glycosylation sites (Asn-X-Ser, Asn-X-Thr or Asn-X-Cys) are available in the extracellular domain of the leptin receptor as determined by its primary structure (Tartaglia *et al.*, 1995). Glycosylation is a multi-step, site-specific enzymatic process. Usually, a 14-sugar chain is first attached to the asparagine in the target protein, then this glycan undergoes extensive processing and modification, such as partial removal of sugar residues, addition of phosphate, and / or acetyl group and elongation with new sugar residues. The molecular weight of SLR is calculated to be 91 kD, based on its primary amino acid sequence. However, studies concerning the molecular characterization of Ob-Re revealed a variable molecular weight ranging between 80 and 300 kD (Sinha *et al.*, 1996; Gavrilova *et al.*, 1997; Liu *et al.*, 1997; Lammert *et al.*, 2001). Comparison of sera from pregnant and non-pregnant mice also revealed Ob-Re with different molecular weight whereas after complete deglycosylation, both migrated at the same position (Lammert *et al.*, 2001). These studies suggest that the extent of glycosylation may contribute to the heterogeneity in the immunoreactivity of Ob-Re and that the diffused band over a broad range of molecular weight above 150 kD in the SLR transfected cell lysate may very well represent SLR undergoing glycosylation processing. Interestingly by contrast, only the 140 kD SLR was detected in the media, suggesting proper glycosylation is necessary for the secretion of SLR. It is reasonable to predict that complete deglycosylation of cell lysate and culture media will reveal SLR at the same migration molecular weight, and probably close to 91 kD, the calculated molecular weight based on amino acid sequence. The reason for the incomplete glycosylation is probably due to an overload of SLR expression. Reducing the transfection amount of pTR-SLR

plasmid into the HEK 293T cells is likely to ameliorate this problem in the *in vitro* Western analysis.

Because the *in vitro* analysis confirmed the proper synthesis, secretion and function of the SLR transgene, it was unexpected that in the follow-up *in vivo* study, no physiological responses were observed after rAAV-SLR delivery for up to 65 days. Food intake and body weight were almost identical between the rAAV-SLR and rAAV-GFP group. High-fat feeding led to a transient hyperphagia and accelerated weight gain, but it failed to induce any difference between the two groups in term of caloric intake, body weight, or body composition changes. One possibility is a lack of transfection of the rAAV *in vivo*. To answer this question, I tested transgene mRNA expression in the hypothalamus of rAAV-SLR injected rats at both day 10 and day 65. Not only was mRNA detected at day 65, SLR expression level was comparable with level at day 10. These results demonstrated that the rAAV-SLR vector was able to transfect cells *in vivo* and also transcribe the transgene producing mRNA.

Another possibility for the lack of response to rAAV-SLR is a low expression level of the transgene. It is likely that this amount of SLR transgene product neutralized only a small fraction of the endogenous leptin in the CSF, thus was without a significant impact on food intake and body weight. In future experiments, it will be of great interest to directly measure CSF SLR levels after both gene delivery and central infusion, in order to estimate the amount of transgene protein necessary to impact food intake or body weight.

There are several factors that could contribute to the low level of SLR transgene product. First, it could be due to the simple fact that the method of rAAV gene therapy is just not efficacious enough to synthesize sufficient SLR to neutralize the amount of leptin in the brain. For example, previous application of rAAV (2.5×10^{11} physical particles per rat) delivering leptin

into the brain increased CSF leptin level by 75%, from 100 pg/mL to 175 pg/mL (Scarpace *et al.*, 2002b). In the current study, a similar amount of rAAV vector (9.3×10^{10} physical particles per rat) was delivered into the brain using the same injection protocol, in animals of same age and similar weight. In this study, however, the level of SLR protein in the cerebrospinal fluid (CSF) was not measured directly. But we can make a rough estimate, assuming the efficiency of leptin and SLR vectors are similar. Because rAAV-leptin increases CSF leptin level by 75 pg/mL, we expect rAAV-SLR to produce a similar amount of the transgene product on a molar basis as rAAV-leptin. Since SLR is almost 6 times larger by molecular weight than leptin (91 kD vs. 16 kD), at best, rAAV-SLR will produce sufficient SLR to achieve a concentration of approximately 450 pg/mL, or 4.7 nM. And based on the previous rAAV-leptin study, the concentration of leptin in the CSF is around 100 pg/mL, or 6.25 nM. SLR binds to leptin with a high affinity similar to the full-length leptin receptor. According the data from several reports (Liu *et al.*, 1997; Sandowski *et al.*, 2002; Mistrik *et al.*, 2004), the average K_D is around 0.5 nM.

$$K_D = \frac{[Leptin_{FREE}][SLR_{FREE}]}{[SLR - Leptin]} \quad (5-1)$$

$$[Leptin_{TOTAL}] = [Leptin_{FREE}] + [SLR - Leptin] \quad (5-2)$$

$$[SLR_{TOTAL}] = [SLR_{FREE}] + [SLR - Leptin] \quad (5-3)$$

Based on the single-site binding model, by substitution and solving, we calculated the ratio of free to total leptin ($Leptin_{FREE} / Leptin_{TOTAL}$) in the CSF to be 62%. The calculation suggests theoretically rAAV should be able to produce sufficient amount of SLR to neutralize a significant portion of CSF leptin. However, it did not seem to be the case according to the physiological responses after rAAV-SLR delivery. Therefore, either the level of SLR produced in the current study was much lower than our theoretical calculation, or the leptin level was

higher than previously measured, or neutralization of leptin did not result in the predicted physiological results. We suggest the first possibility is the most likely.

In the current study, we have administered a considerable amount of rAAV-SLR vector, which is comparable to the amount used in the rAAV-leptin study. It is difficult to further increase the amount of rAAV vectors, as the delivery volume for brain injection is limited. The rAAV-SLR vector was titered at 1.85×10^{10} viral genome/ μL , which is a fairly high concentration that can be achieved following the current protocol for rAAV production and purification. To circumvent this problem, a new protocol for the production of higher titer vector, a new rAAV delivery strategy (for example, infusion instead of single injection), or a completely new gene delivery system may be necessary.

Second, despite previous success in central application of rAAV-leptin, it is possible that transduction efficiency of rAAV-SLR in the brain was compromised and thus was lower than rAAV-leptin. Future experiments will be necessary to compare transduction efficiency between rAAV-SLR and rAAV-leptin. Furthermore, the expression efficiency of rAAV-SLR could be lower, considering SLR (91 kD) is almost 6 times larger than leptin (16 kD) in molecular weight. It is simply more time- and energy- consuming to synthesize the larger molecule, even if all other conditions, such as viral particles delivered and transduction efficiency, were the same between the two vectors.

Third, it is possible that not all synthesized SLR was secreted, thus less SLR was present in the CSF than actually made in the cells. As discussed in the *in vitro* analysis of SLR construct, a diffused band over a wide range of molecular weight was detected in the cell lysate, suggesting that proper glycosylation of SLR is necessary for secretion. It is likely that rAAV-mediated overexpression produced more SLR than the cells can properly process for secretion in a timely

manner. For example, the level of glycosylation-related enzymes may be one of the rate-limiting steps in this process, especially because these rAAV infected cells do not normally synthesize this protein. As a result, a large portion of SLR synthesized may be retained in the cytoplasm, and therefore unable to neutralizing the free leptin in the CSF.

Fourth, it is also possible that SLR could not reach the leptin that had access to the receptors. It is not fully understood how leptin reaches the leptin receptor-bearing neurons that are not lining the cerebroventricle system. The blood-cerebrospinal fluid (CSF) barrier at the choroid plexus and the blood-brain barrier (BBB) at the cerebral endothelium are two major controlling sites for circulating proteins to enter into the brain (Zlokovic *et al.*, 2000). Studies have demonstrated that the blood brain barrier, in contrast to the blood CSF route, is the key pathway for leptin to reach the brain (Kurrimbux *et al.*, 2004). Leptin uptake by the hypothalamus is significantly greater than other BBB regions in the brain, indicating the existence of a high affinity transport system for leptin in the hypothalamus (Zlokovic *et al.*, 2000). It implies that leptin may reach the hypothalamus via two routes, the BBB and the CSF (Kurrimbux *et al.*, 2004). On the other hand, CSF leptin may be a result of CNS sink action, where leptin diffuses out of the brain and accumulates in the CSF. In this study, the rAAV-SLR was delivered to the ventricle, so SLR was most likely being expressed by cells lining the ventricles and secreted into the CSF. In that case, it would only neutralize leptin in the CSF, rendering untouched the fraction of leptin that interacts with the leptin receptors.

