EFFECT OF EXOGENOUS LEPTIN ADMINISTRATION ON TESTICULAR FUNCTION IN ADULT MALE SPRAGUE DAWLEY RATS

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EFFECT OF EXOGENOUS LEPTIN ADMINISTRATION ON TESTICULAR FUNCTION IN ADULT SPRAGUE DAWLEY RATS

by

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AgRP	Agouti-related peptide
ANOVA	Analysis of variance
ARC	Arcuate nucleus
BMI	Body mass index
CART	Cocaine-and-amphetamine-regulated transcript
CRH	Corticotrophin releasing hormone
CSF	Cerebrospinal fluid
db/db	Diabetic rat
DNA	Deoxyribonucleic acid
DPX	Di-n-butylPhthalate in xylene
EIA	Enzyme immunometric assay
ELISA	Enzyme-linked immunosorbent assay
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin releasing hormone
H&E	Hematoxylin and eosin
HMG	3-hydroxy-3-methylglutarate
IRS-1	Insulin receptor substrate-1
IRS-2	Insulin receptor substrate-2
JAK-STAT	Janus-family kinase signal transducer and activator of transcription system
LH	Luteinising hormone
LHRH	Luteinising hormone releasing hormone
MC4R	Melanocortin-4 receptor

MSG	Monosodium L-glutamate
mRNA	messenger ribonucleic acid
NPY	Neuropeptide Y
ob/ob	Obese rat
OBR, LR, LEPR	Leptin receptor
PI3-kinase	Phosphatidylinositol 3-kinase
POMC	Pro-opiomelanocortin
SEH	Seminiferous epithelial height
SHR	Spontaneous hypertensive rat
SOCS3	Suppressor of cytokine signaling-3
STD	Seminiferous tubular diameter
sOB-R, *LEPRe*	Soluble leptin receptor
T_3	Triiodothyronine
T4	Thyroxine
TAF/FFA	Triacylglyceride/free fatty acid
TNFα	Tumor necrosis factor-alpha
UCP	Uncoupling protein
VMH	Ventromedial hypothalamus
WHR	Waist/hip ratio
α-MSH	Alpha-Melanocyte-stimulating hormone

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KESAN PEMBERIAN LEPTIN TERHADAP FUNGSI TESTIS TIKUS SPRAGUE DAWLEY DEWASA

ABSTRAK

Kajian ini mengkaji kesan pemberian leptin terhadap berat organ reproduktif, aras serum gonadotrofin, kiraan dan morfologi sperma, histologi testis, berat badan serta pengambilan makanan dan minuman tikus.

Tikus Sprague Dawley, berumur 10 minggu dengan purata berat badan 200 ± 1.44 g, dirawat dengan suntikan tunggal leptin secara intraperitonial dengan dos 5, 10 atau 30 μg/kg berat badan selama sama ada 7, 15 atau 42 hari (n=10 untuk setiap kumpulan). Berat badan, pengambilan makanan dan minuman diukur setiap dua hari sepanjang tempoh eksperimen. Pada penghujung setiap rawatan tikus dibius dengan eter dan dimatikan dengan serta-merta melalui dislokasi servikal. Laparotomi dijalankan dan epididimis kanan dikeluarkan dan di hancurkan di dalam 2 ml saline normal. Suspensi ditapis dan diwarnakan dengan eosin Y 1 %. Kiraan dan morfologi sperma dijalankan mengikut prosedur piawai. Darah dikumpul daripada vena kava inferior, dibiarkan beku dan diempar untuk memperolehi serum. Aras testosteron, FSH, LH dan leptin serum ditentukan dengan menggunakan teknik ELISA. Berat testis, epididimis, prostate dan vesikel semen dicatat dan berat relative organ dikira. Testis diproses melalui rutin benaman paraffin dan diwarnakan dengan pewarnaan H & E. Keratan tisu diperiksa menggunakan penganalisis imej dan garispusat (STD) tubul seminiferus dan ketinggian epitelial seminiferus (SEH) diukur.

Daripada kajian ini didapati bahawa pemberian leptin dengan dos sama ada 5, 10 atau 30 μg setiap hari secara intraperitonial selama sama ada 7, 15 atau 42 hari tidak mempengaruhi berat badan, pengambilan makanan dan minuman tikus secara signifikan. Tiada juga perbezaan yang signifikan bagi aras testosteron dan leptin serum diantara kumpulan disuntik dengan leptin dengan kumpulan kawalan. Walaubagaimanapun aras FSH dan LH serum bagi kumpulan disuntik dengan leptin lebih tinggi secara signifikan berbanding kumpulan kawalan. Garipusat tubul seminiferus, ketinggian epitelial seminiferus dan kiraan sperma adalah lebih rendah dengan signifikan bagi kumpulan disuntik dengan leptin apabila dibandingkan dengan kumpulan kawalan. Pecahan sperma abnormal juga lebih tinggi dengan signifikan bagi kumpulan disuntik dengan leptin berbanding kumpulan kawalan.

Secara kesimpulan didapati bahawa pemberian leptin setiap hari pada dos 5, 10 atau 30 μg, meningkatkan aras FSH dan LH serum secara signifikan di dalam tikus jantan. Tambahan lagi, pemberian leptin juga mengurangkan kiraan sperma dan meningkatkan pecahan sperma abnormal, dimana ia berkemungkinan mempunyai kaitan dengan nilai STD dan SEH yang rendah di dalam tikus yang dirawat leptin. Kesan leptin terhadap berat badan, pengambilan makanan dan minuman yang tidak seragam menunjukkan kesan pemberian leptin terhadap fungsi testis tidak dipengaruhi oleh perubahan berat badan.

EFFECT OF EXOGENOUS LEPTIN ADMINISTRATION ON TESTICULAR FUNCTION IN ADULT SPRAGUE DAWLEY RATS

ABSTRACT

This study examines the effect of exogenous leptin administration on reproductive organ weight, serum gonadotrophins, sperm count and morphology, testis histology, body weight, food and water intake in the rat.

