Quantitative Expression of Genes Involved in the Leptin Receptor-Mediated STAT Signalling Pathway in Rodent Models of Obesity

by

Shahid Hanif, B.Sc., M.Sc.

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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Dedicated to my mother and father

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1. Briscoe, C.P., **Hanif, S.**, Arch, J.R.S. and Tadayyon, M.

   Leptin receptor long form-signalling in a human liver cell line

   Cytokine 14(4): 225-229, 2001.

2. Briscoe, C.P., **Hanif, S.**, Arch, J.R.S. and Tadayyon, M.

   Fatty acids inhibit leptin signalling in BRIN-BD11 insulinoma cells.

   J. Mol. Endocrinol. 26(2): 145-154, 2001.

Abstracts

1. **Hanif, S.**, Bond, B.C., Tadayyon, M., Arch, J.R.S. and Briscoe, C.P. (2001)
   Quantitative expression of leptin receptor signalling components in the pituitary and pancreas of diet-induced obese and genetically obese *ob/ob* mice. *Diabetologia* 44, Supplement 1, A63.

2. Briscoe, C.P., **Hanif, S.**, Arch, J.R.S. and Tadayyon, M. (2001)
   Quantitative expression of leptin receptor signalling components in BAT and small intestine of diet-induced obese and genetically obese *ob/ob* mice. *Diabetes* 50, Supplement 2, A376.

3. **Hanif, S.**, Tadayyon, M., Moore, G.B.T., Lister, C.A., Arch, J.R.S., Allen, J.M. and Briscoe, C.P. (2000)
   Ob-Ra, Ob-Rb, SOCS3 and CIS expression in the hypothalmus of *ob/ob* and diet-induced obese mice. *Int. J. Obesity* 24, Supplement 1, S49.

4. **Hanif, S.**, Tadayyon, M., Lister, C.A., Arch, J.R.S., Briscoe, C.P. and Allen, J.M. (1999)
   Leptin receptor isoform expression in ZDF rats.
   190th meeting of the Society for Endocrinology, London, U.K.

Abbreviations

α-MSH	α-melanocyte stimulating hormone
ACTH	Adrenocorticotropin hormone
ARC	Arcuate nucleus
AGRP	Agouti-related protein
BAT	Brown adipose tissue
BBB	Blood-brain barrier
CART	Cocaine- and amphetamine-regulated transcript
CIS	Cytokine-inducible SH2-containing protein
CNTF	Ciliary neurotrophic factor
CRH	Corticotropin-releasing hormone
CSF	Cerebrospinal fluid
C_T	Threshold cycle
DIO	Diet-induced obesity
ERK	Extracellular factor-regulated kinases
FSH	Follicle stimulating hormone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GRB-2	Growth factor receptor bound protein-2
HPRT	Hypoxanthine phosphoribosyltransferase

IL	Interleukin
IRS	Insulin receptor substrate
JAK	Janus kinase
K_{ATP}	ATP-sensitive K^+ channel
LH	Luteinizing hormone
LIF	Leukaemia inhibitory factor
MAPK	Mitogen-activated protein kinase
MC-4	Melanocortin-4
MCH	Melanin-concentrating hormone
NPY	Neuropeptide Y
OB-R	Leptin receptor
PDE3B	Phosphodiesterase 3B
PI 3-kinase	Phosphoinositide 3-kinase
POMC	Proopiomelanocortin
PVN	Paraventricluar nucleus
SH2	Src homology 2
SHP-2	SH2 domain-containing protein tyrosine phosphatase
SOCS-3	Suppressor of cytokine signaling-3
STAT	Signal transducers and activators of transcription
TSH	Thyroid stimulating hormone
UCP	Uncoupling protein
VMH	Ventromedial hypothalamus
WAT	White adipose tissue

Summary

In most cases of human obesity serum leptin levels are elevated, suggesting that reduced sensitivity to endogenous leptin may contribute towards the increase in body weight. There are several potential areas that may be involved in reduced leptin sensitivity, including the blood-brain-barrier (BBB) transport system, changes in expression of the functional leptin receptor, OB-Rb, and components of the leptin receptor-mediated STAT signalling pathway in which members of the SOCS family, SOCS-3 and CIS, act as negative regulators. The aim of this thesis was to examine how the mRNA expression of the leptin receptor and components of the leptin receptor-mediated signalling pathway alters in obesity models and thereby establish to what extent such genes are involved in leptin sensitivity. A novel fluorogenic method of quantitative RT-PCR, TaqMan, was utilised to examine the changes in mRNA expression.

Gene expression was examined in central and peripheral tissues of obesity-prone AKR/J mice fed either a chow or a palatable diet for 14 weeks and compared to C57BL/6 lean and *ob/ob* mice fed a chow diet. The expression of OB-Ra mRNA was reduced in the hypothalamus and pituitary of AKR/J mice fed a palatable diet compared to those fed chow, which may implicate reduced OB-Ra-mediated signalling. In WAT of palatable diet-fed mice compared to those fed on chow, the expression of STAT5 and CIS mRNA was increased but SOCS-3 mRNA was reduced, which suggests this tissue may have increased sensitivity to leptin, but the increased CIS mRNA may compensate for the

increased STAT5 mRNA. In contrast, in the small intestine, all of the components of leptin receptor-mediated signalling were reduced, except for CIS, which suggests this tissue has low leptin sensitivity and may have reduced leptin signalling.

In the hypothalamus of *ob/ob* mice compared to leans, the expression of STAT3 and SOCS-3 mRNA were reduced. In the pituitary of *ob/ob* mice compared to leans, the expression of OB-Rb, STAT3, STAT5 and SOCS-3 mRNA was reduced, which may be due a consequence of the leptin deficiency. In the pancreas, the expression of STAT3, STAT5 and SOCS-3 mRNA was increased perhaps reflecting an increase in leptin sensitivity or increased insulin signalling. In BAT, the increased expression of OB-Ra, OB-Rb and STAT3 mRNA and reduced CIS mRNA appears consistent with elevated leptin sensitivity in this tissue although SOCS-3 mRNA was considerably elevated which may be due to obesity-related factors other than leptin.

In order to further expand our understanding of the regulation of leptin sensitivity, the expression of components of the leptin receptor-mediated signalling pathway were analysed in the hypothalamus of *ob/ob* mice intraperitoneally treated with leptin for 24 hours compared to 2 weeks. In comparing *ob/ob* mice to leans in this study, the expression of OB-Rb, STAT3, STAT5 was increased in contrast to the changes reported in the previous study. The treatment of *ob/ob* mice with leptin for 24 hours showed a significant reduction in the hypothalamus of SOCS-3 mRNA, but after 2 weeks of leptin treatment the expression of SOCS-3 mRNA was increased not only in *ob/ob*

mice but also in pair-fed controls. The involvement of SOCS-3 in hypothalamic leptin sensitivity has previously been reported but in this study the increase after 2 weeks of leptin treatment may be due to feeding rather than leptin.

The expression of OB-Ra and OB-Rb mRNA was also examined in ZDF *fa/fa* rats to examine if the mutated leptin receptor has an effect on its expression. An increase of both OB-Ra and OB-Rb mRNA was observed in ZDF *fa/fa* rats compared to ZDF +/? rats, which may be a compensatory change to the reduced leptin receptor-mediated signalling and leptin receptor cell surface expression in these animals.

The gene expression changes in tissues of dietary obese and genetically obese mice followed no consistent pattern in relation to leptin sensitivity and can be interpreted in a number of different ways. The changes suggest, however, that some tissues may be more sensitive to the effects of leptin and changes in sensitivity compared to others but there appears to be no clear gene or set of gene changes predictive amongst the genes examined of leptin sensitivity. Since the expression of STATs and SOCs can be regulated by a number of signals it is difficult to definitively distinguish the effects of leptin, tissue-specific effects and other obesity-related factors in these tissues.

Chapter 1

Introduction

1.1 Obesity as a major health disease

Obesity is widely acknowledged as a serious threat to public health and is considered a social stigma in many cultures. Furthermore, being overweight is strongly associated with the development of adverse medical conditions, such as hypertension, diabetes, heart disease, cancer, and thus it has a strong negative effect on life expectancy (Friedman, 2000; Kopelman, 2000).

Obesity has been increasing in prevalence over the last few decades, especially in Western countries. In the United States, for example, the prevalence of obesity among the adult (age 20-74) population increased from 12.8% in 1960-62 to 22.5% in 1988-94 (Flegal *et al.*, 1998). A similar trend has been seen among children and adolescents, with the prevalence of overweight (6-17 years old) increasing from 5% in 1963-70 to 11% in 1988-94 (Troiano & Flegal, 1998). Today, more than half of all adults can be classified as overweight or obese (Must *et al.*, 1999), and it has been estimated that some 300,000 deaths per year are attributable to obesity (Allison *et al.*, 1999). The direct cost of obesity-related health care in the U.S. has been estimated at $52 billion per year, or 5.7% of total expenditures on health care; indirect costs due to lost productivity are thought to be of equal magnitude (Wolf & Colditz, 1998).

1.2 Leptin

1.2.1 Early implications of a satiety factor

Over the past four decades, considerable clinical and experimental evidence has accumulated to support the existence of sensitive systems for controlling body weight in animals and humans. Kennedy proposed that the amount of energy stored in adipose mass represented the balance between ingested calories and energy expenditure (Kennedy, 1953). He hypothesised that the hypothalamus sensed the concentration of an unknown circulating factor, which provided information on the amount of body fat stores. This adipostatic model of body weight regulation is consistent with the observation that a decrease in adiposity from fasting or surgical resection causes hyperphagia, reduces energy expenditure and eventually restores body weight to the previous level (Faust *et al.*, 1977; Harris *et al.*, 1986; Harris, 1990). Conversely, weight gain from forced overfeeding inhibits voluntary food intake (Harris *et al.*, 1986; Harris1990).

Hervey demonstrated the parabiosis (a long-term surgical anastomosis made between the muscle and/or peritoneal compartments) of two rats, one rendered obese by lesions in the ventromedial hypothalamus (VMH) and one control rat, resulted in death by starvation of the latter (Hervey, 1958). This suggested that increased levels of a circulating satiety factor from the obese rats inhibited food intake in the non-lesioned lean rats. In contrast, the obese rats were incapable of responding to elevated endogenous levels of the presumed satiety factor, because of the hypothalamic lesion.

More convincing evidence for a circulating factor that could control food intake was provided by the discovery of recessive mutations, *obese* (*ob*) and *diabetes* (*db*), both of which led to hyperphagia, reduced energy expenditure and early onset obesity in mice (Ingalls *et al.*, 1950). Coleman (Coleman, 1973; Coleman & Hummel, 1969) and Hausberger (Hausberger, 1959) showed that parabiosis of wild-type and *ob/ob* mice reduced the weight gain in *ob/ob* mice, whereas parabiosis of wild-type and *db/db* mice caused profound hypophagia in lean wild-type mice. These results suggested that the *ob* locus was necessary to produce the satiety factor and that the *db* locus encoded a molecule required for response to this factor.

In 1994, Zhang *et al.* reported the discovery through positional cloning of the gene responsible for the *ob* phenotype (Zhang *et al.*, 1994). The authors identified two mutations at the *ob* locus, which led to underexpression of the 16-kDa protein product, termed leptin (from the Greek word *leptos*, which means thin), in two strains of *ob/ob* mouse. In C57BL/6J *ob/ob* mice, a C→T substitution resulted in a stop codon at position 105 instead of arginine and in synthesis of a truncated protein that is incapable of being secreted (Rau *et al.*, 1999; Zhang *et al.*, 1994). Leptin mRNA expression is increased in C57BL/6J *ob/ob* mice, consistent with the view that the *ob* gene is under negative feedback regulation. In the ob^{2J}/ob^{2J} mouse mutant, a transposon inserted into the first intron of the *ob* gene, prevented the synthesis of mature *ob* mRNA (Zhang *et al.*, 1994). Both *ob/ob* mouse mutants are leptin deficient, hyperphagic,

hypothermic, morbidly obese and have several metabolic and neuroendocrine abnormalities including hyperinsulinaemia, hyperglycaemia and hypercorticism.

1.2.2 Characterisation of leptin

Mouse and human *ob* genes have been localised to chromosome 6 and 7q31.3, respectively (Isse *et al.*, 1995; Zhang *et al.*, 1994). The *ob* gene encompasses 650 kb and consists of 3 exons separated by 2 introns, with the coding region for leptin located in exons 2 and 3. The mouse *ob* gene encodes a 4.5 kilobase mRNA transcript with a highly conserved 167-amino acid open reading frame (Zhang *et al.*, 1994). Human leptin is 84% identical to mouse leptin and 83% identical to rat leptin.

Leptin is synthesised and secreted mainly, but not exclusively, by white adipose tissue (WAT) and circulates as a 16-kDa protein in plasma and is not modified post-translationally (Cohen *et al.*, 1996a; Halaas *et al.*, 1995). Structural analysis indicated that leptin is similar to cytokines (Madej *et al.*, 1995; Zhang *et al.*, 1997) and contains an intrachain disulphide bond that appears to be necessary for its biological activity (Grasso *et al.*, 1997).

There is a strong positive correlation between leptin mRNA and protein levels in adipose tissue and circulating plasma leptin levels (Considine *et al.*, 1996; Frederich *et al.*, 1995; Maffei *et al.*, 1995). Moreover, the plasma level of leptin is highly correlated with adipose tissue mass and falls in both humans and mice after weight loss (Maffei *et al.*, 1995). Leptin deficiency due to mutations in the human *ob* gene are rare (see section 1.4.1) and most human forms of

obesity are associated with increased levels of leptin. Furthermore, leptin levels are generally increased in several genetic and environmentally induced forms of rodent obesity (Maffei *et al.*, 1995).

1.2.3 Localisation of leptin expression

Initial studies indicated that leptin expression was synthesised only in adipose tissue. However, leptin is also now known to be synthesised in some extra-adipose tissues including placenta, gastric epithelium, skeletal muscle, and mammary epithelium where it is secreted in colustrum, and absorbed by the neonate (Bado *et al.*, 1998; Casabiell *et al.*, 1997; Masuzaki *et al.*, 1997).

1.2.4 Regulation of leptin expression

Although leptin levels in blood correlate with total body fat stores (Considine *et al.*, 1996; Frederich *et al.*, 1995; Maffei *et al.*, 1995), it is not known whether increased triglyceride levels, lipid metabolites, or mechanical factors associated with increased adipocyte size, influence leptin expression. Leptin levels increase within hours after a meal in rodents and after several days of overfeeding in humans (Harris *et al.*, 1996; Kolaczynski *et al.*, 1996a; Saladin *et al.*, 1995), whereas levels decrease within hours after initiation of fasting in both species (Boden *et al.*, 1996; Frederich *et al.*, 1995; Saladin *et al.*, 1995). The changes in leptin expression in response to fasting and feeding are out of proportion to the corresponding changes in body weight or body fat

(Boden *et al.*, 1996; Kolaczynski *et al.*, 1996a), suggesting that leptin serves as an indicator of energy stores, as well as a mediator of energy balance.

Regulation of leptin expression by nutrition is probably mediated in part by insulin. Leptin expression increases after peak insulin secretion during the feeding cycle (Saladin *et al.*, 1995; Sinha *et al.*, 1996). Insulin stimulates leptin expression directly in isolated adipocytes (Rentsch & Chiesi, 1996) and increases leptin levels when administered to rodents (Saladin *et al.*, 1995). In contrast, plasma leptin is decreased in low insulin states, such as in streptozotocin-induced diabetes, and increases after insulin treatment (Macdougald *et al.*, 1995). In humans, leptin expression is correlated with insulin levels and is increased several days after insulin infusion (Kolaczynski *et al.*, 1996b; Segal *et al.*, 1996). Conversely, the fall in insulin levels may mediate the decline in leptin levels during fasting (Boden *et al.*, 1997). Moreover, mutational analysis of the region between nucleotides –101 and –83 of the leptin gene promoter showed that this region is critical for glucose/insulin-mediated stimulation of transcription (Fukuda & Iritani, 1999).