Last but not least, when amino acid sequence of pTR-SLR was examined, two ATGs were found upstream of the start codon in the SLR transgene. One ATG starts a different reading frame, which encodes a short peptide of 18 aa. The other ATG falls in the same reading frame of SLR. However, it encodes an immediate stop codon at the 8th amino acid, which is upstream of

the start codon of SLR. This suggests that protein translation under the desired reading frame for SLR is possible to terminate early before the ribosome reaches the SLR sequence. The reading frame analysis indicates that the start codon of SLR may be in disadvantage competing with upstream ATGs for translation initiation, and this likely would result in low translation efficiency of the SLR. In future studies, to optimize translation, site-directed mutagenesis can be used to eliminate unwanted upstream ATGs on the pTR vector. Additionally, a Kozak sequence (ACCATGG) should be incorporated into the starting sequence of SLR to further enhance translation initiation.

There is another overlooked yet possible explanation for the lack of physiological response *in vivo*. It is possible that viral mediated SLR delivery has no effects in young leptin-responsive rats. Previous studies from the Scarpace lab demonstrated that rAAV-POMC produced substantial responses in genetically obese Zucker rats and aged-obese rats (Li *et al.*, 2003; Li *et al.*, 2005; Li *et al.*, 2007b), but it was ineffective in young leptin-responsive rats (Li *et al.*, 2007a). In contrast to obese rats that are leptin-resistant, young rats have an intact leptin signaling pathway, and likely a feedback mechanism that detects the decrease in leptin receptor activation potentially caused by SLR and compensates by augmenting leptin synthesis, increasing leptin transport from the peripheral, or by elevating leptin receptor levels. For instance, when leptin level was elevated due to a leptin infusion, as demonstrated by the SLR infusion study in Chapter 4, leptin mRNA level was reduced in the EWAT, probably through a feedback mechanism in an attempt to offset such increase. When SLR was simultaneously infused with leptin, such inhibition was fully blocked and leptin mRNA level was back to the control level. It will be of great interest in future experiments to test rAAV-SLR in leptin-resistant models, such as diet-obese or aged-obese rats.

One conflicting result I observed in the *in vivo* study was that SLR transgene mRNA level was detected in the hypothalamus at day 10 and day 65 after vector delivery. However, when I tried to measure SLR protein level in the hypothalamus by Western, SLR was detected at day 10, but undetectable at day 65. This suggests that SLR protein level was very low, at least below the detection limit of our method of examining protein extraction of whole hypothalamus. On the other hand, SLR level in hypothalamus was only indicative of, but not equal to, the total SLR protein level in the brain, as rAAV-SLR was injected in to the 3rd ventricle close to the hypothalamus and some vectors may diffuse to other regions of the brain. This may be another reason that SLR protein level was almost undetectable in the hypothalamus, in addition to the low expression level of rAAV-SLR vectors. To confirm successful vector delivery, GFP mRNA and protein levels were also measured in control rats received rAAV-GFP by 3rd ventricle injection. Surprisingly, the results are similar to the rAAV-SLR that mRNA was readily detected in the hypothalamus whereas GFP protein was almost undetectable. This suggests that examining protein level in whole hypothalamus may not be the best method to determine expression level for i.c.v. vector delivery.

In summary, this chapter describes the cloning of rat soluble leptin receptor and the *in vitro* expression of the SLR plasmid, and examined the physiological responses to rAAV-mediated central SLR gene delivery. The SLR transgene was identical to endogenous soluble leptin receptor (Ob-Re) in aa 1-796, with an added c-myc tag (EQKLISEEDL) at C-terminal. *In vitro* analysis of the construct demonstrated proper synthesis, secretion and leptin binding activity of SLR in mammalian cells. We hypothesized that long-term gene delivery of SLR in young leptin-responsive rats will increase food intake and exacerbated weight gain. To our surprise, there was no physiological response after rAAV-SLR administration for 65 days. SLR mRNA was found

in the hypothalamus, whereas protein level was below detection. There may be multiple factors contributing to the low level of transgene expression, which prevented us from obtaining a definitive answer for this hypothesis. For future experiments, it is necessary to test the hypothesis in obese rats, and to optimize gene delivery efficiency as well.

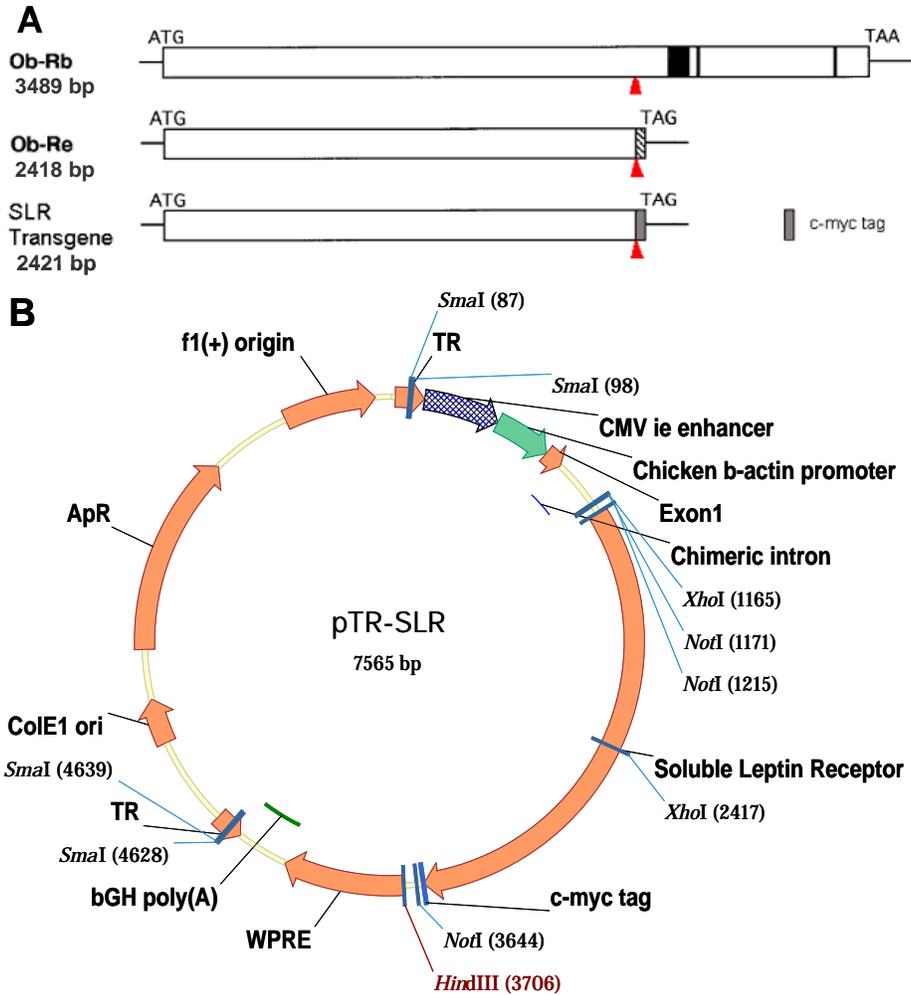


Figure 5-1. Construction of pTR-SLR plasmid. A) Cloning of SLR transgene. Arrow, alternative RNA splicing site (2388 bp). Solid box, transmembrane domain of Ob-Rb. Striped box, distinct C-terminal sequence of Ob-Re. Shaded box, c-myc tag sequence added to the C-terminal of SLR transgene. B) pTR-SLR plasmid map. SLR transgene was subcloned into pTR plasmid backbone by ligation at the XhoI (1165) and HindIII (3706) restriction sites. Flanked by the TRs, the expression cassette of pTR-SLR includes the following components, in 5' to 3' order: 1) the hybrid cytomegalovirus immediate early (CMV ie) enhancer / chicken beta-actin (CBA) promoter / exon 1 / chimeric intron; 2) SLR transgene, containing c-myc tag at the C-terminal; 3) The woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), to enhance SLR expression; 4) the poly A tail from bovine growth hormone.