Sprague Dawley rats, aged 10 weeks, and with a mean body weight of 200 ± 1.44 g, were treated daily with a single intraperitoneal injection of either 5, 10 or 30 μg/kg body weight of leptin for either 7, 15 or 42 days (n=10 for each group). Body weight, food and water intake were measured every two days over the experimental periods. At the end of each treatment, rats were mildly anesthetized with ether and immediately killed by cervical dislocation. Laparotomy was performed, and the right epididymis was removed and minced in 2 ml normal saline. The suspension was filtered and stained with 1 % eosin Y. Sperm count and sperm morphology were conducted as per the standard procedure. Blood was collected from the inferior vena cava, clotted and centrifuged to obtain the serum. Serum testosterone, FSH, LH and leptin were measured using ELISA technique. The testis, epididymis, prostate and seminal vesicles weights were recorded and the relative organ weights were calculated. The testes were processed for routine paraffin embedding and stained with H&E staining. Tissue sections were examined using image analyzer and seminiferous tubular diameter (STD) and seminiferous epithelial height (SEH) were measured.

Daily intraperitoneal administration of either 5, 10 or 30 μg of leptin for either 7, 15 or 42 days in this study did not significantly affect body weight, food intake and water intake of the rats There was also no significant difference in serum testosterone and leptin levels between the leptin-treated groups and their matched controls. Serum FSH and LH levels were however significantly higher in leptin-treated group compared to controls. Seminiferous tubule diameter (STD), seminiferous epithelial height (SEH), and sperm count were significantly lower in leptin-treated groups when compared to age-matched controls. The fraction of abnormal sperms was also significantly higher in leptin-treated groups when compared to controls.

In conclusion, it appears that leptin administration in daily doses of 5, 10 or 30 μg, increases both serum FSH and LH levels in male rats. In addition, it also decreases sperm count while increasing the fraction of abnormal sperms, which might be related to the lower STD and SEH evident in leptin-treated rats. Its effect on body weight, food and water intake was inconsistent, indicating that the effects of exogenous leptin on testicular function are independent of changes in body weight.

CHAPTER ONE
INTRODUCTION

1.1 The discovery of leptin

Body weight in wild animals remains remarkably constant over a considerable period of time, even when there is an abundance of food. This led many to believe that there normally exists a mechanism that regulates food intake, energy expenditure and therefore body weight. The adipostatic model of body weight regulation was proposed to explain this regulation, where the role for depot fat in the hypothalamic control of food intake was hypothesised (Kennedy, 1953).

A few years earlier, however, a strain of recessive mutant mice (*ob/ob*) with hyperphagia, and early onset obesity had been discovered (Ingalls *et al.*, 1950). Parabiotic experiments between these mutant mice and normal wild type mice caused suppression of food intake and weight loss in the mutant mice, indicating the presence of a humoral factor that regulated appetite and body weight in the normal mice (Hausberger, 1959, Coleman & Hummel, 1969). At about the same time it was also observed that experimental lesions in the ventromedial hypothalamus (VMH) resulted in obesity in rats. Moreover, parabiosis between rats rendered obese by experimental lesions in the VMH and normal rats led to death from starvation of the normal rats (Hervey, 1958). Collectively, all these observations suggested a possible interaction between the

hypothalamus and the hypothesised humoral factor that might have its origins in the adipose tissue.

Sometime after the discovery of *ob/ob* mouse, another group of obese mice was discovered where the obesity was once again inherited recessively (*db/db*), but mice in this group were also diabetic (Coleman & Hummel, 1969). Parabiosis between these obese mice and normal mice, this time, led to the death of normal mice by starvation, suggesting the presence in large concentrations of a humoral factor that severely suppressed appetite in normal mice (Tartaglia *et al.*, 1995). The fact that it did not affect the *db/db* mice suggested a possible insensitivity to the hypothesised circulating satiety factor in the *db/db* mice.

While the mechanism by which the deficiency or insensitivity of this circulating satiety factor causes obesity might be more through hyperphagia, there is however also evidence to suggest that weight regulation or reduction by this proposed factor involves more than just the regulation of food consumption. VMH-lesioned mice and *ob/ob* mice were found to still develop obesity even when food intake was matched to that of lean normal controls (Coleman, 1978, Bray & York, 1979). This led some to hypothesise that the satiety factor might also influence, among other things, energy expenditure in these animals. In this regard, sympathetic activity to brown adipose tissue has been reported to be lower in both the VMH-lesioned and the *ob/ob* mice (Bray, 1991). It therefore

appears that long-term body weight regulation by the proposed circulating satiety factor involves both the regulation of appetite i.e. food intake, and energy expenditure.

It was over 40 years after its presence was first suspected that the circulating satiety factor was eventually detected and characterized. Using yeast artificial chromosome, Friedman and his colleagues (1991) managed to clone the *ob* gene, and, that the hypothesised circulating satiety factor was the product of this gene, was subsequently confirmed through positional cloning (Zhang *et al.*, 1994). The product of this gene was called leptin from the Greek root word '*Leptos*' meaning thin. *Ob/ob* mice fail to produce this protein while *db/db* mice are resistant to its action due to an abnormality in the leptin receptor (Zhang *et al.*, 1994).

In humans, the *ob* gene, which is now also sometimes referred to as *LEP* gene, is localized on chromosome 7 (alpha31.3 position). It spans 18 kilobase consisting of 3 exons separated by 2 introns (Isse *et al.*, 1995). The gene encodes a 4.5 kilobase adipose tissue mRNA with 166 amino acid open reading frame and 21 amino acid signal sequence (Zhang *et al.*, 1994). In the mouse it maps to chromosome 6, and consists of 3 exons and 2 introns, which encode a 4.5 kilobase mRNA (Friedman *et al.*, 1991, Zhang *et al.*, 1994). Human leptin nucleotide is a 166 amino acid polypeptide with a putative signal sequence, and

it is 84 % and 83 % identical to that of the mouse and rat, respectively (Masuzaki *et al.*, 1995, Masuzaki *et al.*, 1995a).