Several regulatory elements have been identified within the *ob* gene promoter, including cyclic AMP and glucocorticoid response elements, and CCATT/enhancer and SP-1 binding sites (Gong *et al.*, 1996; Hwang *et al.*, 1996; Miller *et al.*, 1996). Placental leptin expression is stimulated by insulin and glucocorticoids (Mise *et al.*, 1998; Shekhawat *et al.*, 1998). Moreover, glucocorticoids directly stimulate leptin synthesis in cultured adipocytes

(Murakami *et al.*, 1995; Slieker *et al.*, 1996), and leptin expression increases in response to chronic elevation of cortisol in humans (Cizza *et al.*, 1997).

Administration of cholecystokinin or gastrin decreases leptin synthesis in the gastric fundus and increase plasma leptin (Bado *et al.*, 1998). It is speculated that meal-related alterations in gastric and plasma leptin levels may be involved in the short-term regulation of appetite (Bado *et al.*, 1998). The hexosamine biosynthetic pathway also acts as a nutrient-sensing pathway to regulate leptin release and flux through the pathway increases with hyperglycaemia or excess fat intake (Wang *et al.*, 1998a). Glucosamine infusion, which increases the tissue concentrations of the end product of the hexosamine biosynthetic pathway, UDP-*N*-acetylglucosamine (UDP-GlcNAc), results in increased leptin expression in adipose tissue, rat skeletal muscle, and pancreatic islets (Considine *et al.*, 2000; Emilsson *et al.*, 2001; McClain *et al.*, 2000; Wang *et al.*, 1998a).

1.3 Leptin receptor

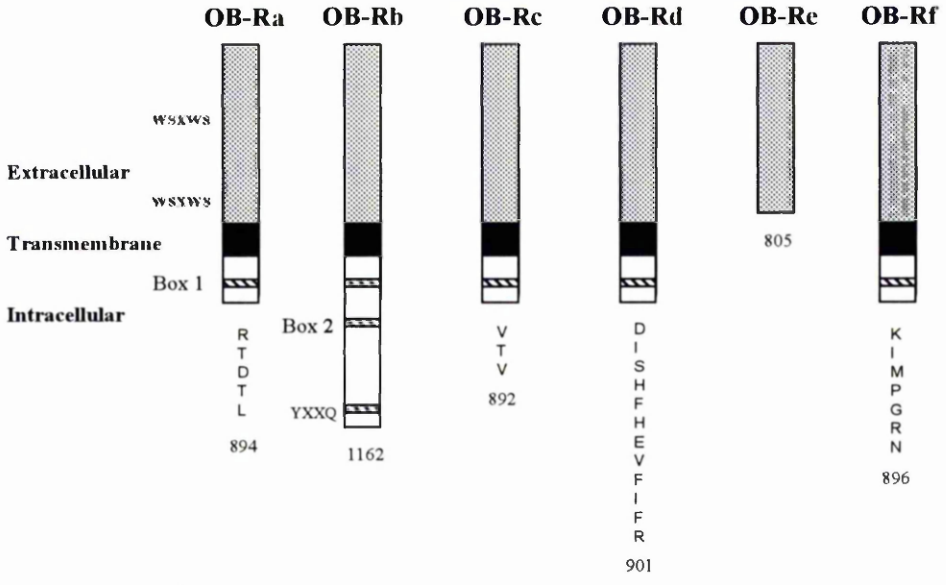
1.3.1 Discovery of leptin receptor

A high affinity leptin receptor (OB-R) was cloned by Tartaglia *et al.* from mouse choroid plexus using an expression cloning strategy and was genetically mapped to the same interval of mouse chromosome 4 that contains the *db* locus (Tartaglia *et al.*, 1995).

1.3.2 Structure of receptor/alternative splicing

To date, six common splice variants of the leptin receptor have been identified as a result of alternative splicing by RT-PCR, namely OB-Ra to OB-Rf (Figure 1) (Lee *et al.*, 1996; Wang *et al.*, 1996). Leptin receptor isoforms share an identical amino-terminal extracellular ligand-binding domain but differ in the length of the intracellular carboxy terminus. Five isoforms, OB-Ra, OB-Rb, OB-Rc, OB-Rd and OB-Rf have transmembrane and intracellular domains; however, only OB-Rb contains intracellular motifs required for activation of the Janus kinase and signal transducers and activators of transcription (JAK-STAT) signal transduction pathway (Bjorbaek *et al.*, 1997; Ghilardi *et al.*, 1996; Vaisse *et al.*, 1996). The fact that OB-Rb is the receptor isoform not expressed in obese *db/db* mice proves that this is the fully functional receptor and that therefore, activation of the JAK-STAT pathway is essential for leptin effects on feeding and energy expenditure.

Figure 1.1 Leptin receptor isoforms



Investigation of the structure of leptin reveals a four-helix bundle similar to that of the long-chain helical cytokine family (Zhang *et al.*, 1997). Confirmation of this similarity was attained when the leptin receptor was determined to be similar to the gp130 signal transduction arm of class I cytokine receptor family members, which includes receptors for various interleukins (IL-2, -3, -4, -6, and –7) (Bazan, 1989; Idzerda *et al.*, 1990; Itoh *et al.*, 1990), granulocyte colony stimulating factor (G-CSF) (Gearing *et al.*, 1989), growth hormone, prolactin, erythropoeitin (EPO) (Bazan1989), and leukaemia inhibitory factor (LIF) (Tartaglia *et al.*, 1995).

The structure of OB-R consists of a signal sequence, two immunoglobulin domains, two cytokine receptor homology domains each containing a Trp-Ser-X-Trp-Ser motif (where the X residue is non-conserved),

fibronectin type III domains, a transmembrane region and intracellular domain (except in OB-Re) (Haniu *et al.*, 1998). The leptin receptor contains a short 23 amino acid transmembrane domain, which is followed by a cytoplasmic domain whose length varies among the isoforms (Lee *et al.*, 1996). OB-Ra and OB-Rb are the two major forms of the receptor expressed. The OB-Ra isoform found in most tissues has a short cytoplasmic domain of 34 amino acids and contains only box 1 that can bind JAK2, but the receptor is not able to activate STAT signalling (Bjorbaek *et al.*, 1997). The longest form of the leptin receptor, OB-Rb has a cytoplasmic domain of 302 amino acids that contains the JAK binding domains, box 1 and box 2, and a potential consensus sequence (YXXQ) for the binding of STATs (Ghilardi *et al.*, 1996; White & Tartaglia, 1996).

The intracellular portion of murine OB-Rb contains three different tyrosine residues that have been implicated in OB-Rb signalling (Banks *et al.*, 2000). Within the folded protein, Tyr^{985} and Tyr^{1138} have been shown to be surrounded by hydrophilic sequences, whereas Tyr^{1077} is surrounded by hydrophobic sequences, suggesting Tyr^{985} and Tyr^{1138} are involved in leptin-mediated STAT signalling (Banks *et al.*, 2000). Recent research has not only demonstrated that Tyr^{985} and Tyr^{1138} are accessible to JAK2 tyrosine kinase, but that mutation of these residues with intact Tyr^{1077} prevents tyrosine phosphorylation of OB-Rb (Banks *et al.*, 2000). Using a model system consisting of an EPO receptor with mutant intracellular OB-Rb domains, researchers have been able to further demonstrate that Tyr^{985} is necessary for the tyrosine phosphorylation of the SH2 domain-containing protein tyrosine

phosphatase (SHP-2), and Tyr^{1138} controls STAT3 activation (Banks *et al.*, 2000).

1.3.3 Leptin receptor expression

In vitro hybridisation studies revealed that both OB-Ra and OB-Rb mRNA are widely expressed in the human, mouse and rat brain (Burguera *et al.*, 2000; Elmquist *et al.*, 1998; Guan *et al.*, 1997; Mercer *et al.*, 1996). Ultrastructural localisation studies showed OB-Rb to be predominantly located in the hypothalamus, a region of the central nervous system long known to be involved in body weight regulation. In particular, OB-Rb has been detected in the arcuate, ventromedial, paraventricular, dorsomedial, and ventral premammillary nuclei, as well as the lateral hypothalamic area (Elmquist *et al.*, 1998; Fei *et al.*, 1997; Mercer *et al.*, 1996). Furthermore, the expression of OB-Rb mRNA has been demonstrated in the Purkinje cells and dentate nuclei of the cerebellum, medulla, amygdala and neurons from both the neocortex and entorrhinal cortex (Burguera *et al.*, 2000; Mercer *et al.*, 1998). The expression of OB-Rb mRNA in the human and rat cerebellum (Burguera *et al.*, 2000; Couce *et al.*, 1997; Elmquist *et al.*, 1998; Savioz *et al.*, 1997) is intriguing and its significance is open to speculation. The cerebellum is involved in the control of posture and movement, as well as in the learning of complex motor tasks, and probably receives information from the periphery, such as that regarding total body fat content, in order to perform its function.

A number of studies have show that OB-Ra mRNA is highly expressed in the choroid plexus epithelium and the ependymal lining (Burguera *et al.*, 2000; Guan *et al.*, 1997; Tartaglia *et al.*, 1995). The authors suggest that leptin in the cerebrospinal fluid (CSF) could bind to OB-Ra at the ependymal layer and cross through the ependymal barrier into the brain parenchyma and reach the nearby hypothalamic nuclei. There are also studies that have shown the presence of OB-R isoforms in the brain capillaries and endothelium (Boado *et al.*, 1998; Burguera *et al.*, 2000; Couce *et al.*, 1997). The presence of specific OB-R isoforms in endothelial cells may permit leptin access to specific hypothalamic nuclei and other locations where leptin exerts its effects. By this mechanism, leptin would not leave the bloodstream until reaching the particular target areas. This proposal is supported by the observation that Ob-Ra and OB-Rb mRNA is expressed in the brain microvessels (Bjorbaek *et al.*, 1998a).

Leptin receptor expression, in particular the OB-Rb isoform, has been observed in a number of peripheral tissues suggesting leptin may exert tissue-specific functions. A study by Ghilardi *et al.* showed, using a RNase protection assay of total RNA from C57BL/6J lean mice, that the short isoforms of the leptin receptor were ubiquitously expressed in a range of peripheral tissues: including the liver, kidney, stomach, small intestine, colon, pancreas, skeletal muscle lung, heart, adipose tissue, lymph nodes, ovary, uterus, mammary gland and testis (Ghilardi *et al.*, 1996). Although the expression of OB-Rb mRNA was only detected in a few peripheral tissues in this study, subsequent studies have shown the expression of OB-Rb mRNA in more peripheral tissues and cell

lines. Leptin function in some of these tissues has also been ascribed; for example the kidney plays a role in the clearance of leptin (Sharma & Considine, 1998), leptin may have a paracrine and/or autocrine effect on gastric epithelial cell function (Mix *et al.*, 2000), leptin increases glucose transport and utilisation in skeletal muscle (Ceddia *et al.*, 1998) and leptin is a growth factor for colonic epithelial cells (Hardwick *et al.*, 2001). The mRNA expression of leptin receptor isoforms, in particular OB-Rb, in the human (Dieterich & Lehnert, 1998; Jin *et al.*, 1999) and rat pituitary (Sone *et al.*, 2001) indicates the pituitary might be involved in leptin regulation of pituitary hormone secretion. In the pancreas, expression of OB-Rb mRNA is expressed in the pancreatic β-cells of mice (Emilsson *et al.*, 1997; Kieffer *et al.*, 1996; Kieffer *et al.*, 1997) where leptin has been shown to inhibit insulin secretion. OB-Rb insulin-secreting cell lines, such as RINm5F and BRIN-BD11, have been shown to increase STAT3 activation upon leptin treatment (Briscoe *et al.*, 2001a; Morton *et al.*, 1999). Leptin receptor isoforms are also expressed in WAT and brown adipose tissue (BAT) (Kutoh *et al.*, 1997; Siegrist-Kaiser *et al.*, 1997), where leptin regulates lipolysis and glucose utilisation. Furthermore, OB-Ra and OB-Rb isoforms are also expressed in the small intestine of mice and rats where leptin has a rapid inhibitory effect on sugar absorption (Lostao *et al.*, 1998; Morton *et al.*, 1998).

Little is known about the expression, or indeed the function, of the OB-Rc, OB-Rd, OB-Re and OB-Rf isoforms of the leptin receptor. There are a number of studies that have described the expression of the OB-Ra isoform, however the expression of each of the remaining short OB-R isoforms (c, d and

f) containing short intracellular domains is low amongst the tissues tested (Fei *et al.*, 1997). OB-R isoforms c, d and f have a box 1 motif similar to Ob-Ra and may perform reduced signalling. The expression of the OB-Rc isoform has been reported in the choroid plexus (Guan *et al.*, 1997), rat adrenal gland (Tena-Sempere *et al.*, 2000) and the rat testis (Tena-Sempere *et al.*, 2001). The expression of OB-Rf mRNA has been detected at higher levels in the rat liver and spleen compared to the brain, hypothalamus, stomach and kidney amongst other tissues, in contrast to the expression of OB-Ra and OB-Rb mRNA (Wang *et al.*, 1996).

The mRNA expression of the shortest splice variant, OB-Re, which is expected to encode a soluble form of the leptin receptor, was detected in relatively high amounts in many tissues (Lollmann *et al.*, 1997). Expression of OB-Re mRNA is comparable to that of leptin in fat tissue, and it has been proposed that OB-Re may be secreted in sufficient amounts to act as a buffering system for freely circulating leptin (Lollmann *et al.*, 1997). Furthermore, the high mRNA expression of OB-Re in the placenta of pregnant mice may be attributable to the binding of leptin (Gavrilova *et al.*, 1997).

1.3.4 Leptin receptor signalling

Class I cytokine receptor members typically lack intrinsic tyrosine kinase activity and are activated by formation of homo- or heterodimers upon ligand binding (Watowich *et al.*, 1996). Recent research has demonstrated that leptin receptors expressed in COS and Ba/F3 cells exist as both homodimer and

homooligomer complexes without any evidence for heterodimer formation (Devos *et al.*, 1997; Nakashima *et al.*, 1997; White *et al.*, 1997a). The fact that the expressed extracellular domain of OB-Rb is a dimer suggests that OB-Rb exists as a preformed homodimer, even in the absence of ligand (Devos *et al.*, 1997). OB-Rb therefore appears to be activated via ligand-induced conformational changes and homodimerisation rather than heterodimerisation or oligomerisation with other receptors of the class I cytokine family (Devos *et al.*, 1997).

Leptin binding to its receptor induces a conformational change in the receptor that allows transphosphorylation of JAKs. The activation of JAK is followed by the phosphorylation of specific tyrosine residues on the intracellular domain of the receptor that provide docking sites for members of the STAT family and other Src Homology 2 (SH2) -containing proteins e.g. SHP-2. Phosphorylation of STATs by JAK is followed by their dimersiation and translocation to the nucleus where they regulate transcription of various genes.

1.3.4.1 JAK-STAT pathway

Given the number of different OB-R isoforms that exist, signals specific to OB-Rb's intracellular tail are crucial for the biological action of leptin. The JAK family was independently identified by low stringency hybridisation (Firmbach-Kraft *et al.*, 1990) and by studies designed to identify novel protein tyrosine kinases using PCR (Partanen *et al.*, 1990; Wilks, 1989). There are presently four identified mammalian Janus kinase (JAK) family members:

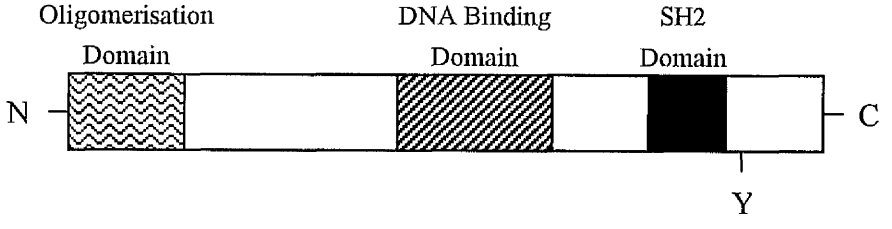
JAK1, JAK2, JAK3 and TYK2, which range from 120 to 140 kDa (Pellegrini & Dusanter-Fourt, 1997). Cytokine receptors have no intrinsic catalytic activity and rely on JAKs, which are constitutively associated with their cytoplasmic regions, to transduce the extracellular ligand-binding event to an intracellular signal. Motifs called 'box 1', usually proline rich, and 'box 2' in the membrane proximal regions of the receptors are important for JAK association. JAKs can be roughly divided into an amino-terminal region, followed by a catalytically inactive kinase-like domain and a tyrosine kinase domain. The amino-terminal region is important for receptor recognition and association. The tyrosine kinase domain is responsible for catalytic activity and the catalytically inactive kinase-like domain is likely to play a regulatory role, which is not yet well-defined.