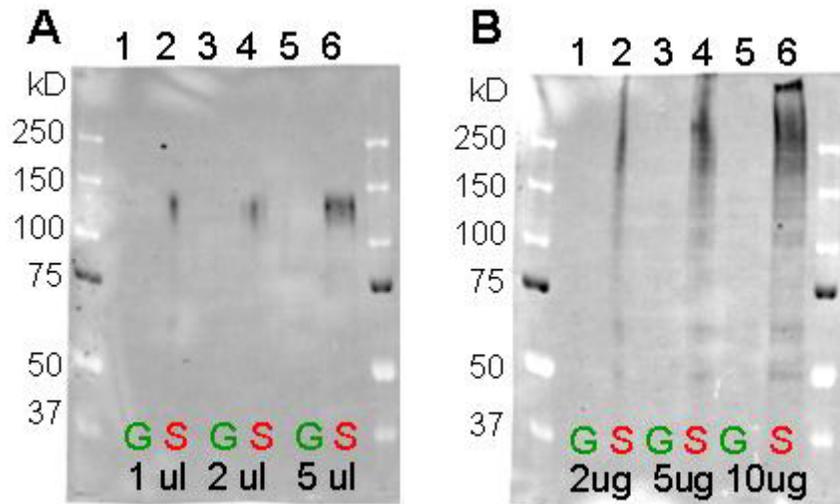


Figure 5-2. Synthesis and secretion of SLR *in vitro*. A) Western analysis for SLR using c-myc tag antibody, in culture media of cells transfected with pTR-SLR (lane 2, 4, 6) or GFP plasmid (lane 1, 3, 5). The culture media were 25-fold concentrated and 1, 2 or 5 μ L were used for Western. B) Western analysis for SLR using c-myc tag antibody, in lysate of cells transfected with pTR-SLR(lane 2, 4, 6) or GFP plasmid (lane 1, 3, 5). The amount of lysate used for Western was 2 μ g, 5 μ g, or 10 μ g.

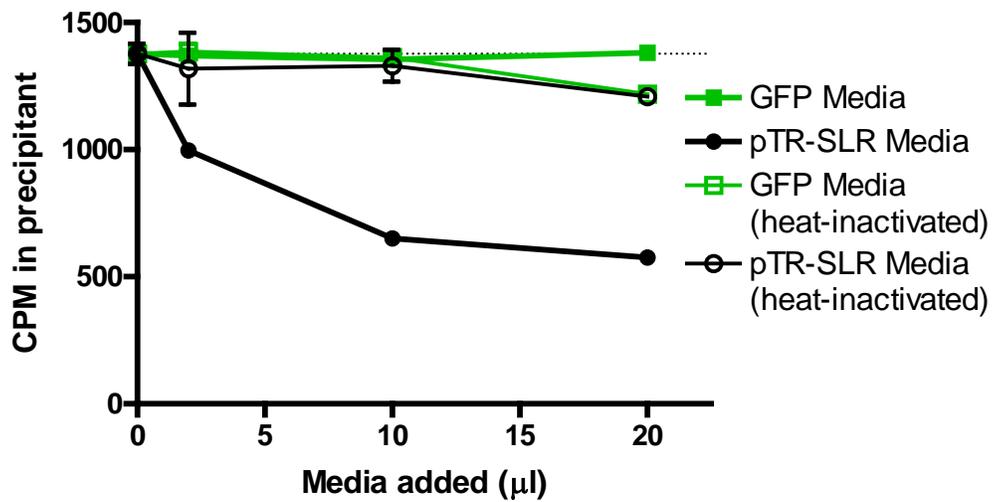


Figure 5-3. Leptin binding activity in culture media as measured by the interference of ^{125}I -leptin immunoprecipitation. Increasing amount of 50-fold concentrated media was incubated with fixed amount of ^{125}I -leptin and radioactivity in pellets was counted following immunoprecipitation. Solid square, media from GFP transfected cells. Solid circle, media from pTR-SLR transfected cells. Open square, heat inactivated GFP media. Open circle, heat inactivated SLR media.

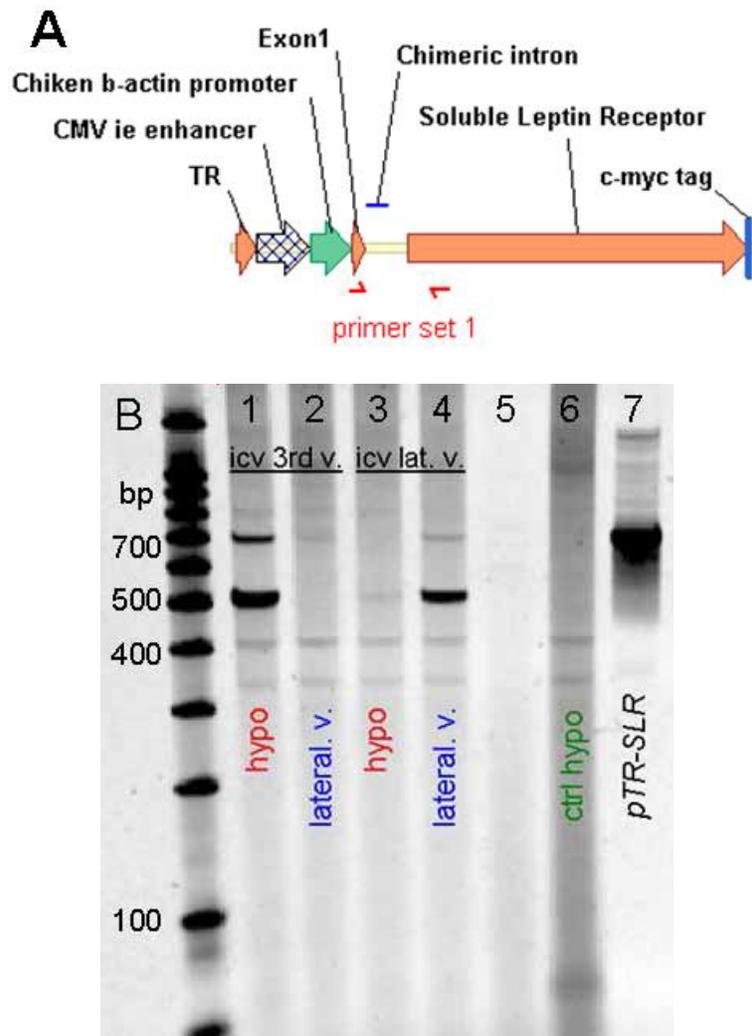


Figure 5-4. Verification of SLR expression *in vivo* by RT-PCR using primer set 1. A) Primer set 1 was targeted toward the exon 1 sequence unique to the pTR vector and the N-terminal sequence of SLR. As the target region includes an intron, size of PCR product will be 611 bp with the intron or 457 bp when intron is removed by mRNA splicing. B) SLR expression levels 10 days after vector delivery. In the rat that was injected rAAV-SLR into the 3rd ventricle, SLR expression was detected only in the hypothalamus (lane 1), but not in the tissues surrounding the lateral ventricle (lane 2). By contrast, in the rat that was injected rAAV-SLR into the lateral ventricle, SLR expression was only detected in lateral ventricle (lane 4) but not in the hypothalamus (lane 3). Lane 5, negative control (no PCR template). Lane 6, hypothalamic cDNA from untreated rat. Lane 7, pTR-SLR plasmid as DNA template.