1.2 Secretion of leptin

Leptin gene is expressed mainly in white adipose tissue (Masuzaki *et al.*, 1995, Gong *et al.*, 1996) although low leptin mRNA expression has also been reported in brown adipose tissue. However, this may be due to contamination of mRNA expression from white adipose tissue (Cinti *et al.*, 1997). A number of non-adipocyte tissues have also been shown to synthesize and secrete leptin, albeit in small amounts. These include the gastric mucosa (Bado *et al.*, 1998, Mix *et al.*, 1999, Cinti *et al.*, 2000), mammary epithelial cells (Smith-Kirwin *et al.*, 1998), and myocytes (Wang *et al.*, 1998). The placenta has also been found to secrete significant quantities of leptin (Senaris *et al.*, 1997, Singh *et al.*, 2005).

It has been suggested that when fat cells increase in number and size, the *ob* gene starts to produce leptin, which is secreted into the circulation. There is a strong positive correlation between leptin mRNA expression and plasma leptin concentration, and total body fat (Frederich *et al.,* 1995, Maffei *et al.*, 1995, Considine *et al.,* 1996). Leptin secretion follows a 24-hours cycle with higher rates during the evening, peaking in the middle of the night hours, and lower rates in the morning, somewhat opposite to those seen in cortisol levels in humans (Laughlin & Yen, 1997). Leptin secretion is mainly constitutive. Leptin is synthesized and extruded into the secretory pathway for release by mass action

(as opposed to being packaged into specialised vesicles for regulated release in response to an acute stimulus). Higher rates of leptin secretion during the night may relate to the time of food intake and hyperinsulinaemia during the day (Sinha & Caro, 1998). Although peak leptin and cortisol levels appear opposite to each other, studies both *in vivo* and *in vitro* in rodents and man have shown that glucocorticoids enhance leptin gene transcription and leptin levels (De Vos *et al.*, 1995, Slieker *et al.*, 1996, Trayhurn *et al.*, 1998). Leptin levels are also elevated in rats given dexamethasone (De Vos *et al.*, 1995). The reason for the pattern of leptin secretion is therefore unclear, whether it is related to food intake is unclear.

Serum leptin concentrations are higher in females when compared with males (Schrauwen *et al.*, 1997). The reason for this gender based difference is not entirely clear but has been observed *in vivo* from early infancy (Garcia-Mayor *et al.*, 1997). This gender difference persists even after correction for fat mass (Hassink *et al.*, 1996). Interestingly, 17 β-estradiol was found to increase leptin secretion into the culture medium of adipose tissue from female rats (Casabiell *et al.*, 1998). The administration of GnRH agonists to women undergoing *in vitro* fertilization treatment increases leptin levels, and serum leptin levels have been reported to correlate with estradiol levels (Stock *et al.*, 1999). Leptin secretion, on the other hand, is inhibited by testosterone, as evidence from inhibition of leptin secretion following administration of testosterone to orchidectomised rats (Kus *et al.*, 2007). Moreover, serum leptin levels have been found to correlate negatively with testosterone in males (Carraro & Ruiz-Torres, 2006). It is

therefore possible that the increased response to oestrogens, to an extent, might contribute to the gender difference. Catecholamines also reduce serum leptin levels (Trayhurn *et al.*, 1998).

Leptin expression and circulating levels increase in parallel with the amount of adipose tissue during the fed state (Lonnqvist *et al.*, 1995) and the relationship between leptin levels and fat mass is curvilinear, rather than linear, with a wide range of individual leptin values at a specific level of body fat (Considine *et al.*, 1996). There is a higher positive correlation between serum leptin levels and total mass of adipose tissue rather than body mass index (BMI) (Maffei *et al.*, 1995). In addition to total tissue fat mass and the size of adipocytes, the pattern of adipose tissue distribution may also influence leptin levels (Tritos & Mantzoros, 1997). Leptin mRNA expression is higher in subcutaneous than in visceral fat depots (Hube *et al.*, 1996). Omental adipocytes express more *β*-1, 2 and 3 adrenergic receptors than subcutaneous adipocytes (Lonnqvist *et al.*, 1995). The different receptor profile makes the former more responsive to the lypolytic actions of catecholamines and less responsive to the antilipolytic actions of insulin (Lonnqvist *et al.*, 1997).

Serum leptin levels are also affected by nutritional status, and fasting reduces leptin levels by approximately 30 %, while excessive food consumption leads to an increase in the secretion of leptin by 50 %. Leptin levels increase more when food rich in fat is taken (Houseknecht & Portocarrero, 1998). Leptin

secretion however, declines during aging. This reduction is higher in women than in men, and is independent of BMI and other age-related endocrine changes (Isidori *et al.*, 2000).

For the ensuing, it is evident that a number of factors influence leptin secretion. Although serum leptin levels in the main correlate well to fat mass, there nevertheless also appears that leptin is not only a static index of fat mass but it also acts as a sensor of energy balance.

1.3 Leptin in circulation

Once secreted into the circulation, leptin circulates in the plasma either in the free form or bound to the soluble leptin receptor (*sOB-R* or *LEPRe*) (Houseknecht *et al.*, 1996, Lammert *et al.*, 2001). In humans and animals, increased leptin levels with adiposity are due to augmented *ob* gene expression and increased leptin production (Considine *et al.*, 1995, Hamilton *et al.*, 1995, Maffei *et al.*, 1995, Ogawa *et al.*, 1995). The possible mechanism for the increase in leptin levels possibly involves an enlargement of adipocytes. A study *in vitro* had shown that leptin secretion is closely related to fat cell size in genetic and diet-induced obese mice (Houseknecht *et al.*, 1996). In humans, small adipocytes express less *ob* mRNA than larger ones from the same individual (Hamilton *et al.*, 1995). As the leptin level varies in proportion to fat mass, it could conceivably serve as an afferent signal that provides sensory input about the degree of adiposity to the central nervous system. In response, adjustment

of food intake and energy expenditure would be made to ensure long term body weight stability.