Research has demonstrated that the JAK2, and possibly JAK1, tyrosine kinases are activated during OB-Rb signalling (Baumann *et al.*, 1996; Ghilardi & Skoda, 1997). JAK activation is followed by the phosphorylation of tyrosine residues located on the intracellular tail of OB-Rb (Bjorbaek *et al.*, 1997). Of all the OB-R isoforms, OB-Rb is the only form to contain tyrosine residues on its intracellular tail, partially explaining OB-Rb's unique role as the major signalling isoforms (Banks *et al.*, 2000; Bjorbaek *et al.*, 1997).

The STAT family of proteins are DNA binding transcription factors that contain serine-rich SH2 domains that interact with the cytokine receptors through phosphorylated tyrosine residues (Ghilardi *et al.*, 1996). Seven members of the STAT family have been identified in mammalian cells: STAT1,

STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (Leonard & O'Shea, 1998). All STAT proteins isolated so far have a conserved domain structure (Figure 1.2). Differences in the SH2 domains of different STATs determines the selectivity of STAT binding to various cytokine receptors. A conserved tyrosine approximately 700 residues from the N-terminus is rapidly phosphorylated by activated JAKs following receptor binding, allowing STAT proteins to form dimers, based on the interaction between the SH2 domain of each STAT and the phosphorylated tyrosine on the other. Although STAT dimerisation is important for DNA binding activity, the highly conserved N-terminal domain is involved in oligomerisation of STAT dimers to form tetramers and potentially more highly ordered oligomers (Vinkemeier *et al.*, 1996; Xu *et al.*, 1996).

Figure 1.2 The structure of STAT proteins



Leptin signalling via the JAK-STAT pathway has been well documented in several tissues and *in vitro* systems. The i.c.v. administration of recombinant leptin activates STAT3, but not STAT5, in the hypothalamus of wild-type and *ob/ob* mice (Vaisse *et al.*, 1996). However, intravenous administration of leptin

was found to induce STAT5 DNA-binding activity in the small intestine of lean and *ob/ob* mice after 30 minutes, but no effect was observed in the OB-Rb-deficient *db/db* mouse (Morton *et al.*, 1998). This result was corroborated by the activation of STAT5 DNA-binding activity upon leptin treatment of Caco-2 cells, a human model of small intestinal epithelium (Morton *et al.*, 1998). The intravenous injection of leptin in lean (+/?) Zucker rats induced translocation of only STAT1 to the nucleus, as demonstrated by binding to the M67-SIE DNA element (Siegrist-Kaiser *et al.*, 1997), whereas i.p. leptin administration was shown to activate both STAT1 and STAT3, in epididymal adipose tissue of wild-type and *ob/ob* mice (Bendinelli *et al.*, 2000).

Leptin activates STAT3 in pancreatic islets and in insulinoma cell lines; leptin activates STAT3 in RINm5F cells (Morton *et al.*, 1998), STAT3 and STAT5 in BRIN-BD11 cells (Briscoe *et al.*, 2001a) and STAT1 and STAT3 in MIN6 cells (Tanabe *et al.*, 1997). Other examples of leptin receptor JAK-STAT signalling *in vitro* include STAT3 and STAT5 activation in the human liver cell line WRL68 cells (Briscoe *et al.*, 2001b) and COS cells, but results for STAT1 and STAT6 were equivocal (Baumann *et al.*, 1996; Ghilardi & Skoda1997). Rosenblum *et al.* reported formation of STAT1, STAT3 and mixed STAT1:STAT3 dimers in cells transfected with OB-Rb and treated with leptin (Rosenblum *et al.*, 1996). Tyrosine phosphorylation of STAT1 has also been demonstrated in a human renal adenocarcinoma cell line after treatment with leptin (Takahashi *et al.*, 1996).

1.3.4.2 SOCS and CIS

CIS (Yoshimura *et al.*, 1995) and SOCS-1 (Starr *et al.*, 1997), also known as SSI-1 (STAT-induced STAT inhibitor) (Naka *et al.*, 1997) and JAB (JAK binding protein), are the two founder members of the family of inhibitors of STAT signalling. CIS and SOCS-1 contain a N-terminal regions of variable length, a central SH2 domain, and a conserved C-terminal region termed the SOCS box, consisting of 40 amino acids (Starr *et al.*, 1997). Searches in DNA databases of the conserved SOCS box and SH2 domain identified 6 structurally related genes, which were termed SOCS-2 to SOCS-7 (Hilton *et al.*, 1998), however SOCS-3 appears to be the one mainly involved in leptin-mediated signalling (Bjorbaek *et al.*, 1998b). The SOCS genes have the main characteristics of immediate-early genes: they are small genes, contain a few or no introns and their expression is induced by cytokine stimulation. STAT response elements have been identified in the promoter sequences of the SOCS genes.

The mRNA for these genes is usually present at low levels in unstimulated cells, SOCS expression is induced by, and can inhibit signalling by many cytokines, growth factors and hormones (Krebs & Hilton, 2000). However, there appears to be some divergence in the mechanisms by which individual SOCS protein accomplish this inhibition. Investigation of the human CIS promoter has shown it contains four STAT binding elements that can bind STAT5-containing complexes (Verdier *et al.*, 1998a). The overexpression of CIS can inhibit STAT5 activation (Matsumoto *et al.*, 1997), and CIS has been

demonstrated to bind specifically to phosphotyrosine Y^{401} of the EPO receptor (Verdier *et al.*, 1998b), one of two docking sites for STAT5. This suggests CIS may act by competing with STAT5 for receptor binding sites, preventing its subsequent phosphorylation and activation. However, CIS does not appear to bind to phosphotyrosine Y^{343} of the EPO receptor, which can also be involved in STAT5 activation (Gobert *et al.*, 1996; Klingmuller *et al.*, 1996) suggesting that the mechanism of inhibition of STAT signalling by CIS may be more complex than simple competition for STAT binding.

Recent evidence has shown that SOCS-3 preferentially binds to phosphotyrosine Y^{985} of OB-Rb to mediate feedback inhibition of OB-Rb signalling (Bjorbaek *et al.*, 2000), and that inhibition of JAK2 activity by SOCS-3 can occur when recruited by the growth hormone receptor (Hansen *et al.*, 1999). This suggests SOCS-3 may act via a distinct mechanism to that of CIS. The recruitment of SOCS-3 to activated receptors may bring it into close proximity to JAKs possibly allowing inhibition of the catalytic activity of JAKs through direct interaction with the kinase domain (Nicholson *et al.*, 1999; Yasukawa *et al.*, 1999). The fact that the binding sites for SOCS-3 on gp130 and the leptin receptor are identical to the SHP-2 binding sites (Bjorbaek *et al.*, 2000; Nicholson *et al.*, 2000) suggests that SOCS-3 may also influence the Ras/mitogen-activated protein kinase (MAPK) signalling pathway (refer to section 1.3.4.5).

1.3.4.3 MAPK pathway

Many cytokines stimulate a kinase cascade involving Ras, Raf-1, MEKK (MAPK kinase), and MAPK (Schiemann & Nathanson, 1998; Winston & Hunter, 1995; Yamashita *et al.*, 1998), which in turn phosphorylate a number of nuclear transcription factors and cytoplasmic proteins involved in various aspects of cellular function including cell proliferation (Aliaga *et al.*, 1999; Begum *et al.*, 1998; Crawley *et al.*, 1997; Sun *et al.*, 1999).

Leptin treatment of $C_3H10T_{1/2}$ cells, a mouse embryonic cell line, resulted in the phosphorylation of MAPKs (Takahashi *et al.*, 1997). In CHO cells stably expressing OB-Rb, leptin stimulation has been shown to increase tyrosine phosphorylation of STAT3 and MAPK , whereas in CHO cells expressing OB-Ra only the phosphorylation of MAPK was detected (Bjorbaek *et al.*, 1999; Yamashita *et al.*, 1998). OB-Rb-stimulated MAPK activation is thought to be mediated through insulin receptor substrate-1 (IRS-1) by the adapter protein, growth factor receptor bound protein 2 (GRB-2), which binds to JAK2 via the SH2 domain it contains (Banks *et al.*, 2000; Bjorbaek *et al.*, 1997). The OB-Ra isoform has a box 1 motif in the intracellular domain that is known to bind JAK2, therefore the phosphorylation of MAPK in OB-Ra-mediated signalling is also thought to be mediated through JAK2 (Bjorbaek *et al.*, 1997). MAPK acts together with STATs, in the leptin-stimulated gene expression of the immediate early response genes c-fos, c-jun and jun-B (Banks *et al.*, 2000; Murakami *et al.*, 1997). These findings suggest that MAPKs in addition to STATs are involved in leptin signal transduction.

1.3.4.4 PI 3-kinase

The phosphoinositide 3-kinase (PI 3-kinase) family is ubiquitously expressed and acts by phosphorylating membrane inositol lipids. Through the generation of phospholipid second messengers, PI 3-kinase plays a key role in the regulation of many cellular processes, including cell proliferation and carbohydrate metabolism. The primary enzymatic activity of PI 3-kinase is the phosphorylation of inositol lipids at the 3 position with the major substrate being phosphatidylinositol (4,5)-bisphosphate ($PtdIns(4,5)P_2$). Hence $PtdIns(4,5)P_2$ is converted to phosphatidylinositol (3,4,5)-trisphosphate ($PtdIns(4,5)P_3$):

$$PtdIns(4,5)P_2 \xrightarrow{\text{PI 3-kinase}} PtdIns(3,4,5)P_3$$

Leptin has been reported to stimulate glucose transport in C_2C_{12} myotubules via a PI 3-kinase-dependent process, which was inhibited by Wortmannin (a PI 3-kinase inhibitor) (Berti *et al.*, 1997). Leptin-stimulated PI 3-kinase activation in the C2C12 cells was found to employ JAK2 and IRS-2 (Kellerer *et al.*, 1997). There have been several reports that leptin reduces insulin secretion from pancreatic β-cells (Emilsson *et al.*, 1997; Fehmann *et al.*, 1997a; Kulkarni *et al.*, 1997; Seufert *et al.*, 1999a). One mechanism proposed to explain the leptin-induced reduction in insulin secretion is via activation of ATP-sensitive K^+ (K_{ATP}) channels (Harvey *et al.*, 1997; Kieffer *et al.*, 1997). Harvey *et al.* have recently shown that leptin activation of K_{ATP} channels in

CRI-G1 cells is sensitive to both Wortmannin and LY 294002 (inhibitors of PI 3-kinase), suggesting the possible involvement of a PI 3-kinase (Harvey *et al.*, 2000a). Most of the cellular roles of PI 3-kinase have been attributed to the lipid products of these enzymes, which bind to target molecules via specific lipid binding domains.

1.3.4.5 SHP-2

Recent data suggests that Tyr^{985} of OB-Rb is required for maximal activation of the MAPK pathway by leptin (Banks *et al.*, 2000). The SH2-domain containing protein tyrosine phosphatase, SHP-2, binds to Tyr^{985} allowing the protein to be tyrosine phosphorylated by JAKs following leptin treatment (Banks *et al.*, 2000; Carpenter *et al.*, 1998; Li & Friedman, 1999).

The relative roles of the phosphatase activity and tyrosine phosphorylation of SHP-2 in regulation of signalling pathways are not well characterised and may differ in different signalling systems. SHP-2 has been shown to play a positive role in mediating MAPK activation by cytokine receptors and by receptor tyrosine kinases (Gu *et al.*, 1998; Kim & Baumann, 1999; Shi *et al.*, 1998a). Studies have suggested that SHP-2 acts as both a negative and a positive regulator of STAT signalling in a cytokine receptor-specific manner (Kim & Baumann1999; You *et al.*, 1999).

One study suggested that SHP-2 is a negative regulator of leptin-induced STAT3-mediated transcription (Carpenter *et al.*, 1998). This conclusion was based on indirect evidence showing that the Tyr^{985} mutant of OB-Rb does not

bind SHP-2 and that this receptor exhibits increased STAT-mediated gene transcription following 24 hours of leptin treatment. However, several reports have shown that mutation of Tyr^{985} on OB-Rb, which results in loss of SHP-2 binding, does not affect STAT3 tyrosine phosphorylation (Banks *et al.*, 2000; Carpenter *et al.*, 1998; Li & Friedman1999). Moreover, Bjorbaek *et al.* found dominant negative SHP-2 had no effect on leptin-induced STAT3 tyrosine phosphorylation (Bjorbaek *et al.*, 2001). Recently, it was demonstrated that SOCS-3 binds to Tyr^{985} and that SOCS-3 requires this residue to mediate inhibition of OB-Rb signalling (Banks *et al.*, 2000). These studies suggest that signalling effects that have previously been attributed to SHP-2, following mutagenesis of tyrosine docking sites, may be due to the inability of SOCS-3 to bind to OB-Rb and inhibit STAT signalling. Bjorbaek *et al.* have demonstrated functional competition between SHP-2 and SOCS-3 for Tyr^{985} of OB-Rb suggesting SHP-2 is an indirect positive regulator of STAT signalling, acting by preventing SOCS-3 from inhibiting leptin signalling via Tyr^{985} (Bjorbaek *et al.*, 2001). The authors further speculate that SOCS-3 can inhibit MAPK signalling by two mechanisms; one involving prevention of SHP-2 binding to Tyr^{985} and the other by inhibition of JAK activity after binding to Tyr^{985} (Bjorbaek *et al.*, 2001). Therefore, SHP-2 is a positive regulator of MAPK activation and may also act as an indirect positive mediator of STAT signalling, by inhibition of SOCS-3 action via competition for Tyr^{985}.

1.3.5 Cross-talk with other signalling pathways

A number of *in vitro* and *in vivo* studies support the existence of cross-talk between the leptin and insulin signalling pathways. Briefly, insulin binds to its receptor and activates the intrinsic protein tyrosine kinase leading to autophosphorylation of the receptor on multiple tyrosine residues (Taha & Klip, 1999). This enables the receptor to bind to and phosphorylate the IRS proteins, which can recruit PI 3-kinase amongst other enzymes and adapter proteins. The downstream effects of these enzymes eventually mediate the effects of insulin on metabolism and gene expression. Therefore, the leptin and insulin signalling pathways may cross-talk through PI 3-kinase activated through JAKs for OB-Rb and through IRS for insulin.

The cross-talk between the leptin and insulin signalling pathways is supported by the observation that the intravenous infusion of leptin in mice increases glucose turnover and stimulates glucose uptake in skeletal muscle and BAT (Kamohara *et al.*, 1997). Furthermore, in rats, intraperitoneal leptin enhances the inhibitory effect on hepatic glucose output, while antagonising insulin action on the gene expression of glucokinase and phosphoenolpyruvate carboxylase (PEPCK) (Liu *et al.*, 1998; Rossetti *et al.*, 1997).

In HepG2 human hepatoma cells, leptin antagonises insulin down-regulation of PEPCK expression and decreases insulin-stimulated tyrosine phosphorylation of IRS-1 but enhances IRS-1-associated PI 3-kinase activity (Cohen *et al.*, 1996b). In C_2C_{12} cells, leptin stimulates a non-IRS-1-associated PI 3-kinase and mimics insulin action on glucose transport and glycogen

synthesis (Berti *et al.*, 1997). Insulin occludes leptin activation of K_{ATP} channels in the CRI-G1 insulinoma cell line(Harvey & Ashford, 1998). However, there is also a study that reports leptin does not play a role in insulin action. The leptin treatment of OB-Rb-transfected HepG2 cells resulted in the recruitment of p85 to IRS-2 but did not modulate the response to insulin (Wang *et al.*, 1997a). These studies point toward cell- and tissue-specific interactions between leptin and insulin signalling.