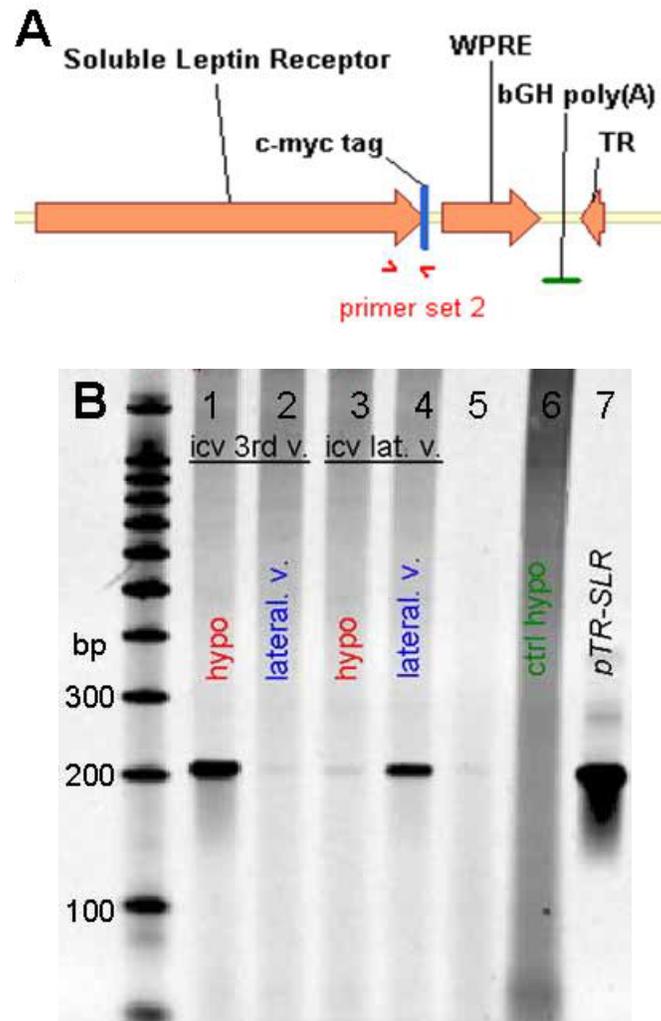


Figure 5-5. Verification of SLR expression *in vivo* by RT-PCR using primer set 2. A) Primer set 2 was targeted toward the C-terminal sequence of SLR and the c-myc tag. B) SLR expression levels 10 days after vector delivery. In the rat that was injected rAAV-SLR into the 3rd ventricle, SLR expression was detected only in the hypothalamus (lane 1), but not in the tissues surrounding the lateral ventricle (lane 2). By contrast, in the rat that was injected rAAV-SLR into the lateral ventricle, SLR expression was only detected in lateral ventricle (lane 4) but not in the hypothalamus (lane 3). Lane 5, negative control (no PCR template). Lane 6, hypothalamic cDNA from untreated rat. Lane 7, pTR-SLR plasmid as DNA template.

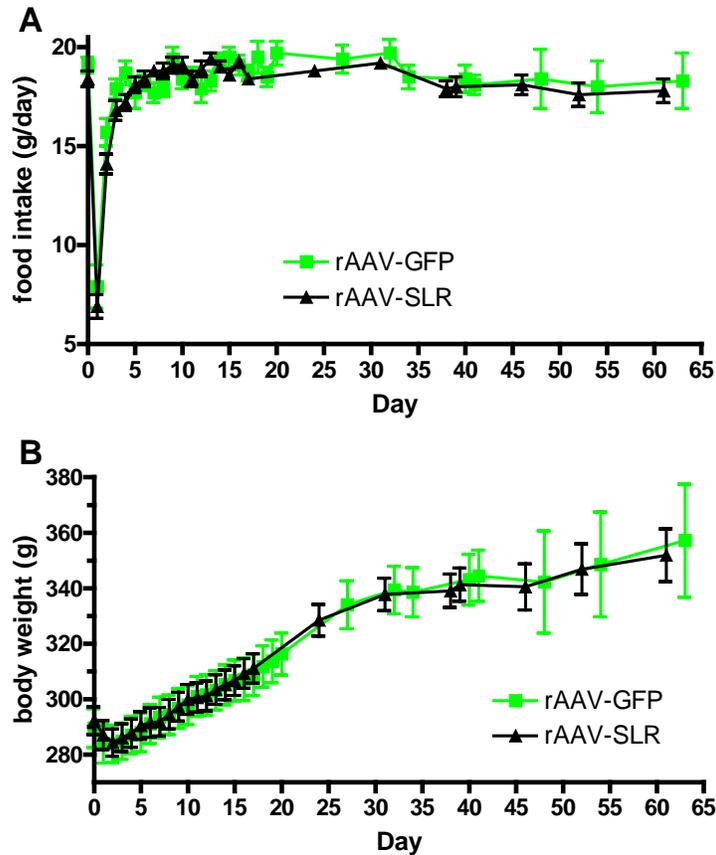


Figure 5-6. Food intake and body weight in rats following administration of rAAV-SLR or rAAV-GFP. Vectors were delivered at day 0 and rats were maintained on chow for 65 days. A) Food intake. B) Body weight. Values represent the mean \pm SE of 12 rAAV-GFP and 23 rAAV-SLR rats from day 0 to day 41. At day 41, a subset of rats from each group was switched to a HF-diet. As a result, from day 42 to day 65, values represent mean \pm SE of 6 rAAV-GFP and 15 rAAV-SLR rats that were still on chow.

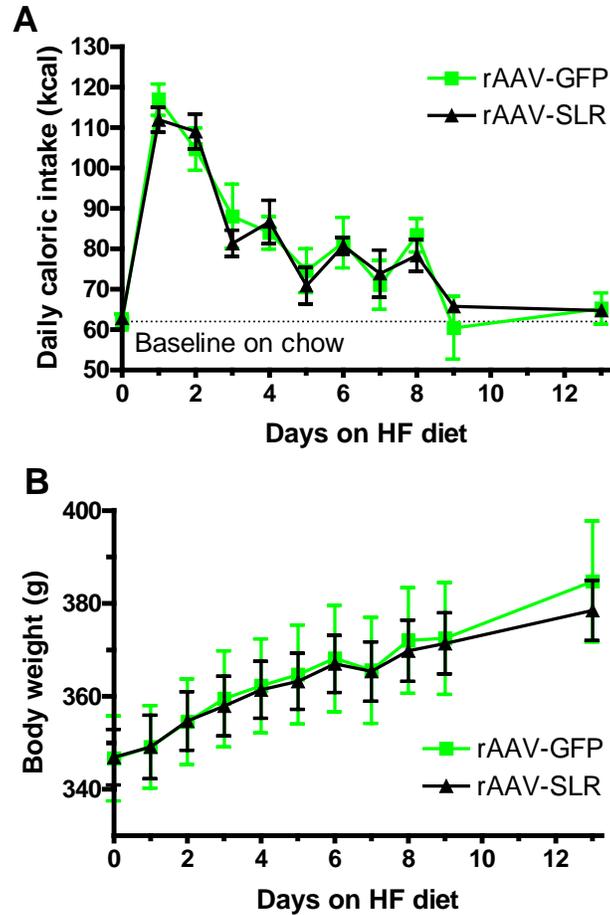


Figure 5-7. Caloric intake and body weight in rats on a high-fat diet following vector administration. At day 0, rats were switched to a high-fat diet after 41 days on chow following rAAV-SLR or rAAV-GFP administration. A) Daily caloric intake. B) Body weight. Values represent the mean \pm SE of 6 rAAV-GFP and 8 rAAV-SLR rats.

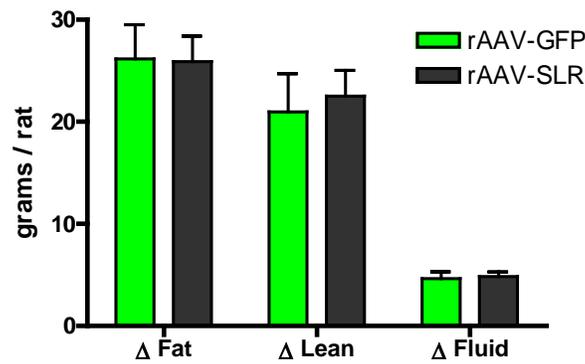


Figure 5-8. Body composition change in response to 13 days of high-fat feeding. Whole body fat, lean and fluid mass were measured at day 0 and day 13 of high-fat feeding. The changes were compared between 6 rAAV-GFP and 8 rAAV-SLR rats.