Leptin levels appear to differ considerably in humans with similar fat mass and there is significant heterogeneity among subjects with similar BMI (Maffei *et al.*, 1995). Women have higher leptin levels than men at any percent of body fat or fat mass. The *ob* gene expression in obese women was reportedly found to be 75 % higher than in obese men (Lonnqvist *et al.*, 1995). Serum leptin levels in normal healthy adults range from 0.5 to 37.7 ng/ml for male and 2.0 to 45.2 ng/ml for female (Lida *et al.*, 1996). Leptin concentration in cerebrospinal fluid in women is also higher than in men after controlling for age, BMI and plasma leptin level (Schwartz *et al.*, 1996). A sex difference has also been described in mice. Female mice had higher plasma leptin levels and adipose tissue *ob* mRNA than male (Frederich *et al.*, 1995). Therefore, it appears that female fat cells produce more leptin than male fat cells with similar body composition. As mentioned earlier, this may be related to the stimulatory effect of oestrogen in the female or the inhibitory effect of testosterone in the male, although leptin in postmenstrual women remains significantly higher than that in men of similar age and is not different from that in younger women after adjusting for body fat (Saad *et al.*, 1997). The difference in fat distribution may also play a role in this difference in leptin levels between the two sexes, as subcutaneous fat expresses more leptin mRNA than intra-abdominal fat (Masuzaki *et al.*, 1995). Central android adipose tissue may produce less leptin than peripheral gynecoid fat, accounting for the

differences between men and women. However, levels of leptin do not appear to be related to the waist/hip ratio (WHR).

Serum leptin levels increase with age during childhood and adolescence as levels of the *sOB-R* decline in both sexes. These developmental changes, which compositely represent an increase in circulating leptin bioavailability, precede the pubertal rise in serum testosterone in boys and estradiol in girls. Leptin might potentially serve as a metabolic signal to inform the central nervous system that energy reserves are adequate to support pubertal development.

1.4 Leptin receptor

Leptin acts directly through the leptin receptor (*OBR or LR or LEPR*). The *LEPR* gene is located on chromosome 1 (1p31) in humans and is constituted of 18 exons and 17 introns, and encodes a protein consisting of 1162 amino acids. The leptin receptor was first isolated from the mouse choroid plexus using expression cloning (Tartaglia *et al.*, 1995) and has been found to belong to the class 1 cytokine receptor family (IL-6 receptor family). The *LEPR* gene is known to encode at least five alternatively spliced forms or isoforms of the leptin receptor (Figure 1.1). Included in these variants are the soluble or secreted isoform (*LEPRe*), the long (*LEPR1 or LRb)* and short isoforms (*LEPRa* or *LRa*). The extracellular and transmembrane domains are identical between *LEPRa* and *LEPR1* and differences are due to changes in the length of the cytoplasmic domain. The cytoplasmic domain of the *LEPR1* has 302 amino acids compared

with that of *LEPRa,* which is 32 to 40 amino acids in length. The secreted or soluble form (*LEPRe*) only contains the extracellular domain of the receptor and not the intracellular motifs or the transmembrane residues (Kieffer *et al.*, 1996, Houseknecht & Portocarrero, 1998). The long form of the receptor is believed to be responsible for the actions of leptin and the short form is more to aid its transport across cell membrane, and the soluble form, for its transportation in the circulation.

Isoforms of the leptin receptor have been identified primarily in the hypothalamus (Houseknecht & Portocarrero, 1998), in the endocrine part of the pancreas, in the ovaries and testes (Kieffer *et al.*, 1996), in the cells of the granular layer of the cumulus oophorus (Cioffi *et al.*, 1997), in the uterus (Cioffi *et al.*, 1997), as well as in other peripheral tissues like kidneys (Sharma & Considine, 1998), heart (Bernardis & Bellinger, 1998), lungs (Sharma & Considine, 1998), liver (Bernardis & Bellinger, 1998) and skeletal muscles (Bernardis & Bellinger, 1998). The long form receptor is expressed mainly in the two hypothalamic nuclei, i.e. the arcuate and the paraventricular nuclei (Woods & Stock, 1996). Three isoforms of leptin receptor are expressed in the human hypothalamus, including the full length receptor (Eikelis *et al.*, 2007). *Ob-Rb* is expressed highly in neurons of the hypothalamic nuclei, including the arcuate, dorsomedial hypothalamic and ventromedial hypothalamic nuclei (Elmquist *et al.*, 1998, Baskin *et al.*, 1999). Within these basomedial hypothalamic nuclei, *Ob-Rb* mRNA is expressed with the highest level in the arcuate nuclei (Elmquist *et al.*,

1999, Schwartz *et al.*, 2000). In addition to the hypothalamus, leptin receptors have also been located in other parts of the brain (Elmquist *et al.*, 1999, Grill & Kaplan, 2002). High expression levels of *Ob-Ra* and *Ob-Rc* are found in the choroid plexus, meninges and brain micro vessels, which may play a role in the transport of leptin across the blood-brain barrier (Tartaglia *et al.*, 1995, Bjorbaek *et al.*, 1998). The wide distribution of leptin receptors in extra-hypothalamic sites in the thalamus and cerebellum suggests that leptin might act on sensory and motor systems too, in addition to its role in neuroendocrine function. Repeated immobilization stress e.g. has been reported to induce an increase in leptin expression in the hypothalamus of female mice, and a decrease in the thalamus of both male and female mice, associated with enhanced expression of leptin receptors in the hypothalamus and thalamus, both in male and female mice (Manni *et al.*, 2007).