1.4 Leptin function

Leptin was first described as a hormone of energy balance, however it soon became evident following identification of OB-Rb expression in many peripheral sites that its effects were more extensive. For example, leptin has important actions on the reproductive system, restoring the fertility of female *ob/ob* mice (Chehab *et al.*, 1996) and advancing the time of first estrus in normal mice (Chehab *et al.*, 1996). The effects of leptin on the reproductive axis have led to a reappraisal of its overall role, suggesting that the hormone may not solely provide a precise homeostatic control for energy balance, but rather that it acts primarily to maintain energy supplies for both reproduction and the normal care and maintenance functions of the animal (Ahima *et al.*, 1996).

In the pancreas, leptin inhibits insulin gene transcription and insulin gene expression (Pallett *et al.*, 1997), causes naturesis in the kidney (Jackson & Li, 1997) and stimulates sugar transport in the intestine (Lostao *et al.*, 1998).

Some of the metabolic functions of leptin are mediated by the opening of the membrane ATP-sensitive potassium channel found in many tissues (e.g. pancreas (Harvey *et al.*, 1997), hypothalamic neurons (Spanswick *et al.*, 1997), intestinal mucosa (Liu *et al.*, 1999)). It has also been postulated that leptin's primary function is to prevent triglyceride overload by stimulating fatty acid metabolism and up-regulating enzymes of fatty acid oxidation (Shimabukuro *et al.*, 1998a; Unger *et al.*, 1999).

Amongst other effects, leptin has also been implicated in activating the immune system (Lord *et al.*, 1998), haematopoiesis (Gainsford *et al.*, 1996) and angiogenesis (Bouloumie *et al.*, 1998; Sierra-Honigmann *et al.*, 1998). Leptin may also play a role in the growth and development of the brain (Steppan & Swick, 1999) and fetus (Hoggard *et al.*, 1997a).

1.4.1 What we know from human mutations

There are several animal models of monogenic obesity that involve mutations in leptin or its receptor. The obese phenotype of *ob/ob* mice results from single mutations in either the coding region or the 5' non-coding end of the leptin gene (Zhang *et al.*, 1994). The obese phenotype of *db/db* mice, *fa/fa* Zucker and fa^k/fa^k Koletsky rats is due to mutations in the leptin receptor gene (Chen *et al.*, 1996; Chua *et al.*, 1996a; Lee *et al.*, 1996; Takaya *et al.*, 1996). However, human obesity is clearly a multifactorial and multigenic disease and, until very recently, there was no evidence to implicate mutations of leptin or its receptors as causative agents. However, very rare forms of human obesity have

now been identified where mutations in leptin or its receptors play a major role in the development of the disease.

Two independent groups were the first to publish reports of mutations in the leptin genes of obese patients expressing very low or undetectable leptin in their serum. Sequencing of the leptin genes in these patients revealed, in the case of two siblings of Pakistani origin, a deletion of a guanidine at codon 133 corresponding to residue 112 (Montague *et al.*, 1997). This resulted in the appearance of a stop codon at position 145 and thus the synthesis of a truncated protein of 121 residues. In a Turkish obese patient with a very low serum leptin level, sequencing of the leptin gene revealed a missense mutation in codon 105 (C→T), the same codon that is mutated in *ob/ob* mice (Strobel *et al.*, 1998). In mice this mutation creates a stop codon, however, in this patient the mutation leads to the substitution of an Arg for a Trp at position 84 of the mature protein. A protein of normal size is synthesised but is not secreted in serum. Two other morbidly obese members of the same Turkish family are both homozygous for the mutation and also lack immunoreactive leptin in their serum, but heterozygotes have normal leptin levels and are not obese (Strobel *et al.*, 1998).

Support for the role of leptin in the control of appetite, body weight and sexual development in humans was provided by the study of the Pakistani leptin-deficient children treated with recombinant leptin (Farooqi *et al.*, 1999). Leptin treatment of the elder sibling resulted in reduced food intake and body weight, increased physical activity and the commencement of pubertal changes at an appropriate age.

A systematic search for mutations in the human leptin receptor gene was launched by Gotoda *et al.*, with a sensitive RT-PCR procedure using lymphocyte RNA (Gotoda *et al.*, 1997). None of the five single-point polymorphisms found in the human leptin receptor gene were found to be associated with obesity. Clement *et al.* recently reported the discovery of a G→A base substitution in the splice donor site of exon 16 in the receptor gene of an obese North African patient (Clement *et al.*, 1998). This mutation resulted in the synthesis of a truncated receptor lacking both the transmembrane and the intracellular domains, similar to the OB-Re splice variant. The patient had serum leptin concentrations six-to-ten-fold higher than expected for obese patients with a similar body fat mass. Heterozygotes in the family are also overweight or obese, and the only homozygotes found so far are sisters, the mutation in the leptin receptor gene is the most likely cause of the obesity in this family.

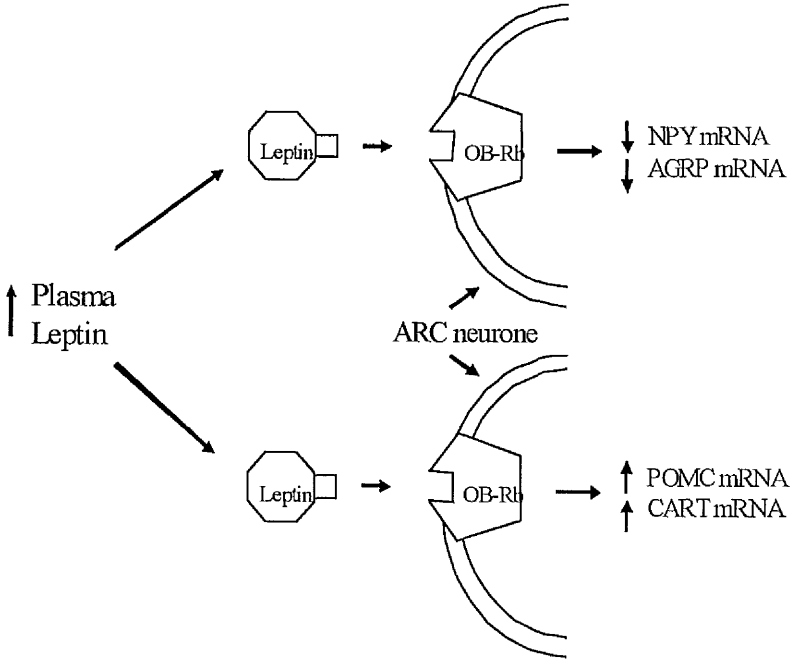
1.4.2 Central leptin function

1.4.2.1 OB-Rb function

Leptin is secreted primarily from WAT and stimulates OB-Rb receptors in the hypothalamus to mediate effects on food intake and energy expenditure. Moreover, leptin interacts with a number of orexigenic and anorexigenic peptides that are involved in maintaining energy balance. The orexigenic peptides favour food intake, reduce energy expenditure and facilitate fat storage, whereas the anorexigenic peptides reduce food intake, increase energy

expenditure, and facilitate the loss of fat stores. Leptin inhibits many of the orexigenic neuropeptides (e.g. neuropeptide (NPY), orexins, agouti-related protein (AGRP)) and favours many of the anorexigenic ones (e.g. proopiomelanocortin (POMC), corticotropin-releasing hormone (CRH), cocaine- and amphetamine-regulated transcript (CART)).

Figure 1.3 Leptin effects on orexigenic and anorexigenic peptides in the hypothalamus



OB-Rb receptors are found in a variety of hypothalamic nuclei including the arcuate nucleus (ARC) and paraventricular nucleus (PVN) (Elmquist *et al.*, 1998; Hakansson *et al.*, 1998; Mercer *et al.*, 1996). Leptin was shown to inhibit expression of hypothalamic AGRP mRNA in mice (Mizuno & Mobbs, 1999)

and hypothalamic NPY mRNA in rats (Schwartz *et al.*, 1996a). Furthermore, AGRP and NPY are coexpressed in the same neurons (Hahn *et al.*, 1998). Leptin increases expression of the anorexic peptides POMC (Schwartz *et al.*, 1997), CART genes (Kristensen *et al.*, 1998) and CRH (Uehara *et al.*, 1998). Similar to the orexigenic neuropeptides AGRP and NPY, the anorexigenic peptides POMC and CART are colocalised in the same neurons (Elmquist *et al.*, 1999). Leptin thus modulates activity in the neurons of the arcuate nucleus to release a range of orexigenic and anorexigenic neuropeptides.

Although leptin activates STAT1, 3, and 5 through OB-Rb in *in vitro* systems, intravenous leptin activates only STAT3 in the mouse hypothalamus (Ghilardi *et al.*, 1996; Vaisse *et al.*, 1996). STAT3 protein is expressed in NPY and POMC neurons in the arcuate hypothalamic nucleus, consistent with the concept that leptin regulates the transcription of these genes at least in part through the JAK-STAT signal transduction pathway (Hakansson & Meister, 1998). Leptin also regulates the expression of other STAT3 target genes in hypothalamus, including c-*fos* and SOCS-3 (Bjorbaek *et al.*, 1998b; Elmquist *et al.*, 1997).

Leptin inhibits glucose-responsive neurons in the hypothalamus through effects on ATP-sensitive potassium channels (Kieffer *et al.*, 1997; Spanswick *et al.*, 1997). Furthermore, leptin depolarises paraventricular hypothalamic neurons (Powis *et al.*, 1998) and regulates vagal afferents in the stomach (Wang *et al.*, 1997b). The mechanisms underlying these rapid electrophysiological actions of leptin are not known but are not likely to involve STAT-mediated

transcription and may involve leptin-stimulated PI 3-kinase activation and cross-talk with the insulin signalling pathway (Kieffer *et al.*, 1997; Spanswick *et al.*, 1997).

1.4.2.2 Other OB-R isoforms

The distribution of mRNA encoding OB-R isoforms was examined by *in situ* hybridisation using isoform-specific probes. A differential expression pattern of leptin receptors was observed in several brain areas (Guan *et al.*, 1997). The OB-Ra and OB-Rb isoforms, but not OB-Rc and OB-Rf, are abundantly expressed in the hypothalamus, whereas OB-Ra, OB-Rc and OB-Rf, but not OB-Rb, are significantly expressed in the choroid plexus. In a further study, the OB-Ra isoform was demonstrated to be widely expressed in the brain and high levels were detected in the choroid plexus epithelium and the ependymal lining (Burguera *et al.*, 2000).

The OB-Ra isoform has been hypothesised to be involved in leptin transport, a proposal supported by a number of observations. Firstly, leptin enters the rat brain by a saturable transport mechanism (Maness *et al.*, 1998), possibly by receptor-mediated transcytosis across the blood-brain barrier, as is the case for some other large proteins (Pardridge, 1986). Secondly, brain microvessels express high levels of OB-Ra, which is increased in rats fed a high fat diet (Boado *et al.*, 1998), and are capable of binding and internalising leptin (Bjorbaek *et al.*, 1998a; Golden *et al.*, 1997). Furthermore, the Koletsky strain

of rats, which lack the OB-Ra isoform (Takaya *et al.*, 1996), show reduced transport of leptin across the blood-brain barrier (BBB) (Kastin *et al.*, 1999).

The blood-cerebrospinal fluid (CSF) barrier at the choroid plexus and the BBB at the cerebral endothelium are two major controlling sites for the entry of proteins into the brain (Zlokovic *et al.*, 2000). Bjoerbaek *et al.* have suggested the transport of leptin into the CSF via the choroid plexus is not the major transport route by which leptin reaches the hypothalamus and that JAK-STAT signalling is unlikely to occur given the low concentrations of leptin in the CSF (Bjorbaek *et al.*, 1998a). On the other hand, Zlokovic *et al.* have recent evidence by in situ brain perfusion that leptin entry into the CSF via the choroid plexus and hypothalamus occurs by a fast transport system in contrast to the slower uptake at the BBB (Zlokovic *et al.*, 2000).

The OB-Ra isoform may play a role other than leptin transport in the brain. The co-expression of OB-Ra and OB-Rb mRNA in the hypothalamus suggests that there may be a possible interaction between the two isoforms (Guan *et al.*, 1997). Although the OB-Ra isoform cannot signal via activation of STATs, it may signal via the activation of the MAPK pathway or PI 3-kinase although the signalling capacity of OB-Ra *in vivo* is not well defined (see sections 1.3.4.3 and 1.3.4.4). Furthermore, leptin hyperpolarises glucose-receptive hypothalamic neurons of lean Sprague-Dawley and Zucker rats by activation of K_{ATP} channels (Spanswick *et al.*, 1997). The authors suggest that the K_{ATP} channel may function as the molecular end-point of the pathway following leptin activation of the OB-Rb receptor in hypothalamic neurons. In

the β-cells of the pancreas, leptin reduces insulin secretion by the activation of K_{ATP} channels which may involve leptin-stimulated PI 3-kinase activation (Harvey *et al.*, 2000b). Similarly, activation of K_{ATP} channels in the hypothalamic neurons may involve PI 3-kinase stimulated via OB-Ra or OB-Rb.

1.4.3 Peripheral leptin function

Initially, leptin was only believed to signal via the OB-Rb isoform in the hypothalamus to reduce food intake and increase energy expenditure. However, the increasing number of reports of OB-Rb mRNA expression in a number of peripheral tissues suggested leptin may have an important role in these tissues. The expression of the signalling competent OB-Rb isoform was observed in the pituitary, pancreatic β-cells, adipose tissue, small intestine, skeletal muscle, lungs, kidneys (although mainly Ob-Ra), adrenal medulla, placenta, ovaries, and testes amongst others (Hoggard *et al.*, 1997b; Karlsson *et al.*, 1997; Kieffer *et al.*, 1996; Kutoh *et al.*, 1997; Liu *et al.*, 1997; Morton *et al.*, 1998; Sharma & Considine1998; Zamorano *et al.*, 1997). Details of leptin function in the peripheral tissues analysed are presented in chapters 3 and 4 and summarised below.

1.4.3.1 Pituitary

The expression of leptin and the Ob-Rb isoform has been identified in human, mouse and rat pituitaries by RT-PCR and *in situ* hybridisation (Jin *et al.*,

1999; Jin *et al.*, 2000), indicating that leptin has the potential to regulate pituitary function via endocrine, paracrine and/or autocrine mechanisms. Leptin has been shown to control gonadotropin secretion in the anterior pituitary (Yu *et al.*, 1997a). Furthermore, it has been shown that high concentrations of leptin (10^{-8}-10^{-6} M) inhibit cell proliferation in human and rat pituitary cell lines *in vitro* (Jin *et al.*, 2000). Consequently, leptin might directly and positively influence the function of the gonadotropes and be involved in the regulation of the growth and differentiation of pituitary cells.

1.4.3.2 Pancreas

The expression of OB-Rb has been identified in the β-cells of the pancreas, where leptin reduces insulin secretion by the opening of K_{ATP} channels (Emilsson *et al.*, 1997; Kieffer *et al.*, 1997). The OB-Rb-mediated signalling events leading to effects on insulin secretion are believed to involve the activation of PI 3-kinase-dependent phosphodiesterase-3B (PDE3B) and subsequent suppression of cAMP levels (Zhao *et al.*, 1998). However, leptin may also regulate gene expression in pancreatic β-cells by the activation of STAT proteins. Leptin treatment of MIN6 cells produced an increase in the tyrosine phosphorylation of STAT3 (Tanabe *et al.*, 1997) and activated STAT3 in rat isolated islets and RINm5F cells (Morton *et al.*, 1999). Furthermore, the treatment of the rat insulinoma cell line BRIN-BD11 was recently shown to activate STAT3 and STAT5 (Briscoe *et al.*, 2001a).

1.4.3.3 Adipose Tissue

The observation that the functional form of the leptin receptor, OB-Rb, was expressed in adipose tissue suggests leptin may have a role in lipid metabolism (Kutoh *et al.*, 1997). Adipose tissue mass is determined by the rate of rival pathways for triglyceride synthesis, versus fatty acid oxidation. Leptin reduces lipid synthesis and stimulates the enzymes necessary for β-oxidation (Wang *et al.*, 1999a). Furthermore, leptin prevents fatty acid synthesis by specifically inhibiting the fatty acid synthase gene (Fukuda *et al.*, 1999). Leptin is also known to exert its effects by increasing energy expenditure and it has been shown that leptin directly induces expression of uncoupling proteins (UCPs) in WAT and BAT (Scarpace *et al.*, 1998).