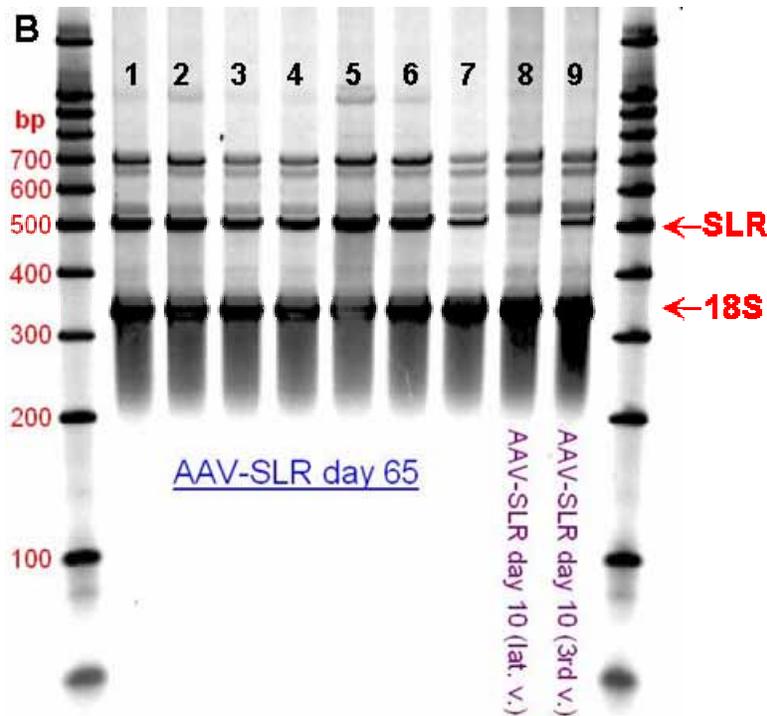
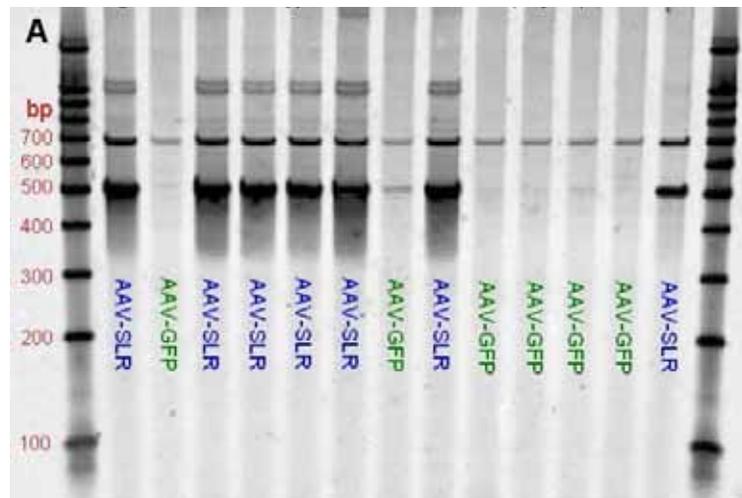


Figure 5-9. SLR expression levels in the hypothalamus by RT-PCR 65 days after vector administration. A) SLR expression levels from rAAV-SLR and rAAV-GFP rats at day 65. The PCR product specific to SLR transgene was only observed in the rAAV-SLR group. B) Comparison of SLR expression levels in the hypothalamus at day 65 and day 10 after rAAV-SLR delivery. Relative-quantitative PCR was performed by co-amplifying SLR with primers for 18S rRNA. Lane 1-7, SLR expression levels at day 65 (3rd ventricle delivery). Lane 8, no SLR expression was detected in the hypothalamus at day 10 post rAAV-SLR delivery into the laterally ventricle. Lane 9, SLR expression level at day 10 (3rd ventricle delivery).

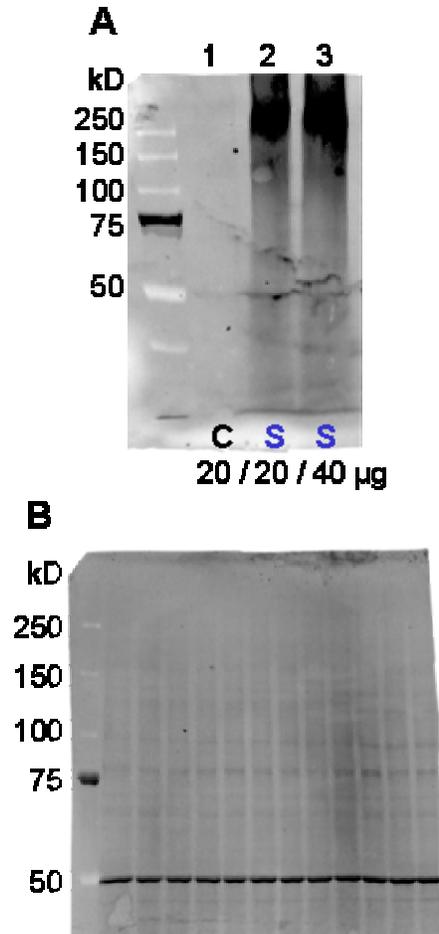


Figure 5-10. Western analysis of SLR protein levels in the hypothalamus using c-myc tag antibody. A) SLR protein levels at day 10 post rAAV-SLR delivery in 20µg (lane 2) and 40 µg (lane 3) of hypothalamic proteins. Lane 1, 20 µg of hypothalamic proteins from an untreated rat as control. B) No SLR protein was detected in 30 µg of hypothalamic proteins from rAAV-SLR and rAAV-GFP rats at day 65.

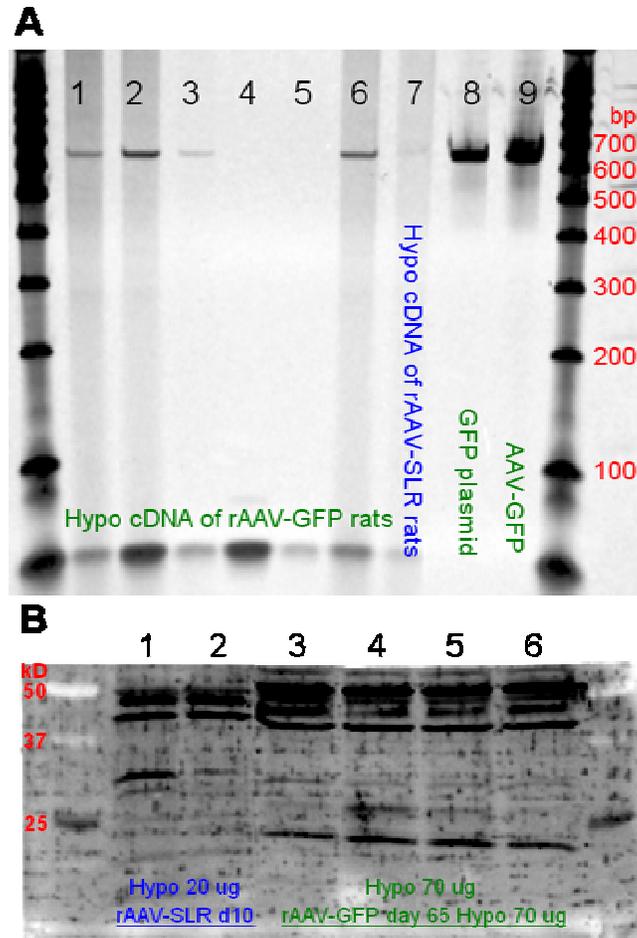


Figure 5-11. GFP expression levels in the hypothalamus at day 65 post vector delivery. A) GFP mRNA levels as measured by RT-PCR at day 65. The PCR product is expected at 650 bp. Lane 1-7, cDNA from rAAV-GFP rats. Lane 8, pooled cDNA from rAAV-SLR rats. Lane 9, GFP plasmid as positive control. Lane 10, rAAV-GFP as positive control. B) Western analysis of GFP protein levels at day 65. GFP band is expected at around 27 kD. Lane 1-2, 20 μ g of hypothalamic proteins from rAAV-SLR rats at day 10. Lane 3-6, 70 μ g of hypothalamic proteins from rAAV-GFP rats at day 65.

CHAPTER 6 GENERAL DISCUSSION AND CONCLUSION

Obesity is a major health issue in the United States and incidence of obesity is also on the rise globally. Excessive body weight not only reduces the quality of life, but also is a predisposing factor for several health conditions, such as type 2 diabetes, cardiovascular diseases, stroke and metabolic syndromes. One of the key regulators in body weight maintenance is leptin, an adipocyte-synthesized hormone, which suppresses food intake and promotes energy expenditure. Upon its discovery, leptin was hoped to be the magic bullet against obesity. It has proved to be effective in the treatment of congenital leptin deficiency or lipodystrophy (Oral *et al.*, 2002; Farooqi and O'Rahilly, 2005; Park *et al.*, 2008). However, benefits of leptin treatment have been limited for obesity in humans and rodents under normal genetic background. In obese individuals and rodents, leptin level rises in proportion to adiposity, but the elevated leptin is no longer able to suppress the progression of obesity. This apparent leptin ineffectiveness is defined as leptin resistance, which is present in most obese rodents.

The increasing prevalence of obesity is often ascribed to the consumption of a high-fat diet. It is of little wonder, as high-fat diets are typically high in energy and highly palatable. Indeed, when rodents are provided a high-fat diet, the immediate response is an increase in caloric intake, and it normally takes weeks before gradually returning to the control intake level (Ramirez, 1990). This process of caloric normalization may very well be mediated by leptin, as evidenced by the increase in serum leptin levels immediately after overfeeding (Kolaczynski *et al.*, 1996).