The short leptin receptor isoforms have been found in the choroid plexus (Lynn *et al.*, 1996) and the brain capillary endothelium (Golden *et al.*, 1997). In the choroid plexus, leptin receptors are believed to aid the transport of circulating leptin into the cerebrospinal fluid (CSF), and leptin receptors in the brain capillary endothelium may also provide a direct transport of leptin from blood to the brain interstitium (Caro *et al.*, 1996). Short forms of leptin receptors are also found in the lungs and kidneys, where they might be involved in leptin clearance (Cumin *et al.*, 1996).

LEPRe, also known as the soluble leptin receptor is the major leptin binding protein in blood (Lammert *et al.*, 2001) and is derived from ectodomain shedding of membrane-bound receptors (Ge *et al.*, 2002). It forms one of the circulating leptin binding proteins that confer some degree of metabolic stability and affect the leptin transport in blood and its tissue availability (Houseknecht *et al.*, 1996a, Kieffer *et al.*, 1996).

Resting energy expenditure and muscle sympathetic activity are more positively correlated to bound than free leptin concentration (Brabant *et al.*, 2000, Tank *et al.*, 2003), and examination of *LEPRe* concentration is important to separate the key role of total, free and bound leptin (Venner *et al.*, 2006). Unlike the mutation in the leptin *ob* gene that leads to impairment of leptin secretion (Farooqi *et al.*, 2001), mutation in leptin receptor gene had not been found to lead to any differences in soluble leptin receptor concentrations in lean versus obese subjects (Lahlou *et al.*, 2002). Higher *LEPRe* concentrations are found in lean compared to obese individuals (van Dielen *et al.*, 2002). Thus it may be important when investigating the effect of leptin on body weight or correlating its concentration in serum to body weight, one might need to measure both free and bound components, and possibly also the soluble receptor concentration as well. When leptin binds to *LEPRe*, there may be a delay in leptin clearance and degradation from the circulation, and this sometimes increases the concentration of available circulating leptin (Huang *et al.*, 2001, Zastrow *et al.*, 2003). Only free leptin can act on target sites to elicit biological responses.

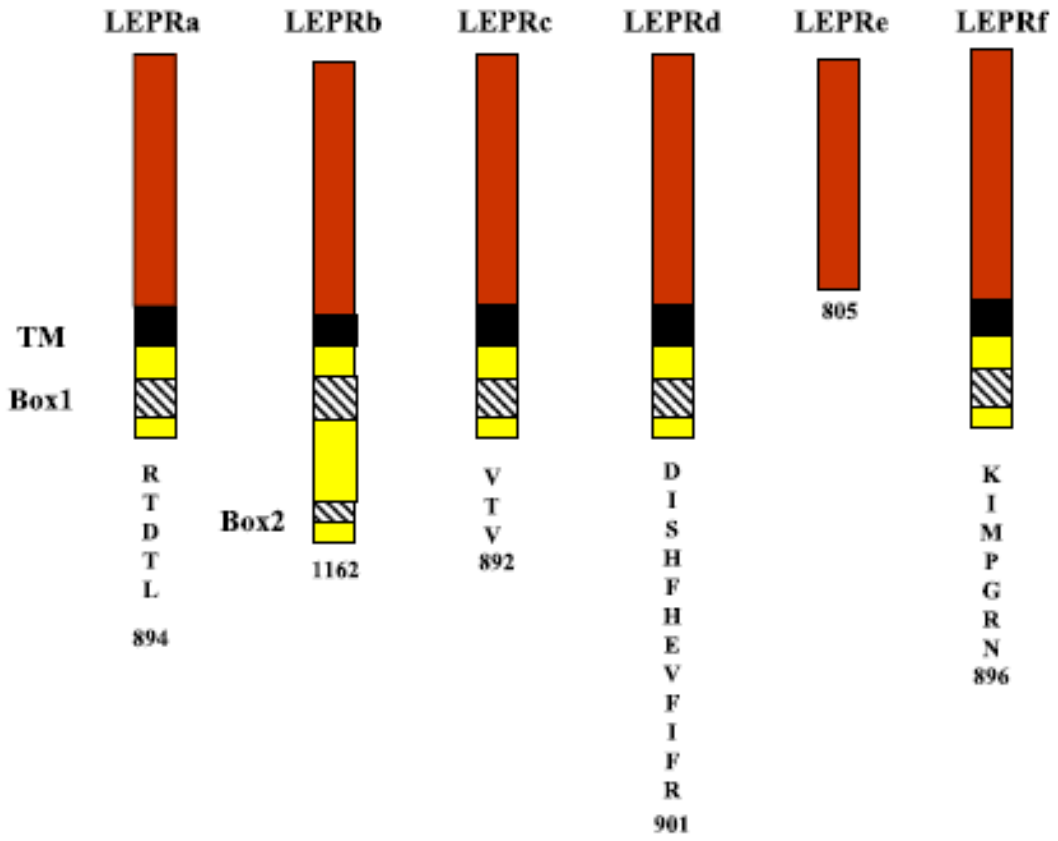

Figure 1.1: Domain structures of alternatively sliced leptin receptor isoforms. The long form, LEPRb, has a long cytoplasmic region containing several motifs required for signal transduction. The four short forms, LEPRa, LEPRc, LEPRd and LEPRf, have a shorter intracellular tail. LEPRe is known as the soluble leptin receptor and the major binding protein in blood circulation (Adapted from Ahima & Osei, 2004).

1.5 Leptin JAK-STAT signal transduction

The mechanism of leptin action involves the Janus-family kinase signal transducer and activator of transcription system (JAK-STAT). Leptin receptors, particularly *LEPR1*, form homodimers which are capable of activating JAK-STAT system (Lee *et al.*, 1996, Myers, 2004). *LEPR1* has three intracellular conserved tyrosine residues (Y985, Y1077 and Y1138). Y985 and Y1138 are phosphorylated upon leptin binding, while Y1077 is not phosphorylated and does not contribute to leptin signaling. Its role remains to be identified. Phosphorylation of Y985 activates the SHP2 signaling pathway, whose exact action is still unclear. Phosphorylation of Y1138 recruits STAT 3 to the *LEPR1*/JAK2 complex, resulting in the tyrosine phosphorylation and subsequent nuclear translocation of STAT 3 to mediate transcriptional regulation. Tyrosyl-phosphorylated STAT 3 undergoes homodimerization and nuclear translocation, and regulates the expression of gene that encodes neuropeptides and other target genes. Replacement of serine in Y1138 (Y1138S) disrupts STAT 3 activation and causes hyperphagia, impairment of thermoregulation and obesity but does not affect sexual maturation and growth (Bates *et al.*, 2003). Moreover, Y1138S mice are less hyperglycemic with normal expression of neuropeptide Y (NPY).