1.4.3.4 Small Intestine

The functional isoform of the leptin receptor, OB-Rb, was identified in the jejunum and in the ileum of lean and *ob/ob* mice by RT-PCR, suggesting leptin may be involved in nutrient absorption (Morton *et al.*, 1998). This was confirmed by the observation that leptin produced a rapid inhibitory effect on sugar absorption in rat intestinal rings (Lostao *et al.*, 1998). Furthermore, the intravenous administration of leptin resulted in a two-fold reduction in apolipoprotein of chylomicrons (APO-AIV) mRNA levels in the jejunum of *ob/ob* mice (Morton *et al.*, 1998), suggesting leptin may reduce the transport of triglycerides into plasma.

1.5 Leptin insensitivity

The majority of obese humans exhibit elevated plasma leptin levels in proportion to their body fat mass (Considine *et al.*, 1996; Schwartz *et al.*, 1996b) and leptin mRNA has been shown to be increased in obese humans relative to non-obese humans (Maffei *et al.*, 1995). As a result, it has been suggested that obese humans may be insensitive to the leptin signal. Mechanisms thought to underlie leptin insensitivity include dysregulation of leptin synthesis and/or secretion, defects in the transport of leptin in the brain, and abnormalities of leptin receptors and/or post-receptor signalling.

1.5.1 Leptin access to the hypothalamus

The reduced ratio of CSF/plasma leptin observed in obese humans (Caro *et al.*, 1996; Schwartz *et al.*, 1996b) and the ability to respond to central but not peripheral leptin administration in diet-induced and New Zealand obese mice (Halaas *et al.*, 1997; Lin *et al.*, 2000; Van Heek *et al.*, 1997), suggests the transport of leptin is not functioning optimally in obesity. There are two proposed mechanisms that could explain the reduced leptin transport in obesity. The leptin transporter could be saturated by the increased plasma leptin levels or the BBB could have a reduced capacity for leptin transport (Banks *et al.*, 1999). The injection of radioiodinated leptin into 12-month old obese CD-1 mice resulted in the reduced rate of leptin transport across the BBB compared to leans (Banks *et al.*, 1999). Moreover in a follow-up experiment using a perfusion method containing a buffer with low levels of radioactive leptin, the obese mice

still had lower rates of leptin transport compared to leans (Banks *et al.*, 1999). These findings suggest that the reduced BBB transport rate associated with high levels of leptin in obesity is not due to the saturation of transport, but the reduced capacity of the BBB to transport leptin. In support of this proposal, intravenous administration of radioiodinated leptin in *ob/ob* and *db/db* mice showed there was no significant difference in the rate of leptin transport compared to leans (Maness *et al.*, 2000). This suggests that the impaired leptin transport is not directly explained by the absence of leptin or OB-Rb, which as a result of their respective genes result in obesity in mice. Therefore, obesity may not always be related to a defect of the transport of leptin across the BBB and alternative factors may be involved in the transport of leptin, emphasising the dynamic regulatory role of the BBB in leptin transport.

1.5.2 Leptin receptor expression

Leptin insensitivity could also reside at the level of receptor expression or steps downstream from the initial step of receptor interaction. A number of studies have shown that leptin receptor expression is increased in the hypothalamus of the hypersensitive *ob/ob* mice compared to leans (Baskin *et al.*, 1998; Emilsson *et al.*, 1999; Huang *et al.*, 1997). However, there is no change in leptin receptor expression in the liver, small intestine or WAT of *ob/ob* mice compared to leans. A recent study showed no change in the expression of hypothalamic OB-Rb mRNA in diet-induced obese mice (El

Haschimi *et al.*, 2000). Therefore, changes in the leptin receptor expression may occur as a result of leptin sensitivity in a tissue-specific manner.

1.5.3 Post-receptor signalling

Leptin sensitivity may also involve a number of components downstream of the receptor signalling pathway. In diet-induced obese mice, there was no change in the expression of hypothalamic STAT3 mRNA, however reduced STAT3 activation was observed by electrophoretic mobility shift assays (EMSA) in the hypothalamus (El Haschimi *et al.*, 2000). A recent study that examined the expression of STAT3 mRNA in the hypothalamus of *ob/ob* mice compared to leans showed that STAT3 mRNA was reduced. In contrast, Emilsson *et al.*, observed no change in STAT3 or STAT5 mRNA expression in the hypothalamus, liver, small intestine or WAT of *ob/ob* mice compared to leans (Emilsson *et al.*, 1999).

The increased expression of SOCS-3 mRNA after leptin treatment of lean and *ob/ob* mice has suggested SOCS-3 may be a potential mediator of leptin insensitivity (Bjorbaek *et al.*, 1998b). The observation that the expression of SOCS-3 and CIS mRNA was increased in the hypothalamus of *ob/ob* mice compared to leans (Emilsson *et al.*, 1999) is inconsistent with their proposed roles as negative regulators of leptin-mediated signalling. The authors suggest that the increased expression of OB-R mRNA may counteract the effect of the increased expression of SOCS-3 and CIS mRNA in *ob/ob* mice. In diet-induced obese rodents, there was no change in the expression of SOCS-3 mRNA in the

hypothalamus (El Haschimi *et al.*, 2000) or in the WAT, BAT and liver (Peiser *et al.*, 2000). However, in a different study of dietary obese rats and acquired obesity caused by ventromedial hypothalamus lesioning, the expression of SOCS-3 mRNA was increased in the adipose tissue (Wang *et al.*, 2000). This suggests that effects on SOCS-3 expression may be tissue-specific.

1.6 Animal models used to study leptin function

1.6.1 Diet-induced obese mice

There are few examples of human monogenic disorders such as a mutation in leptin or its receptor, as observed in *ob/ob* and *db/db* mice (Clement *et al.*, 1998; Gotoda *et al.*, 1997; Montague *et al.*, 1997; Strobel *et al.*, 1998). Obesity in humans is the result of a complex interaction of many factors; two major influences being genetic predisposition and diet.

Leptin insensitivity has been described in diet-induced obese mice fed a high fat diet for 8 weeks, which closely reflects the human condition (Van Heek *et al.*, 1997; Widdowson *et al.*, 1997). A recent study describes the development of high fat diet-induced obesity in C57BL/6J mice (Lin *et al.*, 2000). After 8 weeks of feeding, C57Bl/6J mice were insensitive to the leptin effect on food intake but still retained central leptin sensitivity. However, after 19 weeks of feeding a high fat diet a reduction of central leptin sensitivity was observed. The high fat diet-induced obesity model is a more accurate depiction, compared to genetic disorders, of the human situation and therefore provides researchers with an invaluable tool to study obesity.

1.6.2 *ob/ob* mice

The leptin gene is one of the most extensively studied in obesity research, because a spontaneous mutation ($C^{428} \rightarrow T^{428}$) of this gene in mice prohibits secretion of mature leptin causing hyperphagia, hypothermia, hypercorticosteronaemia, hyperglycaemia, hypothyroidism, growth hormone deficiency, hyperinsulinaemia reduced energy expenditure, decreased linear growth and, infertility and early-onset morbid obesity (Friedman & Halaas, 1998; Zhang *et al.*, 1994). Leptin administration of *ob/ob* mice normalises all aspects of the obesity and diabetes syndrome and restores reproductive function (Campfield *et al.*, 1995; Halaas *et al.*, 1995; Pelleymounter *et al.*, 1995).

1.6.3 ZDF *fa/fa* rats

Obese Zucker rats have a mutation in the extracellular domain of the leptin receptor and exhibit morbid obesity, dislipidaemia, hyperphagia, hypothyroidism, reduced fertility and impaired glucose tolerance, as recessive traits (Unger, 1995; Unger & Orci, 2001). The *fa/fa* mutation observed in Zucker rats, an $A^{880} \rightarrow C^{880}$ nucleotide missense mutation resulting in $Gln^{269} \rightarrow Pro^{269}$ amino acid substitution, reduces the expression of the leptin receptor on the cell surface, with marked intracellular retention, decreased leptin binding and diminished signal transduction (Chua *et al.*, 1996b; Crouse *et al.*, 1998; Da Silva *et al.*, 1998; Yamashita *et al.*, 1997). ZDF rats were isolated from a colony of obese Zucker rats and become diabetic after about 8 weeks when presented with a high fat diet (Clark & Palmer, 1982).

1.7 Aims

The phenotype of *ob/ob* and *db/db* mice makes it clear that leptin plays a major role in obesity. Furthermore, it is now clear that leptin functions not only in the hypothalamus but also in peripheral tissues. Investigation into the signalling mechanism of the functional form of the leptin receptor, OB-Rb, has identified key components of the leptin receptor-mediated signalling pathway that might be involved in leptin sensitivity.

The aim of this thesis was to examine the relationship between changes in expression of genes involved in leptin receptor signalling and leptin sensitivity. Gene expression was analysed in central and peripheral tissues of leptin-insensitive dietary obese mice compared to leptin-hypersensitive *ob/ob* obese mice. To gain further insight into some of the mRNA changes in genes involved in leptin receptor-mediated signalling that might be involved in leptin sensitivity, expression was quantified in *ob/ob* mice treated with leptin for 24 hours and 2 weeks. Research into the factors that are involved in leptin sensitivity may help identify therapeutic targets for the treatment of obesity.

Chapter 2

Materials and Methods

2.1 Materials

Ambion, Austin, U.S.A.

Glyco Blue.

Anachem, Luton, England.

2M sodium acetate pH 4.2.

Crystal Chem, Chicago, I.L., U.S.A.

Leptin and insulin ELISA.

Harlan Olac Ltd, Bicester, England.

AKR/J and C57BL/6J mice and Zucker diabetic fatty (ZDF) rats.

Life Technologies, Paisley, Scotland.

TRIzol reagent, chloroform:isoamyl alcohol, DNase I amplification grade, RNase OUT (RNase inhibitor), Superscript II.

Linco Research Inc., St. Charles, Missouri, U.S.A.

Rat leptin RIA kit.

MJ Research Inc. Waltham, M.A., U.S.A.

Peltier Thermal Cycler (PTC-225) supplied by Genetic Research Instrumentation, Braintree, England.

Molecular Devices Ltd., Wokingham, England.

Thermo plus microplate reader.

Oncor Appligene, Parc d'Innovation, France.

AquaPhenol, ReadyRed.

Perkin-Elmer Applied Biosystems, Foster City, C.A., U.S.A.

2X Universal PCR Master Mix, TaqMan primers and probes, MicroAmp optical 96-well PCR plate, MicroAmp optical cap strips and ABI PRISM 7700 Sequence Detector.

Promega, Southampton, England.

Guanidine thiocyanate and mineral oil.

Roche Diagnostics Ltd., Lewes, England.

Unimate 5 Glucose HK and COBAS MIRA Plus.

Sigma-Aldrich Company Ltd, Dorset, England.

Isopropanol, sodium citrate, β-mercaptoethanol, 7.5M ammonium acetate and N-laurylsarcosine.

Stratagene, La Jolla, C.A., U.S.A.

Random 9-mers.

Wako Chemicals, Richmond, V.A., U.S.A.

NEFA C test kit.

2.2 Animals

This work was conducted in compliance with the Home Office Guidance on the operation of the Animals (Scientific Procedures) Act 1986, and was reviewed and approved by the SmithKline Beecham Procedures Review Panel.

2.2.1 Diet-induced obesity

Five-week old female AKR/J and C57BL/6J (lean and *ob/ob*) mice were housed in groups of 10 and maintained at 24 ± 2 °C on a 12 hour light/12 hour dark light cycle (lights on at 06:00hr), for at least 1 week before the experiment in order to acclimatise to the new research environment. Mice were provided with a standard laboratory diet and water *ad libitum* for the acclimatisation period. After the acclimatisation period, the mice were weighed and divided into groups of approximately equal mean body weight, in two cages of 4 and 6 mice.

AKR/J mice were fed either a normal chow diet or a palatable diet and C57Bl/6 lean and *ob/ob* mice were fed a standard laboratory diet for a period of

14 weeks. The palatable diet consisted of 33% powdered laboratory diet, 33% condensed milk (Nestle), 7% sucrose and 6% corn oil by body weight.

Body and food weights were measured using an analytical balance. Body weights were taken every week and food weights taken over a 24 hour period each week, for the duration of the 14-week study. The difference in food weight was assumed to represent the amount of food eaten per day, taking into account any spillage of food in the cages. Mice were sacrificed by increasing the CO_2 concentration in a gas chamber; then the tissues were removed and immediately frozen in liquid nitrogen and stored at -80°C.

2.2.2 Leptin treatment of *ob/ob* mice

Five-week old female C57BL/6J (lean and *ob/ob*) mice were maintained and acclimatised as described in 2.2.1, then weighed and divided into groups of approximately equal mean body weight and housed in cages of 2 mice in each. Mice were provided with a standard laboratory diet and water *ad libitum* for the duration of the study.

The *ob/ob* mice were injected intraperitoneally (i.p.) twice a day (08.00 and 17.00 hours) with 1 mg/kg recombinant murine leptin or saline vehicle for 2 days and 14 days. Body weights were measured daily, as was the food intake of the *ob/ob* mice (11.00 hours) undergoing the 14-day leptin infusion. The equivalent food intake to that of *ob/ob* mice over 24 hours was given to the pair-fed group to account for changes in food intake as a result of the leptin treatment. Mice were sacrificed and tissues removed as described in 2.2.1.

2.2.3 Zucker diabetic fatty rats

Five-week old female ZDF rats were obtained from Harlan Olac Ltd. The rats were maintained and acclimatised as described in 2.1.1, then weighed and divided into groups of approximately equal mean body weight in two cages of 4 mice each. Mice were provided with a standard laboratory diet and water *ad libitum* for the duration of the study. At the age of 24 weeks, the rats were sacrificed and the tissues removed as described in 2.2.1.

2.3 Assays

Following termination of the animal, truncal blood was collected for measurement of leptin and insulin by enzyme-linked immunosorbent assay (ELISA). As well as removing the tissues for RNA extraction, a small section of the pancreas was homogenized in 5 mls of acid-ethanol [ethanol/hydrochloric acid/water 50:1:10] in order to determine the pancreatic insulin content by ELISA.

2.3.1 Leptin and insulin ELISA

The major steps of the mouse leptin and insulin ELISA assays are described in Figure 2.1. All the solutions were supplied as part of a kit, the components of which are anonymous unless otherwise indicated.

(a) Serum leptin ELISA

Microplate wells, coated with a rabbit anti-mouse leptin antibody, were washed twice with 300 µl washing buffer and incubated overnight with 50 µl guinea pig anti-mouse serum, 45 µl sample diluent and 5 µl sample to be analysed (or standards 0-12.8 ng/ml). Each well was then washed five times with 300 µl washing buffer to remove any unbound material. 100 µl of horse radish peroxidase (POD)-conjugated anti-guinea pig IgG antibody was then added to each well for 3 hours at 4°C. Subsequently each well was washed seven times with 300 µl washing buffer to remove any unbound materials, before adding 100 µl of 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate solution to detect the bound POD conjugate. The plate was incubated in the dark for 30 minutes at room temperature before the enzyme reaction was stopped by the addition of 100 µl 1 N sulphuric acid. The absorbance was measured on a Thermo plus microplate reader plate reader (measuring wave length: 450 nm, subtracting wave length: 630 nm). The leptin concentration in each sample was determined from the standard curve.

(b) Serum insulin ELISA

The insulin ELISA assay was essentially the same as the leptin ELISA assay except that an anti-insulin antibody was used as the primary antibody, o-phenylenediamine (o-OPD) was used as the substrate solution, the standard curve was generated using 0-10 ng insulin/ml and there were 5 washes following addition of POD-conjugated antibody. The washing buffer comprises of phosphate buffered saline (PBS) powder and 20% Tween 20.