Leptin circulates in both free and bound forms, but only the free leptin is considered biologically active. In obese subjects, total leptin level is elevated and the majority is in the free form, whereas in lean subjects, leptin primarily exists in the bound form. Whether the elevated leptin, particularly the free leptin, is secondary to the obesity or a causative factor of obesity is

still under debate (Scarpace and Zhang, 2007). Soluble leptin receptor is the major leptin binding protein in the circulation and its level does not increase with obesity. It is demonstrated that soluble leptin receptor prevents leptin-induced signal transduction *in vitro* (Yang *et al.*, 2004) and it also inhibits leptin transport across the blood brain barrier *in vivo* (Tu *et al.*, 2008). Whether neutralization of leptin by soluble leptin receptor, *in vivo*, modulates food intake and body weight was not previously studied. Neutralization of elevated leptin associated with obesity is potentially beneficial, although direct evidence is lacking.

In this dissertation, the major objective was to further understand the role of leptin in food intake and body weight regulation. The availability of a leptin antagonist and SLR are powerful tools to study the role of leptin in physiological processes. Specifically, we used a leptin antagonist and soluble leptin receptor to block leptin action *in vivo* and examined the consequences in leptin-responsive rats. Therefore we put forward two major hypotheses. First, leptin is necessary for the normalization of high-fat feeding induced hyperphagia and this counter-regulatory response will be blocked by central delivery of a leptin antagonist. Second, soluble leptin receptor neutralizes leptin *in vivo*, thus increasing food intake and body weight in leptin-responsive rats. The goals of this dissertation were, first, to characterize the leptin antagonist, *in vivo*; second, to examine the effects of leptin antagonist on caloric normalization following high-fat feeding in young adult rats; third, to characterize soluble leptin receptor, *in vivo*; and finally, to examine the long-term effects of leptin neutralization by soluble leptin receptor overexpression on energy regulation in leptin-responsive rats.

Major Findings and Conclusions

In Chapter 3, I first verified the antagonist properties of this leptin antagonist compound *in vivo*, as only *in vitro* characterization had been examined before. The leptin antagonist fully blocked the leptin-mediated STAT3 signaling in a dose-dependent manner. Moreover, there was

no evidence of inverse agonist activity, as i.c.v. administration of the leptin antagonist alone did not alter basal STAT3 level. Central infusion of the leptin antagonist alone increased food intake and body weight, while simultaneous infusion of the antagonist with leptin blocked the anorexic and weight-reducing responses to the exogenously administered leptin. The leptin antagonist also prevented the leptin-mediated UCP1 increase in BAT. It is concluded from these data that the leptin antagonist acts as a true antagonist *in vivo*, and it is able to block leptin receptor under pharmacological and physiological settings.

The leptin antagonist was then administered simultaneously with the initiation of high-fat feeding. Normally in response to HF feeding, caloric intake rises abruptly and is followed by a gradual restoration to normal levels over time. The leptin antagonist completely prevented this process of caloric normalization and resulted in prolonged hyperphagia and exaggerated weight gain. These results revealed that caloric normalization following high-fat feeding is mediated by leptin, highlighting the importance of leptin in the homeostatic regulation of everyday food intake and body weight. Moreover, this result is consistent with the exacerbated weight gain observed with high-fat feeding in leptin resistant animals. Upon exposure to a high-fat diet, leptin resistant animals exhibit hyperphagia initially and fail to normalize the elevated caloric intake, or display a prolonged normalization process. As a result, they consume more calories and gain more weight than the non-leptin resistant controls (Scarpace *et al.*, 2005; Judge *et al.*, 2008; Shapiro *et al.*, 2008). The similar outcomes of high-fat feeding between a leptin receptor blockade and leptin resistance suggest that one component of leptin resistance is the impairment of functional leptin receptor activity. This reduction could result from decreased leptin receptor level, diminished receptor-mediated signaling transduction, or an uncoupling between receptor signaling and downstream physiological responses (Scarpace and Zhang, 2008).

In addition to caloric intake and body weight, thermogenesis in the brown adipose tissue was measured as UCP1 protein level after high-fat feeding. UCP1 was elevated by nearly two fold after 7 days of high-fat feeding, which is consistent with numerous observations that an increase in caloric intake leads to a parallel increase in energy expenditure (Arnold and Richard, 1987). On the contrary, this increase in UCP1 induced by high-fat feeding was completely abolished by the infusion of the leptin antagonist, despite the fact that the leptin antagonist resulted in a greater hyperphagia upon exposure to the high-fat diet than that displayed by the control infused rats. These results suggest the BAT thermogenic effect in response to high-fat feeding is leptin-dependent. Although total energy expenditure was not measured in this study, it is reasonable to predict a parallel increase in whole body energy expenditure following high-fat feeding, which is likely mediated by leptin and can be prevented by the leptin antagonist.

In response to high-fat feeding, the peak of caloric intake was observed after 2 days of exposure. In the follow-up experiment, maximal leptin signaling in the hypothalamus was examined after 2 days of high-fat feeding. Central administration of leptin induced robust increase in STAT3 phosphorylation level in both chow and high-fat fed rats, but maximal signaling was blunted with high-fat feeding. This acute desensitization may have occurred in response to the HF-induced elevated leptin. It was previously demonstrated by the Scarpace lab that high-fat feeding reduces the maximal signaling capacity with a parallel reduction in leptin receptor expression. In that study, however, HF feeding was prolonged (115 days) and resulted in obesity (Wilsey and Scarpace, 2004). In comparison, the study in Chapter 3 suggests that this putative desensitization occurs rapidly and is likely not related to the development of obesity. Because the desensitization occurs within the time frame of the caloric normalization, the loss of leptin responsiveness, itself, may be contributing to the time course of the normalization. In that

case, this desensitization should disappear quickly when the caloric intake has restored to the control level and the extent of the desensitization may vary along the course of caloric normalization. Further studies comparing levels of maximal leptin signaling at different stages of the normalization process may provide insights into the physiological significance of the desensitization.

The employment of this leptin antagonist enabled us to establish a pharmacological “knockdown” of the leptin receptor. Central infusion of leptin antagonist at 25 $\mu\text{g}/\text{day}$ prevented the caloric normalization following high-fat feeding, demonstrating an apparent full blockade of central leptin receptor. In lean rats on a chow diet, the same dose of leptin antagonist should completely block leptin receptor activation induced by the endogenous CSF leptin. Surprisingly, central infusion of leptin antagonist alone did not result in massive hyperphagia and body weight gain. Antagonist infusion increased food intake to 22.22 ± 1.85 g/day as compared with 18.38 ± 0.86 g/day in the controls. By comparison, age-matched obese Zucker rats, which are genetically devoid of functional leptin receptor, exhibit an average food intake of 35 g/day on a chow diet (Li *et al.*, 2003). The difference in food intake between the two observations suggests that consequences of genetic leptin receptor mutation are more severe than those of a pharmacological receptor blockade. One possibility is that not all the central leptin receptors were fully blocked. Also, the lack of leptin receptors during fetal development may result in more severe consequences, such as damages of other regulatory pathways.

In Chapter 4, I set out to characterize the ability of SLR to neutralize leptin *in vivo*. Leptin exists in free and bound form, but only free leptin is functional. SLR is a truncated isoform of leptin receptor and it is the major leptin binding protein in the circulation. SLR is able to inhibit leptin-induced signaling, similar to the leptin antagonist used in Chapter 3, but they are

fundamentally different in modes of action. The leptin antagonist competes with leptin for leptin receptor binding, whereas SLR is unique in that it neutralizes leptin action by binding free leptin and preventing it from activating leptin receptors. Studies show that obese subjects have similar level of plasma SLR, but much higher leptin when compared with the lean subjects. Therefore, obesity is associated with elevated leptin to SLR ratio and as a result, excessive amount of free leptin. Increasing SLR level may be a potential strategy to normalize elevated free leptin levels with obesity. We suggest that by binding excessive leptin, we may even reverse leptin resistance. However, neutralization of a substantial fraction of leptin in the circulation would require a large amount of SLR, and such an amount of SLR is limited by the cost. Instead, we targeted the site of administration to the CSF, where the amount of leptin is considerably less compared to peripheral levels and thus requiring less SLR to neutralize.

In Chapter 4, I first demonstrated that the SLR is able to block leptin-induced STAT3 signaling in the hypothalamus, indicating SLR can neutralize leptin action *in vivo*, presumably by binding leptin. SLR administration alone did not alter basal signaling level, demonstrating the SLR itself does not modulate leptin receptor activity. It also suggests that either the level of endogenous CSF leptin is negligible, or that it does not contribute to the basal STAT3 phosphorylation, at least not at our level of detection. Additionally, central infusion of SLR by itself increased food intake and body weight, which is likely due to the neutralization of endogenous leptin in the CSF. However, there is no direct evidence that SLR does not interact with other molecules in the CSF. In order to prove that SLR does not elevate appetite and weight gain independent of leptin neutralization, the leptin deficient *ob/ob* mice will be the ideal animal model for future experiments. As no leptin neutralization would take place in *ob/ob* mice, SLR infusion should not alter food intake or body weight.