Leptin binding to *LEPR1* also activates insulin receptor substrate 1 (IRS-1) and insulin receptor substrate 2 (IRS-2), mitogen-activated protein kinase, extracellular-regulated kinase and phosphatidylinositol 3-kinase (PI3-kinase)

(Niswender *et al.*, 2004). Leptin enhances IRS2-mediated activation of PI3-kinase in the hypothalamus. On the other hand, blockade of PI3-kinase activity prevents the anorectic action of leptin (Niswender *et al.*, 2004). Leptin terminates its signal through the induction of suppressor of cytokine signaling-3 (SOCS3), which belongs to a family of proteins that inhibit JAK-STAT signaling (Howard *et al.*, 2004). SOCS3 deficiency increases leptin sensitivity and prevents obesity (Howard *et al.*, 2004).

In addition to the activation of STAT 3, leptin also induces the activation of STAT 5 and systemic administration of leptin has recently been found to increase the number of nuclear STAT 5 signal in the hypothalamus (Mutze *et al.*, 2007). In the hypothalamus, nuclear STAT 5 activation has also been reported in response to prolactin (Lerant *et al.*, 2001), and tumor necrosis factor-alpha (TNFα) (Rizk *et al.*, 2001). However, the functional relevance of the leptin-induced nuclear STAT 5 activation in the hypothalamic cells is still unknown.

1.6 Functions of leptin

1.6.1 Regulation of appetite and body weight

The fundamental role of leptin as a 'lipostat' in the regulation of body weight has been a focus of much research. Daily injection of recombinant leptin has been shown to cause significant weight loss and reduced food intake in *ob/ob* and lean wild-type mice, whereas no changes were observed in *db/db* mice (Campfield *et al.*, 1995). This reduction in food intake by leptin is now

known to be mediated primarily through the hypothalamus. It has been shown to regulate appetite through changes in the release of NPY, agouti-related peptide (AgRP) and α-melanocyte-stimulating hormone (α-MSH) from the hypothalamic nuclei, in particular the arcuate nucleus (ARC). *LEPR1* (*LRb*) mRNA is highly expressed in the two distinct populations of ARC neurons. One population synthesizes NPY and agouti-related peptide, and the other synthesizes pro-opiomelanocortin (POMC), which is processed to produce α-MSH (Elmquist *et al.*, 1999, Schwartz *et al.*, 2000). Leptin down-regulates NPY and AgRP, and causes a reduction in food intake, increases sympathetic nervous system outflow, thus increasing energy expenditure (Figure 1.2). Leptin also stimulates the activity of POMC neurons resulting in increased release of POMC and its conversion to α-MSH that decreases appetite by activating the melanocortin-4 receptor (MC4R). AgRP is an antagonist of α-MSH/MC4R signaling as well as an inhibitor of endogenous MC4R activity (Schwartz *et al.*, 2000, Cowley *et al.*, 2001).