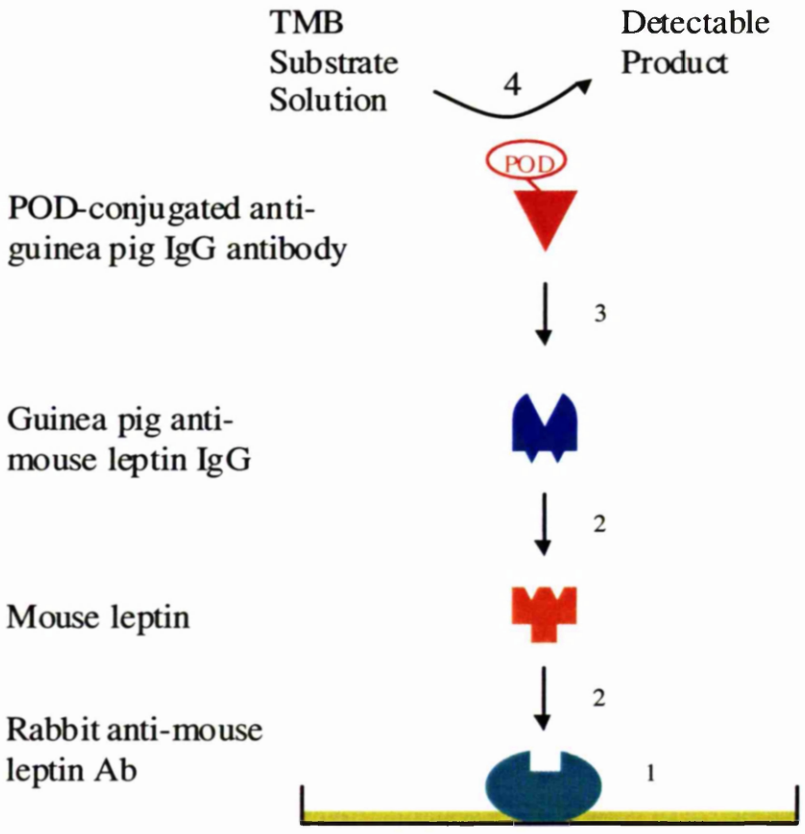
Figure 2.1 Mouse leptin and insulin ELISA assay

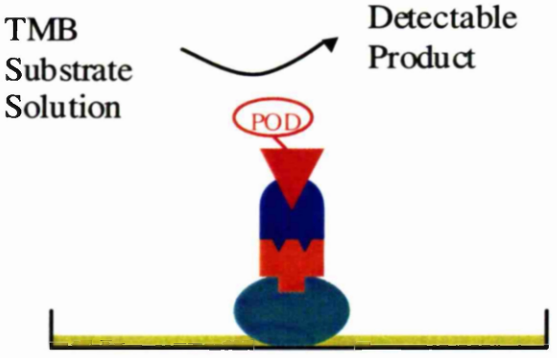
1. Microplate well coated with a rabbit anti-mouse leptin (or mouse anti-insulin) antibody.

2. Leptin (or insulin) in the sample simultaneously binds to the rabbit anti-mouse leptin (or mouse anti-insulin) antibody and the guinea pig anti-mouse leptin IgG (or guinea pig anti-insulin antibody).

3. POD-conjugated anti-guinea pig IgG antibody (no IgG in insulin assay) is binds to the guinea pig anti-mouse leptin IgG (or guinea pig anti-insulin antibody) complex immobilised on the microplate well.

4. TMB (or o-OPD) substrate solution is added and the absorbance measured (measuring wavelength: 450 nm, subtracting wavelength: 630 nm) to determine the mouse leptin concentration in the sample.


TMB
Substrate
Solution
4
Detectable
Product
POD
POD-conjugated anti-
guinea pig IgG antibody
3
Guinea pig anti-
mouse leptin IgG
2
Mouse leptin
2
Rabbit anti-mouse
leptin Ab
1


TMB
Substrate
Solution
Detectable
Product
POD

(c) Pancreatic insulin ELISA

Analysis of pancreatic insulin by ELISA was essentially as described, except that 50 µl of diluted sample (1:100 for AKR/J mice on the normal diet and C57Bl/6 lean mice and 1:1000 for AKR/J mice on the palatable diet and C57Bl/6 *ob/ob* mice) was added to 50 µl of the anti-insulin antibody, and the standards ranged from 0-2.5 ng/ml.

2.3.2 Rat leptin radioimmunoassay (RIA)

Rat leptin RIAs were performed in borosilicate glass tubes (12 X 75 mm) and the assay procedure is described in table 2.1. The assay buffer consisted of 0.05M phosphosaline, pH 7.4, containing 0.025M EDTA, 0.08% sodium azide, 0.05% Triton X-100 and 1% RIA grade BSA. After the addition of the precipitating reagent, vortex and incubation for 20 minutes at 4°C, all the tubes (except total count tubes) were centrifuged at 2000-3000 xg for 20 minutes at 4°C, where

$$xg = 1.11*10^{-5} (r) ((rpm)^2$$

and r = radial distance in cm (from axis of rotation to the bottom of the tube),

and r.p.m = rotation velocity of the rotor.

The supernatant was removed from all the tubes, except the total count tubes, by draining for 15-60 seconds. The tubes were counted in a gamma counter for 1 minute, and the concentration of rat leptin (ng/ml) was calculated in unknown samples from the standard curve using the gamma counters' processing data reduction capabilities.

Table 2.1 Rat leptin radioimmunoassay procedure flow chart

This table describes the RIA method as a flow chart beginning from left to right.

(Abbreviations: NSB = Non-Specific Binding; QC = Quality Control)

Tube Number	Sample	Buffer	Standard/QC Sample	Rat Leptin Antibody	Vortex, cover and incubate 20-24 hours, at RT	^{125}I-rat Leptin	Vortex, cover and incubate 20-24 hours, at RT	Precipitating Reagent	Centrifuge, decant, and count pellet. Vortex and incubate for 20 minutes at 4°C.
1,2	Total Count	-	-	-		100 µl		-	
3,4	NSB	300 µl	-	-				1.0 ml	
5,6	Reference (B_0)	200 µl	100 µl of 0.5 ng/ml	100 µl					
7,8	Standard	100 µl	100 µl of 1 ng/ml						
9,10			100 µl of 2 ng/ml						
11,12			100 µl of 5 ng/ml						
13,14			100 µl of 10 ng/ml						
15,16			100 µl of 20 ng/ml						
17,18			100 µl of 50 ng/ml						
19,20			100 µl of QC 1						
21,22	QC 1		100 µl of QC 2						
23,24	QC 2		100 µl of unknown						
25,26	Unknown		100 µl of unknown						
27-n	Unknown								

2.3.3 Non-esterified fatty acids (NEFA)

The NEFA C test kit utilises an *in vitro* enzymatic colorimetric method for the quantitation of NEFA. The enzymatic method relies upon the acylation of Coenzyme A (CoA) by the fatty acids in the presence of added acyl-CoA oxidase (ACOD) with concomitant generation of hydrogen peroxide. Hydrogen peroxide, in the presence of POD, permits the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple coloured adduct, which can be measured using a spectrophotometer at 550 nm.

50 µl of the sample serum, standards and deionised water was pipetted into the appropriate tubes (see Table 2.2). All the tubes were added with 1.0 ml of Colour Reagent A [3 U/10 ml acyl-coenzyme A synthetase (ACS), 30 U/10 ml ascorbate oxidase (AOD), 7 mg/10 ml CoA, 30 mg/10 ml adenosine triphosphate (ATP), 3 mg/10 ml 4-aminoantipyrine, 0.05 mol/l phosphate buffer pH 6.9, 3mmol/l magnesium chloride, surfactant and stabilisers], mixed by inversion and incubated for 10 minutes at 37°C. A further 2.0 ml of Colour Reagent B [132 U/10 ml ACOD, 150 U/10 ml POD, 1.2 mmol/l MEHA (3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline) and surfactant] was added to the tubes, which were then mixed and incubated for another 10 minutes at 37°C. The tubes were removed from the incubator and equilibrated to room temperature for 5 minutes. Finally, the optical density of all the tubes was read using a spectrophotometer at 550 nm versus the Reagent Blank (B).

Table 2.2 Procedure outline for the measurement of NEFA

The table below describes the steps for the quantitative determination of NEFA in serum using the ACS-ACOD method.

	Specimen	**Standard (STD)**	**Reagent Blank** (B)
Sample	Serum 50 µl	Standard Solution 50 µl	Deionised Water 50 µl
Colour Reagent A	1.0 ml	1.0 ml	1.0 ml
Mix well and incubate at 37°C for 10 minutes			
Colour Reagent B	2.0 ml	2.0 ml	2.0 ml
Mix well and incubate at 37°C for 10 minutes			
Spectrophotometer with filter of 550 nm	Measure the optical density of all tubes versus the Reagent Blank (B) at 550 nm		

2.3.4 Glucose tolerance test

Glucose (3 g/kg in a volume not exceeding 10 ml/kg) was injected into mice after taking an initial blood sample (0 minutes). Further blood samples were then taken after 45 minutes, 90 minutes and 135 minutes. Blood samples (10µl) were added to 0.5 ml of haemolysis reagent (50 mg/l digitonin, 100 mg/l maleimide). Quantitative determination of blood glucose concentration was performed using a Unimate 5 Glucose HK (hexokinase) *in vitro* diagnostic reagent system. The principle of the method is outlined in the following two reactions:

$$\text{D-Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{D-Glucose-6-P} + \text{ADP}$$

$$\text{D-Glucose-6-P} + \text{NAD}^+ \xrightarrow{\text{G6P-DH}} \text{D-gluconate-6-P} + \text{NADH} + \text{H}^+$$

The formation of NADH is directly related to the glucose concentration and is measured spectrophotometrically using the COBAS MIRA Plus analyser.

The area under the [blood glucose] versus time curve (glucose tolerance curve) was calculated trigonometrically using the trapezoid rule.

$$\text{Area Under Curve (AUC)} = (Glu_0+(2*Glu_{45})+(2*Glu_{90})+Glu_{135})*(t_i/2)$$

where $Glu_{0...135}$ is the blood concentration (mmol/l) at the various time points and t_i is the time (minutes) between blood sampling.

2.4 Extraction of total RNA

The TRIzol method of total RNA extraction was used for the hypothalamus, pituitary, WAT, BAT and small intestine. The guanidine thiocyanate method (Sparmann *et al.*, 1997) was used for total RNA extraction from the pancreas.

2.4.1 Extraction of total RNA using the TRIzol method

The tissue samples were homogenised in TRIzol reagent using 1 ml of TRIzol per 50 mg of tissue. 0.2 ml chloroform:isoamyl alcohol was added for each 1 ml TRIzol reagent used. The tubes were mixed by vortex for 10 seconds and left on ice for 5 minutes. Samples were then centrifuged at 14,000 rpm for 15 minutes at 4°C. The resultant upper aqueous phase was transferred to a tube containing 0.5 ml isopropanol for each 1 ml TRIzol reagent used. The tubes were mixed and incubated at -20°C for 1 hour to precipitate the RNA before being centrifuged at 14,000 rpm for 20 minutes at 4°C to pellet the RNA precipitate. The supernatant was removed and the RNA pellet washed twice by adding 1 ml 75% ethanol for each 1 ml TRIzol Reagent used, centrifuging at 14,000 rpm for 5 minutes at 4°C and removing the supernatant before repeating the procedure. The RNA pellet was air-dried and resuspended in DEPC water.

The quality of the RNA was tested by running 1 μl sample on a 1% agarose gel containing 0.5 μg/ml ethidium bromide (ETBr), which was visualised under U.V. light. An example of the quality of hypothalamic RNA from diet-induced obesity (DIO) mice extracted by the TRIzol method is shown

in figure 2.2(a). The RNA concentration was determined by measuring absorbance of 1:100 dilutions of samples at 280 and 260 nm using a SPECTRAmax Plus microplate spectrophotometer. Samples were stored at -80°C.

2.4.2 Extraction of pancreatic RNA using the Guanidine Thiocyanate method

The pancreas was removed and added to a guanidine buffer [10 ml 4 M guanidine thiocyanate, 25 mM sodium citrate pH 7 and 7 µl/ml β-mercaptoethanol] and chilled on ice. The tissue was homogenised using a large Ultra Turrax dispersing tool for 10-20 seconds, after which 250 µl 20% N-laurylsarcosine was added and the sample mixed by swirling. The lysate was left on ice for 5 minutes, frozen on cardice in 0.7 ml aliquots and stored at -80°C.

When samples were ready to be processed, the lysate was thawed rapidly and shaken to dissolve the precipitated salt. All steps were performed at 0°C to +4°C to avoid RNA denaturation. 0.1 volume of 2 M sodium acetate pH 4.2, 1 volume of AquaPhenol and 0.3 volume of Ready Red chloroform/isoamyl alcohol was added to the lysate, with 10 seconds of vigorous shaking after each addition. Samples were placed on ice for 10 minutes, then centrifuged for 30 minutes at 14,000 rpm. Approximately 75% of the resultant aqueous phase was removed and 0.1 volume sodium acetate, 1 volume AquaPhenol and 0.3 volume Ready Red was added and samples shaken and centrifuged as described above.

Following centrifugation, 50-75% of the aqueous phase was taken to a fresh sterile tube to which was added 0.075 volume 7.5 M ammonium acetate, 4 µl of 15 mg/ml Glyco Blue and 1 volume isopropanol. The samples were mixed and centrifuged for 30 minutes at 14,000 rpm to collect the RNA pellet. The RNA pellet was washed once with 1 ml cold 100% ethanol and twice with 1 ml cold 75% ethanol with samples centrifuged at 14,000 rpm for 5 minutes between each wash. The residual ethanol was removed by air-drying the RNA pellet for 5 minutes before resuspending the RNA in DEPC water.

The quality of the RNA was tested by running 1 µl sample in a 1% agarose gel containing 0.5 µg/ml ETBr, which was visualised under U.V. light. An example of the quality of pancreata RNA from DIO mice extracted by guanidine thiocyanate method is shown in figure 2.2(b). The RNA concentration was determined by measuring absorbance of 1:100 dilutions of samples at 280 and 260 nm using a SPECTRAmax Plus microplate spectrophotometer. Samples were stored at -80°C.

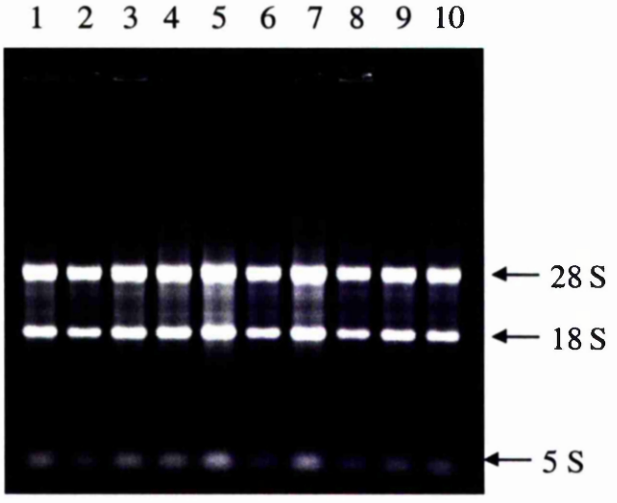
Figure 2.2 Agarose gel of RNA extracted from hypothalami and pancreata of DIO mice

1 μl of RNA was run on a 1% agarose gel containing 0.5 μg/ml ETBr at 100 V for 60 minutes

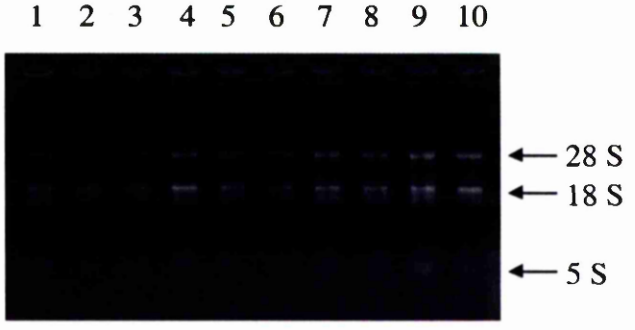
(a) RNA extracted by the TRIzol method from hypothalami of DIO mice

(b) RNA extracted by the guanidine thiocyanate method from pancreata of DIO mice

(a)



(b)



2.5 TaqMan RT-PCR

The cDNA-specific 5' nuclease assay for quantitatively detecting RT-PCR products uses a nonextendable oligonucleotide hybridisation probe. The probe is labelled with a reporter fluorescent dye, 6-carboxyfluorescein (FAM), at the 5' end and a quencher fluorescent dye, 6-carboxytetramethylrhodamine (TAMRA), at the 3' end. When the probe is intact, the reporter dye emission is quenched due to the physical proximity of the reporter and quencher fluorescent dyes. During the extension phase of the PCR cycle, the nucleolytic activity of the DNA polymerase cleaves the hybridisation probe and releases the reporter dye from the probe (see figure 2.3). The resulting relative increases in reporter fluorescent dye emission was monitored in real time during PCR amplification using the 7700 Sequence Detector (Heid *et al.*, 1996).