Following the characterization of SLR, I examined if SLR is able to neutralize leptin-induced anorexia and weight reduction *in vivo* with two experiments, both of which infused the leptin peripherally and the SLR centrally. These experiments were designed so that SLR and leptin were delivered separately and only mixed at the site of action. Two combinations of leptin and SLR doses were used and both demonstrated that SLR partially blocked leptin-induced anorexic and weight-reducing responses, but neither dose of SLR achieved full blockade. Interestingly, when UCP1 level in BAT and leptin expression level in EWAT were assessed, changes in these central leptin-mediated markers were completely inhibited, suggesting SLR achieved full blockade of these biochemical markers despite the incomplete blockade of food intake and body weight.

There are several explanations for the incomplete blockade of physiological responses mediated by exogenous leptin. First and most likely, neither doses of SLR was sufficient to bind all the leptin. Ideally, using higher doses of SLR would be the best way to test this possibility, however, at present time it is not practical due to the high cost of SLR. Second, the separate routes of delivery may have caused a temporal delay in the arrival of SLR into the CSF. It is likely that leptin reached the brain and activated leptin receptors before the first of SLR made an appearance. A straightforward method to eliminate the delay is a co-infusion of leptin and SLR into the CSF. However, as SLR is likely to fully bind leptin when incubated together in the infusion pump, and thus would already exist as a SLR-leptin binding complex when pumped into the brain. Although this method was used for the leptin antagonist study in the previous chapter, it does not test the ability of SLR to neutralize leptin already present in the brain. It will be necessary to improve the experimental protocol for synchronized delivery in future experiments. Third and most intriguing is the possibility that central leptin action was fully blocked and the

residual anorexic responses were due to peripheral leptin action. This is supported by the fact that UCP1 increase in BAT and the decrease in leptin expression in EWAT, both of which are mediated centrally by leptin, were fully blocked despite persistent but reduced anorexic responses. This suggests a role for peripheral leptin action in the energy balance. It is well established that leptin modulates immune responses by a direct peripheral mechanism (Lord *et al.*, 1998; Zhang *et al.*, 2002). However, with respect to energy regulation, most studies so far have only confirmed a central role for leptin and no direct role for peripheral leptin action in energy balance independent of brain function has been established. Fat transplantation studies by Unger's group indicated that leptin-mediated depletion of adipocyte fat in lean rats requires not only the presence of functioning leptin receptor on adipocytes, but also the hypothalamic actions of leptin (Park *et al.*, 2006). Although their results suggest that both centrally-mediated and peripheral leptin actions are involved in adipocyte fat depletion, it did not reveal any independent effect of peripheral leptin action. Thus, the nature of any direct peripheral effects of leptin remains unresolved.

Based on the conclusion that SLR neutralizes leptin *in vivo*, in Chapter 5, we attempted to assess the long-term physiological responses to SLR gene delivery in leptin-responsive rats. First of all, SLR was cloned from the extracellular domain of leptin receptor, with an additional c-myc tag at the C-terminal. *In vitro* analysis of the SLR clone confirmed that SLR was synthesized in the mammalian cells, secreted into the culture media and maintained leptin binding activity. The SLR transgene was then packaged into rAAV serotype 2, and subsequently administered into the 3rd ventricle in leptin-responsive rats. Our choice of rAAV2 as serotype and 3rd ventricle as our delivery site was based on previous successful application of rAAV2 in overexpressing leptin, which is also a secreted protein, in the brain. However, rAAV-SLR did not produce any

physiological responses. Food intake and body weight of rAAV-SLR injected rats were no different from rAAV-GFP controls for up to 65 days. In addition, HF challenge induced almost identical hyperphagia and subsequent caloric normalization between the two groups. Similarly, there was no difference in whole body composition. SLR mRNA expression was confirmed at day 10 and day 65 post rAAV delivery, but SLR protein level in the hypothalamus was only detected at day 10 and no longer detectable at day 65.

There are a couple possibilities for the lack of physiological responses. The most likely is a low expression level of the transgene, which may have neutralized only a small fraction of leptin in the CSF and thus was not sufficient to exert a significant impact on food intake and body weight. The expression level could be compromised by several factors, such as low transduction and / or expression efficiency due to the large molecular weight of SLR. The SLR protein may have not all been secreted, or it may have neutralized the leptin that had no access to the leptin receptor. It is also possible that expression level achieved by rAAV at best was still not sufficient for our purpose. However, according the calculation in Chapter 5, the predicted level of SLR production, should be able to neutralize, theoretically, 62% of the total leptin in the CSF. Therefore, the choice of rAAV was likely not the problem in this study. Lastly, there is another possibility that SLR gene therapy has no effects in young leptin-responsive rats. Due to the lack of direct evidence, it is not clear whether the long-term SLR treatment was by itself not effective in modulating food intake and body weight, or SLR protein level was just not sufficient to induce any responses. In future experiments, expression of transgene need to be enhanced and the effects of chronic SLR gene delivery should be tested in leptin-resistant rats as well.

In summary, this dissertation characterized a leptin antagonist and a soluble leptin receptor, *in vivo*. The leptin antagonist demonstrated pure antagonist activity, *in vivo*, i.e., it inhibited

leptin-mediated signaling dose-dependently in response to acutely administered leptin and also blocked the anorexic and weight-reducing effects of exogenously-infused leptin. Central infusion of the leptin antagonist prevented the normalization of caloric intake and BAT thermogenic effect of food after HF feeding, indicating these processes are leptin-dependent. These results underscore an essential role of leptin in the homeostatic regulation of caloric intake in response to high-fat feeding. In addition, this dissertation also characterized, *in vivo*, the antagonistic-like properties of the soluble leptin receptor, a major leptin binding protein. The SLR fully blocked leptin-induced signaling in an acute injection with leptin, presumably by binding leptin. Central infusion of SLR alone increased food intake and body weight, presumably by neutralizing endogenous leptin. Two doses of SLR partially prevented the anorexia and body weight loss in response to peripherally infused leptin. Interesting, leptin-induced UCP1 increase in BAT and leptin expression decrease in EWAT were fully reversed by the high dose SLR. Finally, effects of long-term SLR delivery were examined in young leptin-responsive rat by central rAAV-SLR gene therapy. However, no physiological responses were observed for 65 days despite persistent SLR expression throughout the experiment. It is unsolved whether long-term SLR treatment can modulate food intake and body weight.

Future Directions and Potential Improvements

This dissertation started out with two major hypotheses. First, leptin is necessary for the normalization of high-fat feeding induced hyperphagia and this counter-regulatory response will be blocked by central delivery of a leptin antagonist. Second, soluble leptin receptor neutralizes leptin *in vivo*, thus increasing food intake and body weight in leptin-responsive rats. In Chapter 3, I confirmed the first hypothesis, by verifying the *in vivo* antagonist activity of the leptin antagonist toward the leptin receptor, and it revealed that the counter-regulatory process following high-fat induced hyperphagia is indeed leptin-dependent. In Chapter 4, I partially

confirmed the second hypothesis by demonstrating that SLR is able to neutralize leptin *in vivo* and infusion of SLR increases food intake and body weight. However, it is still uncertain whether long-term SLR treatment modulates energy balance in leptin-responsive rats, as rAAV-mediated SLR gene therapy resulted in no physiological responses.

In addition in Chapter 3, we observed that maximal leptin signaling capacity is blunted by only 2 days of high-fat feeding, suggesting the rapid occurrence of a receptor desensitization. We propose that this acute desensitization is likely contributing to the time course of the caloric normalization. Future studies could compare the level of maximal leptin signaling at different stages of the normalization process. The maximal signaling capacity may vary along the course of caloric normalization and the extent of the desensitization may modulate the progression of the normalization. For instance, a greater desensitization may indicate a longer normalization process, whereas maximal signaling capacity may return to normal level when the caloric intake has restored to the control intake level. The outcomes may elucidate the physiological relevance of such desensitization. Studies by the Scarpace lab have demonstrated that responses to high-fat feeding differed between rats of various ages. Specifically, older rats displayed a higher peak in caloric intake and a longer time-course in their caloric normalization (Judge *et al.*, 2008). A comparison of the maximal signaling capacity between the aged and the young may indicate some mechanisms underlying the difference in peak caloric intake. It will also be interesting to examine if desensitization occurs during prolonged caloric normalization. If so, a comparison of the extent of desensitization across ages may provide new insights into the mechanism of the age-dependent delay in caloric normalization.