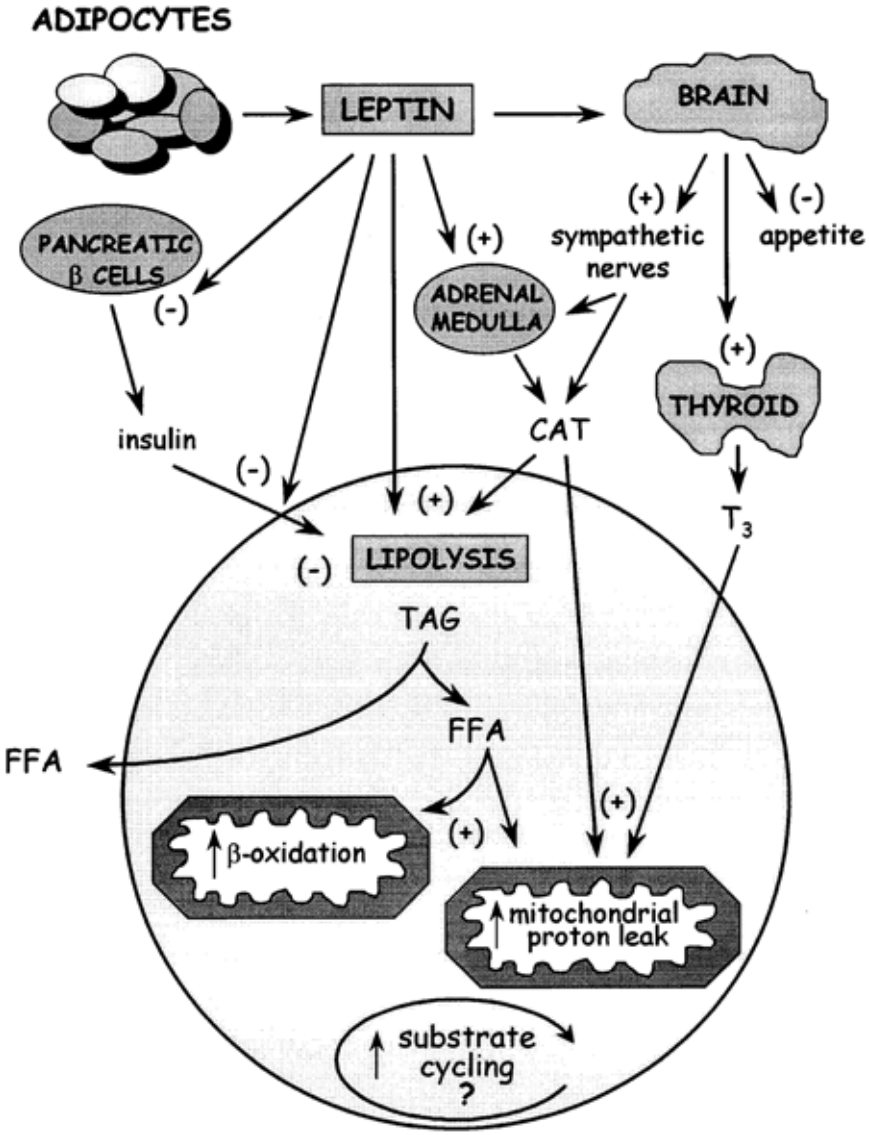

Figure 1.2: Pathways through which leptin effects lipid metabolism, energy expenditure and caloric intake (Adapted from Reidy & Weber, 2000).

Note: (CAT) Catecholamine, (FFA) Free fatty acid, (TAG) Triacylglyceride, (T_3) Triiodothyronine, (+) Stimulatory effect, (-) Inhibitory effect

Leptin also modulates appetite signaling pathways that are independent from NPY. NPY deficient mice, which have normal food intake and body weight, show a decrease in food intake, body mass and fat mass when treated with leptin (Erickson *et al.*, 1996). It is possible that a number of other appetite affecting factors like cocaine-and-amphetamine-regulated transcript (CART) (Friedman & Halaas, 1998, Elmquist *et al.*, 1999), orexin/hypocretin, corticotrophin releasing hormone (CRH) (Flier & Maratos-Flier, 1998), galanin (Beck *et al.*, 1993), cholecystokinin, melanin-concentrating hormone, and neurotensin might also be involved in the leptin induced reduction in appetite.

There is also evidence to suggest that the loss in weight associated with leptin is not entirely due to a reduction in food intake or suppression of appetite. The high rates of adipose tissue loss observed in leptin-treated animals can be also partly attributed to increases in metabolic rate, secondary to increased sympathetic activity (Chen *et al.*, 1996, Levin *et al.*, 1996, Ormseth *et al.*, 1996), and stimulation of substrate cycles (Clark *et al.*, 1973). It has been shown that the triacylglyceride/free fatty acid (TAG/FFA) substrate cycling rate of human adipocytes is negatively correlated with obesity (Bottcher & Furst, 1997). *In vitro,* leptin treatment of adipocytes increases the TAG/FFA cells (Wang *et al.*, 1999), suggesting that the TAG/FFA cycling rate may be increased by leptin. This may be a possible mechanism by which leptin increases the resting metabolic rates above the basal levels. In addition, leptin also has an important impact on the relative contribution of the different oxidative fuels available. In *ob/ob* mice e.g.,

leptin treatment decreased the respiratory quotient in a dose-dependent manner (Hwa *et al.*, 1997).

Leptin has also been found to exert its influence on energy expenditure through its effect on the hypothalamic-pituitary-thyroid axis. The thyroid hormone, triiodothyronine (T_3), is one of the key regulators of metabolic rate, and leptin prevents fasting-induced suppression of prothyrotropin-releasing hormone mRNA in neurons of the hypothalamic paraventricular nucleus (Legradi *et al.*, 1997). In addition to its effects through the hypothalamic-pituitary-thyroid axis, leptin is also thought to be able to change the proton leakiness of membranes, and hence energy expenditure, by varying the mRNA expression and membrane concentration of uncoupling protein (UCP). Different uncoupling proteins are expressed in specific tissues and affected by leptin through different pathways. UCP1 is only expressed in brown adipose tissue (Himms-Hagen, 1989). Leptin administration causes an increase in UCP1 mRNA levels in brown adipose tissue and enhances energy expenditure (Scarpace *et al.*, 1997). This effect is possibly mediated through increased sympathetic activity.

It therefore seems that the role of leptin in the normal regulation of body weight involves both a reduction in food intake and an increase in energy expenditure. The latter might be achieved through a number of mechanisms, which include an increase in sympathetic activity, activation of the hypothalamic-

pituitary-thyroid axis, direct effect on substrate utilization, and perhaps to some extent uncoupling of oxidative phosphorylation.

1.6.2 Leptin and the control of sexual maturation

It is known that the onset of puberty in adolescents, particularly in girls, is linked with attainment of adequate body fat mass. Sexual maturation is delayed when metabolic conditions are not adequate, as in food restriction and low body fat (Kiess *et al.*, 1998). Once when adequate fat stores have been attained there is a signal to the brain that the body is sufficiently developed to afford the pubertal changes or onset of reproductive life (Frisch, 1980). Circulating leptin levels might represent putative signal to the hypothalamus, indicating that nutritional status is compatible with the onset of sexual function (Figure 1.3). In normal children leptin levels increase before puberty and reach their peak at the onset of puberty (Garcia-Mayor *et al.*, 1997), after which they begin to decline in boys but continue to increase in girls, with levels depending on fat mass. There is also an inverse correlation between leptin levels and the age at menarche in women (Matkovic *et al.*, 1997). Increase of leptin levels results in the earlier onset of menstrual cycle in women. The increasing leptin level is believed to permissively activate the hypothalamic–pituitary-gonadal axis and the beginning of puberty (Mantzoros *et al.*, 1997, Kiess *et al.*, 1999, Clayton & Trueman, 2000, Dearth *et al.*, 2000). Nocturnal urinary leptin concentration has been found to show a positive correlation with LH and FSH as children progress into puberty (Maqsood *et al.*, 2007). These observations suggest that leptin is an important

facilitator of the early phases of human puberty. Interestingly, mutations of *ob* and *db* genes result in hypothalamic hypogonadism in humans (Strobel *et al.*, 1998). Similarly, *ob/ob* mice are also infertile (Ingalls *et al.*, 1950), a condition believed to be due to reduced circulating gonadal steroids secondary to insufficient hypothalamic-pituitary drive (Swerdloff *et al.*, 1978). Injection of recombinant leptin evidently restores fertility status in these mice (Chehab *et al.*, 1996, Mounzih *et al.*, 1997).