The sequence detector is a combination thermal cycler, laser, and detection software system that automates 5' nuclease-based detection and quantitation of nucleic acid sequences. A computer algorithm compares the amount of reporter dye emission (R) with the quenching dye emission (Q) during the PCR amplification, generating a ΔR_n value (R/Q). The ΔRn value reflects the amount of hybridised probe that has been degraded. The algorithm fits an exponential function to the mean ΔRn values of the last three data points of every PCR extension cycle, generating an amplification plot. A relative fluorescent emission threshold was set, based on the baseline of the ΔR_n during the first 10-15 cycles. The algorithm calculates the cycle at which each PCR

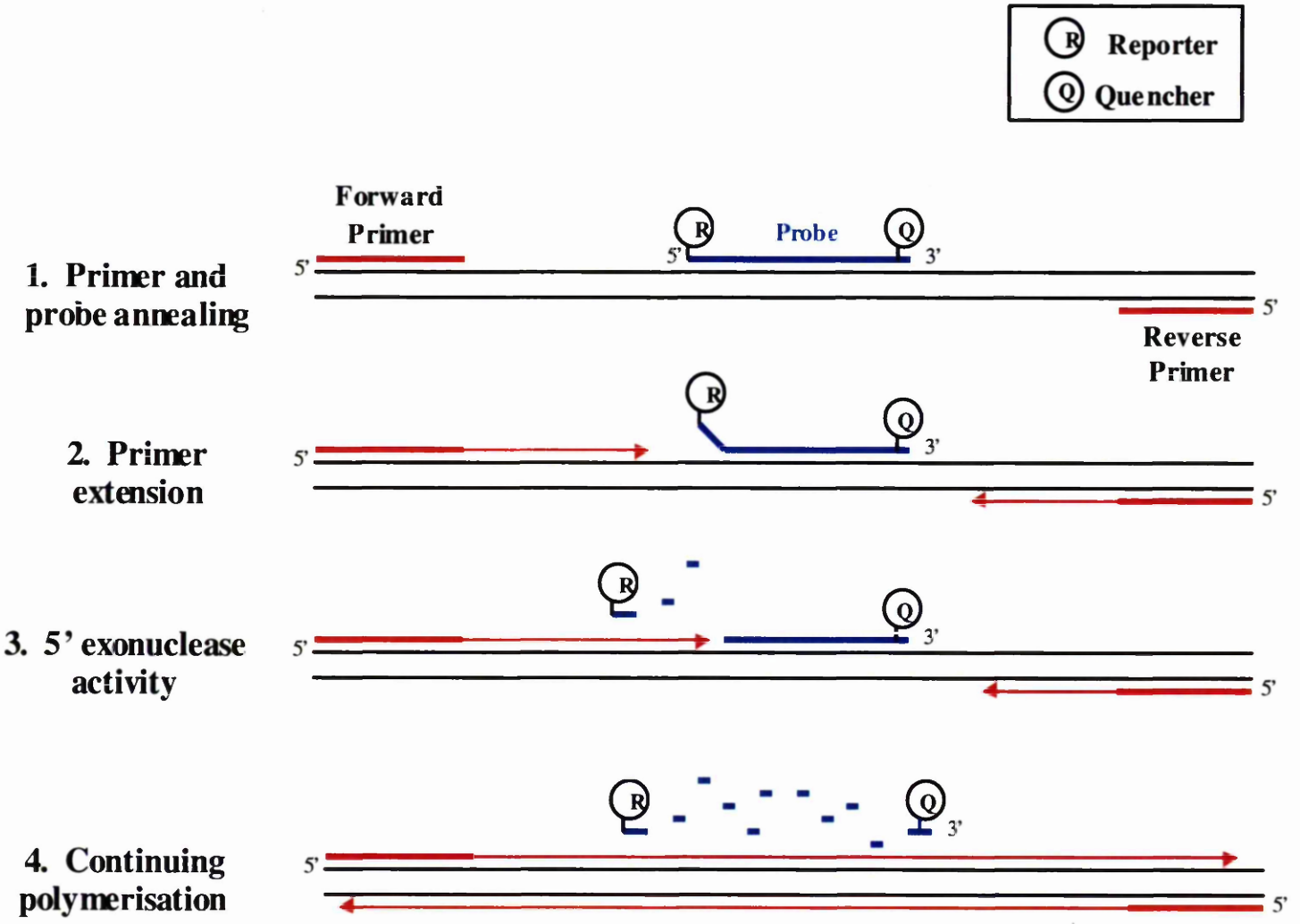
amplification reaches a significant threshold (usually 10 times the standard deviation of the baseline) (C_T) (Heid *et al.*, 1996). Serial dilutions of a known quantity of cDNA were used to construct a standard curve relating threshold cycle to quantity of template cDNA. This standard curve was used to determine relative amounts of amplified cDNA in samples.

2.5.1 DNase treatment

RNA (2 μg) was added to a tube containing 2 μl DNase I Amp Grade and 2 μl 10X DNase I Reaction Buffer and in a final volume of 20 μl. The tube was then incubated for exactly 15 minutes at room temperature. The DNase I was then inactivated by adding 2 μl of 25 mM EDTA solution and heating the tube for 10 minutes at 65°C. The total solution was divided into 2 sets of 10 μl each for reverse transcription (RT) and non-reverse transcription (non-RT) controls.

Figure 2.3 Diagrammatic representation of the steps of a typical TaqMan reaction

1. The primers and probe anneal to the cDNA transcript. There is no fluorescence because the reporter dye emission is quenched.
2. The primers are extended during the extension phase of the PCR cycle.
3. The 5'-3' exonuclease activity of the DNA polymerase cleaves the hybridised probe and releases the reporter dye emission resulting in an increase in reporter fluorescent dye emission.
4. The primers continue to be extended until polymerisation of the amplicon is complete


R Reporter
Q Quencher
Forward Primer
Probe
Reverse Primer
1. Primer and probe annealing
2. Primer extension
3. 5' exonuclease activity
4. Continuing polymerisation
5'
3'

2.5.2 cDNA synthesis

The 10 µl of total RNA solution was combined with 1 µl 500 ng/µl random 9 mers in a MicroAmp optical 96-well reaction plate and sealed with optical caps. The RNA was denatured by incubation for 15 minutes at 70°C on a Peltier Thermal Cycler (PTC-225), then immediately chilled on ice for 5 minutes before a brief centrifugation in a bench-top centrifuge. 9 µl of a reverse transcription master mix [4 µl 5X First Strand Buffer; 2 µl 0.1 M DTT; 1 µl of 40 U/µl RNaseOUT ribonuclease inhibitor; 1 µl 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH] and 1 µl 200 U/µl Superscript II reverse transcriptase or 1 µl DEPC-water for non-RT controls was then added to appropriate wells. The plate was sealed using optical caps, samples mixed by inversion and then centrifuged briefly. The microplate was subsequently incubated using the PTC-225 for 15 minutes at 25°C, then for 50 minutes at 42°C to reverse transcribe the RNA. The reaction was inactivated by heating for 15 minutes at 70°C. The microplate was briefly centrifuged and the cDNA volume was made up to 100 µl by the addition of 80 µl of sterile water.

2.5.3 TaqMan PCR

TaqMan PCR was carried out on the cDNA samples using an ABI Prism 7700 sequence detector in a 25 µl reaction consisting of TaqMan Universal PCR Master Mix [1X TaqMan buffer, 8% glycerol, 0.2 mM dATP, dCTP, dGTP and dUTP, 5.5 mM $MgCl_2$, 0.025 U/µl Amplitaq Gold DNA polymerase and 0.01

U/μl Amp Erase uracil-N-glycosylase], 300 nM of each primer, 100 nM TaqMan probe. Each sample was covered with 20 μl mineral oil. Cycle conditions were 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. Known quantities of mouse brain polyA cDNA was used for standard curves for all tissues except the pancreas, where mouse pancreas polyA cDNA was used in order to construct a standard curve relating C_T to template amount.

2.5.4 Design of TaqMan primers and probes for OB-Ra and OB-Rb

TaqMan primer and probe sequences for all genes (Table 2.3) were designed from sequences in the Genbank database using Primer Express software. A number of important guidelines were followed by this program in order to design suitable TaqMan assays:

- a difference of at least 10°C in the T_m of primers and probes
- the 5' end of the probe must not start with a guanosine (G) nucleotide
- the primers and probes must be approximately 50% guanosine, cytosine (GC) rich
- the ideal amplicon size is around 100 bp and should not exceed 150 bp.

Since the leptin receptor isoforms occur as a result of alternative splicing, the assay was designed such that the forward primer and probe are common to both OB-Ra and OB-Rb, but the reverse primers span the last intracellular splice site. Thus the RT-PCR is specific for OB-Ra or OB-Rb (Figure 2.4).

Transcript	Forward Primer (5'-3')	Reverse Primer (5'-3')	TaqMan Probe (5'-3')
OB-Ra	TGTTTTGGGACGATGTTCCA	GATACTTCAAAGAGTGTCCGCTCTC	CAAGAATTGTTCCTGGGCACAAGGACTTAATT
OB-Rb	TGTTTTGGGACGATGTTCCA	AAAGATGCTCAAATGTTTCAGGC	CAAGAATTGTTCCTGGGCACAAGGACTTAATT
STAT3	CCCCGCACTTTAGATTCATTG	CTCCGAGGTCAGATCCATGTC	ACTCAAACTGCCCTCCTGCTGAGGG
STAT5	AGAACCTCGTGTTCCTGGCA	TTCTCCCGGTTGAACTGGG	AGCAACCACCTCGAGGACTACAACAGCA
SOCS-3	GCACCTTTCTTATCCGCGAC	GGTTCTTGGTCCCCGACTG	CTCGGACCAGCGCCACTTCTTCAC
CIS	CTACAGAAGATGCCGGAGGG	TTGGTGGGGCCACGG	AGACAGCACCCACCCCAGCTACCTGT
NPY	ATACTACTCCGCTCTGCGACACTAC	TCTGTGCTTTCCTTCATTAGAGGTC	CTCATCACCAGACAGAGATATGGCAAGAGATCC
Insulin	ACCTTCAGACCTTGGCGTTG	CAGCACTGATCCACAATGCC	AGGTGGCCCGGCAGAAGCG
PDX-1	CGCGTCCAGCTCCCTTT	GCCCACTGGCCTTTCCA	TGGATGAAATCCACCAAAGCTCACGC
Glucokinase	CAGATCCTGGCAGAGTTCCAG	CGGTCCATCTCCTTCTGCAT	CAGGAGGAAGACCTGAAGAAGGTGATGAGC
GLUT2	CATCCCACAAGGCACCCTC	CATGCCACCCACAGAGAAGA	CTACGCTCTGGGCTCTCTCCGTGG
GAPDH	GAACATCATCCCTGCATCCA	CCAGTGAGCTTCCCGTTCA	CTTGCCCACAGCCTTGGCAGC
Cyclophilin	GATGAGAACTTCATCCTAAAGCATACA	TCAGTCTTGGCAGTGCAGATAAA	CCTGGCATCTTGTCCATGGCAAATG
36B4	GCTTCATTGTGGGAGCAGACA	TGCGCATCATGGTGTTCTTG	TCCAAGCAGATGCAGCAGATCCGC
HPRT	GGTGAAAAGGACCTCTCGAAGTG	ATAGTCAAGGGCATATCCAACAACA	CCAGACTTTGTTGGATTTGAAATTCCAGACAA

Figure 2.4 Illustration of OB-Ra and OB-Rb TaqMan assay design

(a) Alternative splicing of the leptin receptor.

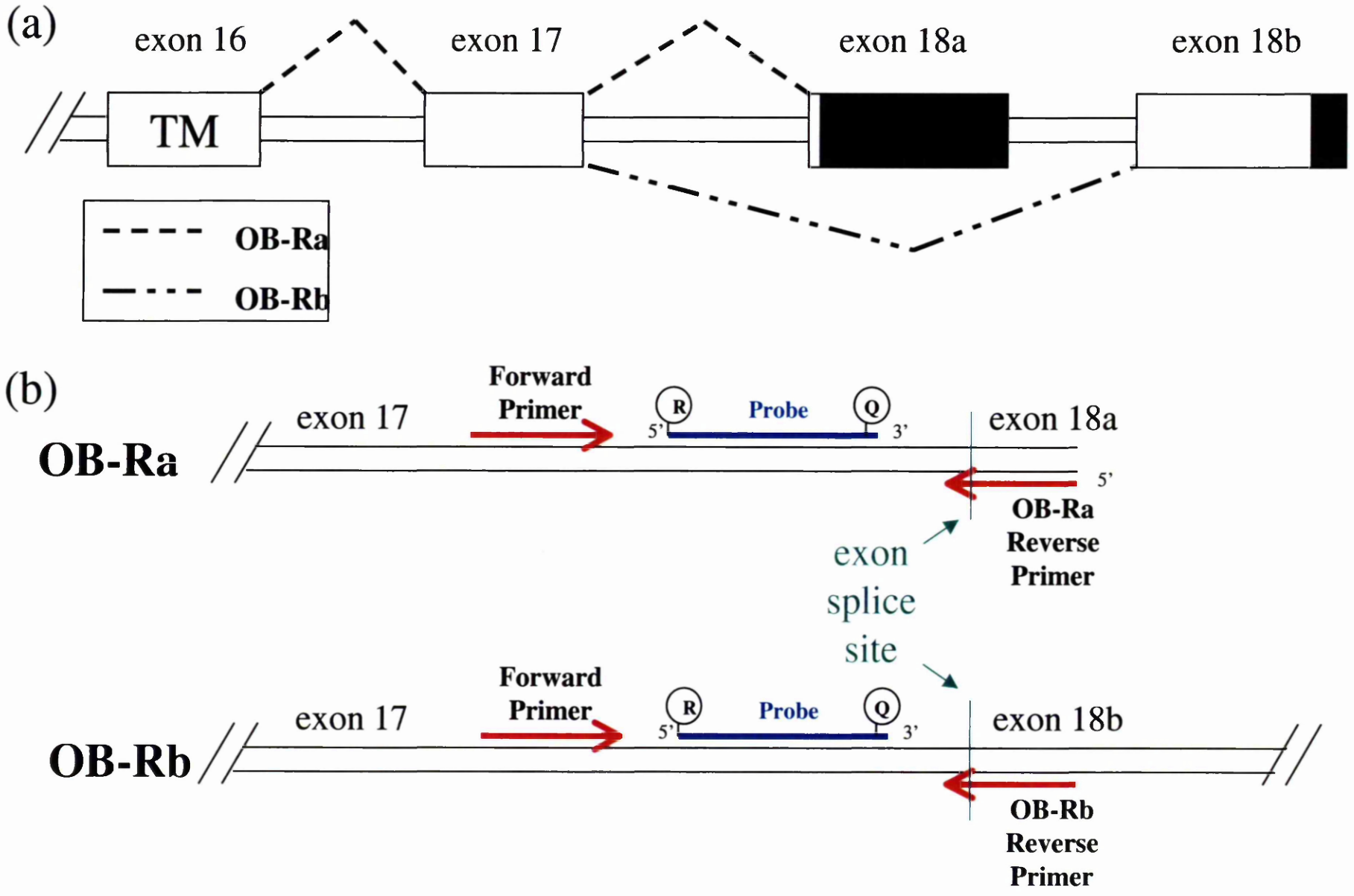
Exons are illustrated as boxes and introns by solid lines. The clear boxes correspond to translated exon sequences, and the dark boxes to 3'-untranslated sequences. Alternative splicing results in transcripts that encode either

OB-Ra (----------) or

OB-Rb (-·-·-·-·-·-).

(b) Design of OB-Ra and OB-Rb primers and probe

The forward primer and probe are common to both forms of the leptin receptor, but the reverse primer spans the exon splice site between the penultimate and the final exon, thus specifically amplifying either OB-Ra or OB-Rb.