As for Chapter 4, it remains an intriguing question if SLR can fully block leptin and if there is a peripheral component of leptin action in energy regulation that cannot be blocked by

central SLR administration. Although SLR is demonstrated to fully block leptin-induced acute signaling, only partial blockade of leptin-induced anorexia was observed in Chapter 4. To verify if SLR can fully block leptin, the best way will be the use of higher doses for central SLR infusion. Considering it is impractical to increase SLR dose due to the high cost, an alternative way to test this hypothesis is by the use of leptin antagonist for central leptin blockade, coupled with a peripheral leptin infusion. It is verified in Chapter 3 that central leptin antagonist infusion fully blocked central leptin-mediated anorexic responses. Therefore, when sufficient leptin antagonist is infused centrally with a simultaneous peripheral infusion of leptin, it is expected that all responses mediated by central leptin will be blocked by leptin antagonist and any remaining responses, if any, are induced by leptin action in the peripheral.

In Chapter 5, rAAV-mediated SLR gene therapy failed to induce any physiological responses in young leptin-responsive rat. It is especially surprising considering rAAV-leptin antagonist increased food intake and body weight in rats of same strain and age. Detailed discussion has been provided in Chapter 5 on the possible explanations. In retrospect, there are several steps that could have been improved or modified to eliminate uncertainty in the outcomes.

- 1) Reduce the amount of plasmid used for the *in vitro* transfection. This may reduce the appearance of large protein aggregates above 250 kD in Western analysis, which is probably due to expression overload in transfected cells.
- 2) Incorporate a Kozak sequence into the start codon of SLR transgene and mutate unwanted upstream ATGs.
- 3) Clone the full sequence of Ob-Re and place the c-myc tag downstream. The current construct is missing the last 9 aa of Ob-Re, although there is evidence that difference in ending sequence does not affect leptin binding activity of SLR (Tu *et al.*, 2008).
- 4) Use another rAAV serotype (e.g. type1 or 8) other than type 2, as serotype 2 has delayed onset and low expression level. Additionally, it is shown

to have poor storage stability and a tendency to aggregate at high titer (Wright *et al.*, 2005). 5) Develop an ELISA assay for measuring rat SLR level in the CSF. Because there was no established ELISA assay available at the time of rAAV-SLR study, CSF SLR level can not be measured and compared to the level induced by SLR central infusion. Therefore, we were not able to conclude if low SLR level was the reason for the lack of physiological responses. 6) rAAV-SLR gene therapy could be tested in mice. Rat SLR shares a 92% homology to murine sequence and mice have less CSF leptin, thus requiring less SLR to neutralize. 7) Seek a new gene delivery system if rAAV mediated SLR gene therapy can not be optimized to produce enough SLR to neutralize CSF leptin in the brain.

One question we did not address in this dissertation is whether the elevated leptin, particularly elevated free leptin, is only a consequence of obesity or also one causative factor of obesity. Obesity is associated with both elevated leptin and leptin resistance. Mounting evidence has indicated that prolonged exposure to elevated leptin level induces leptin resistance (Martin *et al.*, 2000; Sahu, 2002; Scarpace and Zhang, 2007). A leptin-induced leptin resistant model was first established by the Scarpace group, in which rats that received central rAAV-leptin delivery displayed significant anorexic responses at early stage, but responses waned over time despite persistent transgene expression (Scarpace *et al.*, 2002a). Those rats eventually become non-responsive to either overexpressed or pharmacologically delivered leptin. In rAAV-leptin treated rats, diminished leptin responsiveness is also accompanied by reduced maximal leptin signaling and a parallel reduction in leptin receptor levels (Wilsey and Scarpace, 2004). This leptin-induced leptin resistance model with the absence of obesity supports the hypothesis that elevated leptin is also one cause of leptin resistance.

Consistent with this idea, down-regulated central leptin receptor activity due to increased leptin level may be one cause of leptin resistance. A future direction for the leptin antagonist study is to examine the effects of long-term antagonist treatment on leptin receptor level and receptor activity. The leptin antagonist may be able to increase leptin receptor level, and possibly restore leptin receptor activity to normal levels. Central leptin antagonist gene therapy is an ongoing project in the Scarpace lab, and so far it has shown promising results. We predict that central rAAV-leptin antagonist will increase leptin receptor level and also enhance leptin responsiveness in young lean rats. It will be of great interest to test if central rAAV-leptin antagonist gene therapy will eventually reverse leptin resistance in diet-obese or aged-obese rats.

In Chapter 5, in addition to the original hypothesis that long-term SLR treatment enhances food intake and body weight gain, we were also interested in examining if chronic overexpression of SLR would potentially upregulate leptin receptors by reducing free leptin level in the brain. SLR gene therapy in leptin-responsive rats was intended to serve as a basis for SLR gene therapy in obese leptin resistant rats, by normalizing the elevated leptin and thus, reversing the leptin resistance. In other words, reverse leptin-induced leptin resistance by lowering free leptin levels. Future direction for the SLR project should include optimization of SLR gene therapy and rAAV-SLR gene delivery in leptin-resistant rats. If central SLR gene therapy proves to be effective in enhancing leptin sensitivity, it will be a promising strategy to reverse leptin resistance and thus to reinstate leptin as an efficacious treatment for obesity. The ultimate goal for SLR study is peripheral delivery, as SLR is normally generated in the peripheral and intravenous delivery is more clinically applicable. However, leptin level in the blood is considerably higher than in the CSF. The peripheral application is limited by the high cost of recombinant SLR and the mediocre transgene level produced by SLR gene therapy. The

emergence of an optimized gene delivery system would be desirable, providing the advantages such as high expression level, large transgene cassette, ease to produce in large quantity and the ability to transduce peripheral tissues.

Conclusion

Once seen as a symbol of wealth and status, obesity is a serious and costly public health problem in today's society. Obesity not only predisposes individuals to diabetes, cardiovascular diseases, stroke and certain cancers, it also puts a major strain on the health care budget. High-fat diets are implicated as one major cause of obesity and for prevention purposes, it is essential to understand the metabolic responses following high-fat feeding. The hormone leptin is a powerful fat-reducing agent, but obese individuals are resistant to leptin possibly due to the already elevated serum leptin level. Therefore, it is important to reverse leptin resistance in order to fully utilize leptin for the treatment of obesity.

In the research described in this dissertation, we demonstrated the important role of leptin in homeostatic energy regulation by the use of a leptin antagonist and a soluble leptin receptor. We revealed that the counter-regulatory process in response to the consumption of high-fat diets is leptin dependent. We further established the feasibility of *in vivo* leptin neutralization by the soluble leptin receptor. These results contributed to the knowledge of leptin physiology and the fundamental understanding of obesity. It is my hope that these findings will contribute to the prevention of obesity as well as the development of novel interventions.

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BIOGRAPHICAL SKETCH

Jiejing Zhang was born in Shanghai, China in March 1982, to Peili Song and Shenxin Zhang. As the only child of the family, she grew up very close to her first cousins. Starting at an early age, Jiejing was immersed in different interests and hobbies. Her favorite activities were playing the piano and learning English. Jiejing later spent seven years at Shanghai Foreign Language School to pursue her interest in English. After high school, Jiejing matriculated at Fudan University for her undergraduate study, majoring in biological sciences. During her senior year, Jiejing acquired her first research project, studying protein expression during flower development of Arabidopsis, a small plant. In 2004, Jiejing received her Bachelor of Science degree and was accepted into the Interdisciplinary Program in Biomedical Sciences at the University of Florida College of Medicine, in Gainesville, Florida. In 2005, Jiejing joined the laboratory of Philip J. Scarpace in the Department of Pharmacology and Therapeutics, and pursued her doctoral research in obesity. Outside of lab, Jiejing was highly involved in student activities, from hosting and directing Chinese New Year shows, to going to football and basketball games. It was there at the University of Florida that she met and fell in love with Jack Shao, a former chemistry student, who became her husband. Jiejing and Jack married in June of 2008, in Shanghai, the hometown for both of them. After graduating, Jiejing plans to move to Cleveland, Ohio to be with her husband, who is currently attending medical school at Case Western Reserve University. In the future, Jiejing hopes to pursue a career in medical research, as well as raising and caring for her family.