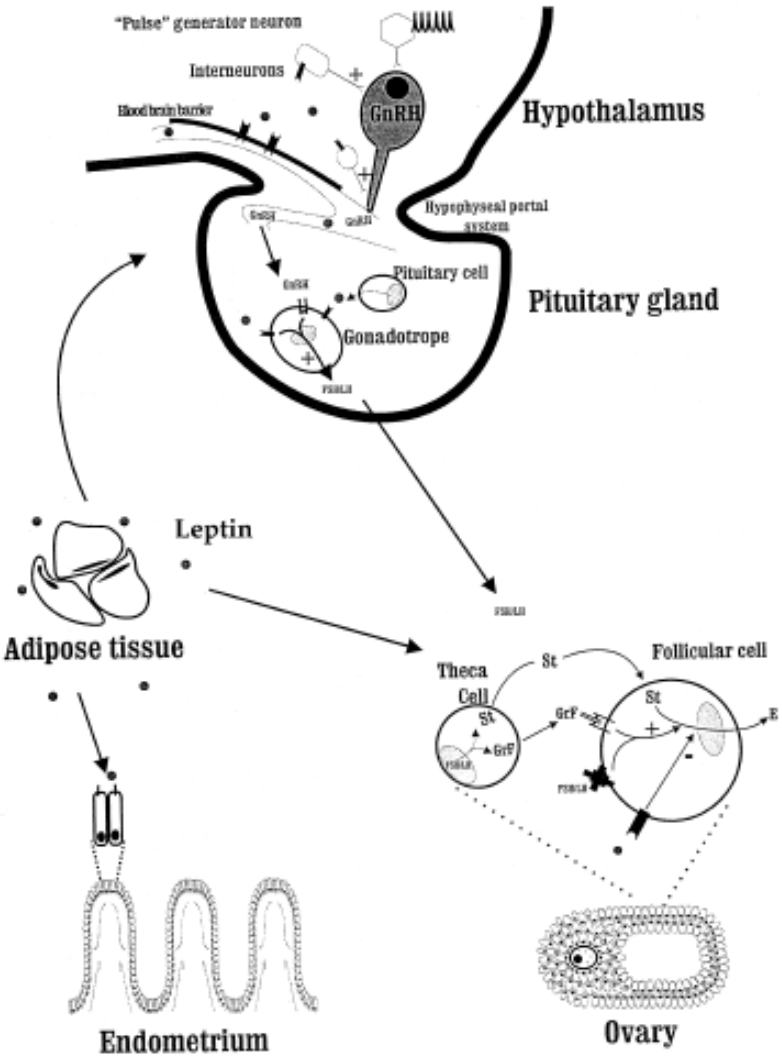

Figure 1.3: Interaction of leptin with the hypothalamic-pituitary-gonadal axis and endometrium (Adapted from Moschos *et al.*, 2002).

The precise mechanism by which leptin helps trigger the onset of puberty is unclear. As leptin receptors are expressed in specific hypothalamic nuclei, leptin might be able to modulate the expression of several hypothalamic neuropeptides (Ahima *et al.*, 2000). In this regard, leptin at very low concentrations was found to stimulate LHRH release from hypothalamic explants, and FSH and LH release from anterior pituitaries of adult male rats*, in vitro.* It was also found to stimulate the release of LH, but not FSH in the same species *in vivo* (Yu *et al.*, 1997). Systemic administration of leptin to *ob/ob* mice increased the secretion of FSH and LH in both male and female mice (Barash *et al.*, 1996). Leptin-treated females had significantly elevated serum levels of LH, increased ovarian and uterine weights, and stimulated aspects of ovarian and uterine histology compared to controls (Barash *et al.*, 1996). Leptin-treated males had significantly elevated serum levels of FSH, increased testicular and seminal vesicle weights, greater seminal vesicle epithelial cell height, and elevated sperm counts compared to controls (Barash *et al.*, 1996). These results demonstrate that leptin stimulates the reproductive endocrine system in both sexes of *ob/ob* mice and suggest that leptin may serve as a permissive signal to the reproductive system of normal animals.

Precisely how leptin stimulates the hypothalamus is unclear. Central infusion of NPY in rats was found to delay sexual maturation (Gruaz *et al.*, 1993), and it may be proposed that the increasing leptin levels around puberty transiently suppress the release of NPY from the hypothalamus, thus releasing

the hypothalamic brake on the onset of puberty (Ahima *et al.*, 1997). Clearly more studies are needed to elucidate the exact mechanism of action of leptin in the initiation of puberty.

The presence of leptin receptors in rat testis (Zamorano *et al.*, 1997) and in the germ cells in mice (El-Hefnawy *et al.*, 2000) suggests there might be a direct action of leptin on the testis too, in addition to its effects on the hypothalamic-pituitary-gonadal axis. Analysis of the cellular location of *LEPR* mRNA shows a scattered pattern of expression in adult testis tissue and specific signals being detected in Leydig and Sertoli cells (Hoggard *et al.*, 1997). Interestingly, mRNA for all the *LEPR* isoforms have been reported in the testes and *LEPR* gene in rat testis is expressed throughout postnatal development (Tena-Sempere *et al.*, 2001a). The precise role of leptin and the receptors in the testes is unclear and remains a focus of study. The presence of *LEPR* in both the Sertoli and Leydig cells suggests that it might have a role in the endocrine function of the testes and in spermatogenesis. There is therefore a need to examine the precise role for leptin in the normal regulation of reproductive function in the male.

1.6.3 Leptin and fertility

The evident positive correlation between gonadotrophins and leptin, particularly during puberty in both the sexes, suggests that leptin has a significant role in reproduction and might exert its influence on reproductive activity via the