(a)
exon 16
exon 17
exon 18a
exon 18b
TM
OB-Ra
OB-Rb
(b)
OB-Ra
exon 17
Forward Primer
R
Probe
Q
5'
3'
exon 18a
5'
OB-Ra Reverse Primer
exon splice site
OB-Rb
exon 17
Forward Primer
R
Probe
Q
5'
3'
exon 18b
OB-Rb Reverse Primer

2.5.5 Primer and probe optimisation

(a) Primer optimisation

The purpose of this procedure was to determine the minimum primer concentrations giving the maximum ΔR_n. To determine the optimal primer concentration, a PCR reaction mix was prepared such that at least three or four replicates of each of the nine conditions shown in Table 2.4 were performed.

The PCR reaction mix for primer optimisation (25 µl) consisted of 1X TaqMan Universal PCR Master Mix, 50-900 nM forward and reverse primer, 200 nM TaqMan probe and 25 ng of genomic DNA or mouse brain polyA cDNA (for OB-Ra and OB-Rb primer optimisation). The cycling conditions were as described in 2.5.3. At the end of the run, the results were tabulated for ΔR_n. The minimum forward and reverse primer concentrations that yielded the maximum ΔR_n were used for future experiments.

Table 2.4 Combinations of forward and reverse primer for primer optimisation

	Forward Primer (nM)		
Reverse Primer (nM)	**50**	**300**	**900**
50	50/50	300/50	900/50
300	50/300	300/300	900/300
900	50/900	300/900	900/900

(b) Probe optimisation

The purpose of this procedure was to determine the minimum probe concentrations that give the minimum C_T for each probe target. To determine the optimal probe concentration, a PCR reaction mix was prepared such that three or four replicates at 25 nM intervals from 25-225 nM were performed, using the optimal forward and reverse primer concentrations as determined in 2.5.5 in the reaction mix.

The PCR reaction mix for probe optimisation (25 µl) consisted of 1X TaqMan Universal PCR Master Mix, optimal forward and reverse primer concentrations, 25-225 nM TaqMan probe and 25 ng of genomic DNA or mouse brain polyA cDNA (for OB-Ra and OB-Rb primer optimisation). The cycling conditions were as described in 2.5.3. At the end of the run, the results were tabulated for C_T. The minimum forward and reverse primer concentrations that yielded the minimum C_T were used for further experiments.

(c) Optimal primer and probe concentrations

From the optimisation experiments described above, the primer and probe concentrations used in RT-PCR are listed in Table 2.5.

Table 2.5 Optimal primer and probe concentrations used for TaqMan analysis

Transcript	Forward Primer (nM)	Reverse Primer (nM)	TaqMan Probe (nM)
OB-Ra	900	50	200
OB-Rb	900	50	200
STAT3	300	300	100
STAT5	300	300	100
SOCS-3	300	300	100
CIS	300	300	100

2.6 Statistical analysis

Analysis of individual genes (both test and housekeeper) was performed by independent t-test on log transformed data (base 10). Differences between treatment means were calculated along with the standard error for the difference between means (sed). These were used in both the calculation of 95% confidence intervals (95%CI) for the difference and the t-statistic (difference/sed). The p-value for each t-statistic was calculated and the difference and 95%CI for the difference were back-transformed to the quantity scale to calculate fold-differences (the ratio of geometric means) and the 95%CI for the fold difference.

For each sample the ratio of test to housekeeper was calculated for every combination of test and housekeeping gene. Each ratio was analysed by independent t-tests. Differences between treatment means, 95%CI, t-statistic and p-value were calculated for each gene. The ratios were log transformed (base 10) and again submitted to an independent t-test. The same statistics were calculated as in the analysis of individual genes.

Analysis of the test genes was performed by an analysis of covariance (ANOCOVA) using, in turn, each one of the 4 housekeeping genes as a covariate. Post-hoc comparisons were performed using the t-test. The same statistics were calculated as in the analysis of individual genes. However, the sed was based on the residual mean square of the ANOCOVA and covariate efficiency factors for both the treatment and residual were calculated.

Chapter 3

Quantitative Expression of Genes Involved in the Leptin Receptor-Mediated STAT Signalling Pathway in the Hypothalamus and Pituitary of Diet-Induced and Genetically Obese Mice

3.1 Introduction

3.1.1 Hypothalamus

Most cases of human obesity are characterised by elevated serum leptin levels, leading to the suggestion that reduced sensitivity to endogenous leptin contributes towards the increase in body weight. Potential sites involved in central leptin insensitivity include the blood-brain-barrier transport system (Caro *et al.*, 1996; Schwartz *et al.*, 1996b), reductions in functional long-form leptin receptor expression (Martin *et al.*, 2000) and changes in expression of components of the leptin signalling mechanism in leptin-responsive neurons in the hypothalamus (Vaisse *et al.*, 1996).

Mice that become obese as a result of feeding on a high-fat diet become hyperleptinaemic and do not reduce their food intake or body weight in response to peripheral administration of leptin (Van Heek *et al.*, 1997; Widdowson *et al.*, 1997). However, these mice remain sensitive to central leptin administration suggesting a defective blood-brain-barrier transport system, which may therefore prevent leptin accessing the hypothalamus to affect food intake and body weight (Van Heek *et al.*, 1997; Widdowson *et al.*, 1997). The molecular mechanism for the apparent defect in central leptin access or transport involves the short OB-Ra isoform, which, as mentioned in Chapter 1, is highly expressed within the blood-brain barrier (Bjorbaek *et al.*, 1998a). A recent report by Maness *et al.* showed no change in leptin transport across the BBB in *ob/ob* (no leptin production) and *db/db* (high leptin levels, but no OB-Rb) mice compared to lean controls (Maness *et al.*, 2000). This suggests impaired leptin transport is

not a result of obesity where animals are leptin-sensitive or where elevated levels of circulating leptin occur.

However, it has been found that DIO mice have alterations in central leptin sensitivity. Mice fed a high-fat diet for 19 weeks, have reduced central leptin sensitivity, compared to those fed a high-fat diet for 8 weeks (Lin *et al.*, 2000). The mechanism causing this central leptin insensitivity is unclear, but it has been suggested that desensitisation of the hypothalamic leptin receptor or saturation of the receptors with high levels of endogenous leptin may have occurred (Lin *et al.*, 2000; Widdowson *et al.*, 1997). Leptin insensitivity could also occur through leptin receptor downregulation as observed by Martin *et al.* (Martin *et al.*, 2000), or disruption of leptin-mediated signal transduction.

In some cases of human obesity a mutation in the *ob* gene results in reduced levels of circulating leptin, hyperphagia and obesity. Due to the lack of circulating leptin, the *ob/ob* mice are hypersensitive to exogenous leptin and have significantly reduced body weight after the administration of exogenous leptin. These mice have been instrumental in the elucidation of the leptin-mediated signalling pathway. Peripheral leptin administration to normal and *ob/ob* mice rapidly induces hypothalamic leptin receptor-mediated signalling through the JAK/STAT pathway, primarily through STAT3 (Vaisse *et al.*, 1996). Negative regulation of STAT3 was found to occur in the hypothalamus through induction of SOCS-3 but not CIS mRNA after an hour of exogenous leptin treatment (Bjorbaek *et al.*, 1998b). However, an increase in both SOCS-3

and CIS mRNA in the hypothalamus was reported following peripheral leptin administration for 48 hours (Emilsson *et al.*, 1999).

3.1.2 Pituitary

There are various studies that implicate leptin in anterior pituitary function. Yu *et al.* first reported that leptin controlled gonadotropin secretion in the anterior pituitary (Yu *et al.*, 1997a) and stimulated nitric oxide release from the anterior pituitary (Yu *et al.*, 1997b). Zamorano *et al.* (Zamorano *et al.*, 1997) showed that the leptin receptor was expressed in the rat anterior pituitary by RT-PCR, whereas other investigators reported that growth hormone (GH) and/or growth hormone releasing hormone (GHRH) increased the leptin receptor gene expression in human GHRH transgenic mice (Cai & Hyde, 1998). Leptin deficiency in humans, due to a homozygous mutation in the human leptin receptor gene that results in a truncated leptin receptor lacking both the transmembrane and the intracellular domains, has been associated with early-onset obesity and pituitary dysfunction (Clement *et al.*, 1998). This observation emphasises the important role of leptin in pituitary function. Moreover, the recent localisation of leptin receptor isoforms in human, mouse and rat pituitaries by RT-PCR and *in situ* hybridisation suggest a functional role of leptin in the pituitary (Jin *et al.*, 1999; Jin *et al.*, 2000). Indeed, leptin inhibits the proliferation of human and rat anterior pituitary cell lines *in vitro*, which suggests leptin is important in the growth and differentiation of anterior

pituitary cells. Few studies have looked at gene expression changes of leptin receptor-mediated signalling components in the pituitary.

In summary, changes in central leptin sensitivity may be manifest at several levels, not only in the blood-brain transport system, but also at the receptor level or in further downstream pathways. In this study I have investigated the quantitative expression of genes involved in the leptin receptor-mediated STAT signalling pathway in different models of obesity using an RT-PCR technique involving a double-labelled fluorescent probe. I have compared the expression of genes in the hypothalamus and pituitary of palatable diet-fed AKR/J mice compared to chow-fed mice and also in the leptin-sensitive genetically obese C57BL/6 *ob/ob* mice relative to lean controls. In this way I hope to clarify how changes in central expression of the leptin receptor or downstream signalling components may alter with obesity and leptin sensitivity.

3.2 Results

3.2.1 Body weights

The body weights of the mice were monitored for the duration of the 14 weeks and are shown in Figure 3.1. The lean (+/?) mice in both groups showed a small, but steady, increase in body weights. However, a sharper increase in body weight was observed for the AKR/J mice fed a palatable diet and C57BL/6 *ob/ob* mice. At the end of the study, the palatable diet-fed AKR/J mice weighed 40.25 ± 3.42 g compared to 27.65 ± 1.02 g for the chow-fed AKR/J mice, and the *ob/ob* mice weighed 54.25 ± 0.96 g compared to 22.86 ± 0.48 g for lean controls.

3.2.2 Leptin, insulin and NEFA

Leptin, insulin and NEFA concentrations were determined from terminal blood samples of all the groups and are shown in Table 3.1. Leptin levels were significantly elevated by about 16-fold in palatable diet-fed AKR/J mice compared to AKR/J mice fed a normal chow diet ($P<0.05$). There was no detectable leptin in *ob/ob* mice. There was no significant difference in insulin levels in diet-induced obese mice compared to those on a normal diet, whereas the *ob/ob* mice had a 4.8-fold elevation in insulin compared to C57BL/6J mice ($P<0.05$). NEFA levels in AKR/J mice fed a palatable diet and *ob/ob* mice fed a chow diet were significantly increased by 31% and 30% compared to AKR/J mice and C57BL/6J +/? mice fed a chow diet, respectively ($P<0.01$).

Figure 3.1 Changes in body weight of AKR/J mice fed a palatable diet and C57BL/6 *ob/ob* mice

Effect of feeding a normal or palatable diet on the body weight of obesity-prone AKR/J mice and a normal diet on C57BL/6J lean and *ob/ob* mice for 14 weeks.

■ AKR/J mice fed a normal diet

□ AKR/J mice fed a palatable diet

● C57BL/6 lean mice fed a normal diet

○ C57BL/6 *ob/ob* mice fed a normal diet

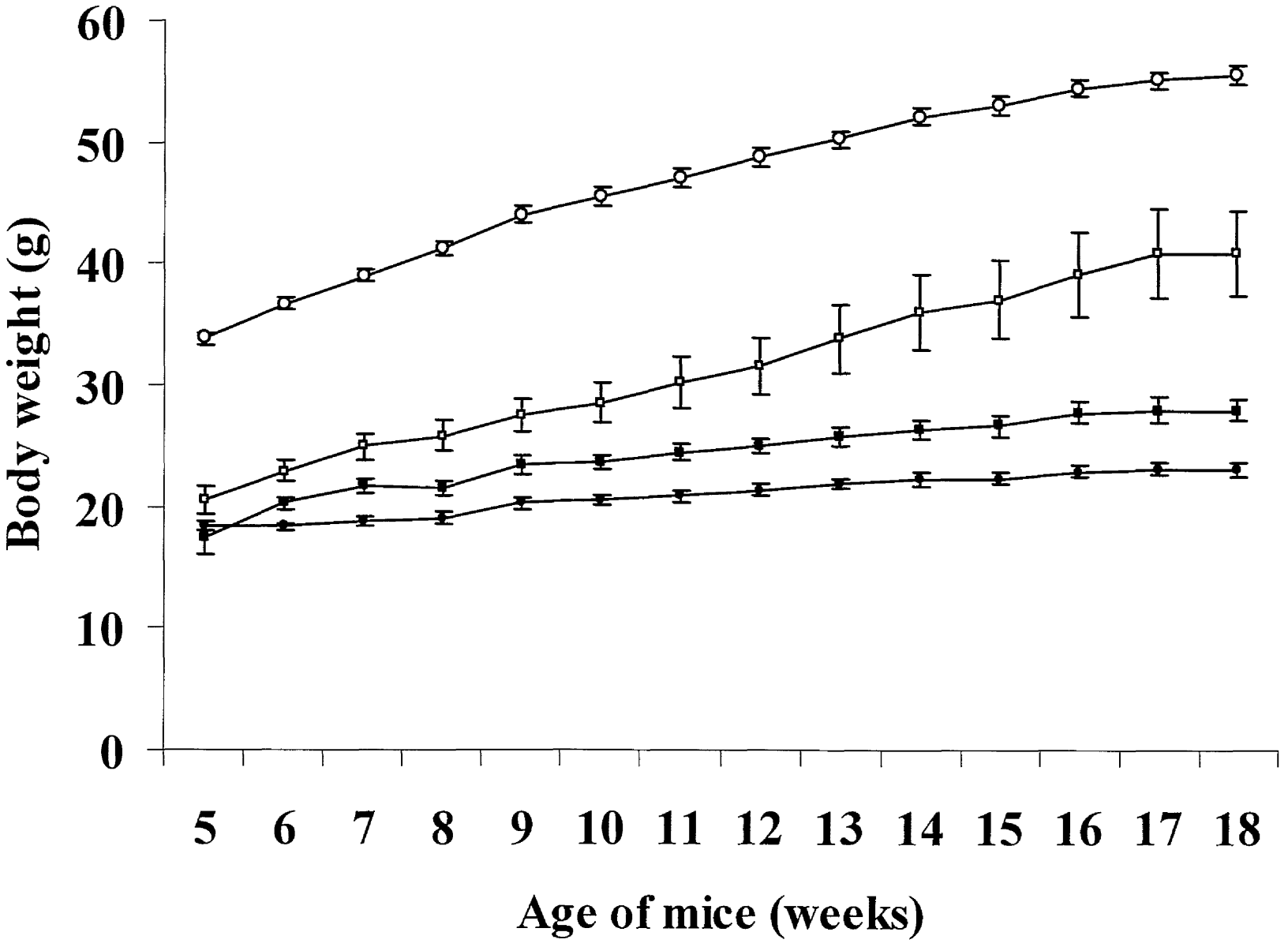

60
50
40
30
20
10
0
Body weight (g)
5
6
7
8
9
10
11
12
13
14
15
16
17
18
Age of mice (weeks)

Table 3.1 Leptin, insulin and NEFA levels in terminal blood samples of AKR/J mice fed either a chow or palatable diet, and C57BL/6 lean and *ob/ob* mice fed a chow diet for 14 weeks

Leptin, insulin and NEFA concentrations in terminal blood samples of AKR/J mice fed a chow or palatable diet for 14 weeks, and C57BL/6 lean and *ob/ob* mice fed a chow diet were measured by ELISA. Leptin was not detectable (ND) in *ob/ob* mice. Leptin, insulin and NEFA values are represented as mean ± S.E.M in ng/ml or mmol/l. * $P<0.05$ ** $P<0.01$

Group	Leptin (ng/ml)	Insulin (ng/ml)	NEFA (mmol/l)
AKR/J +/? chow diet	7 ± 1	1	0.09 ± 0.01
AKR/J +/? palatable diet	117 ± 49*	1	0.13 ± 0.01**
C57BL/6J +/? chow diet	3 ± 1	2	0.08 ± 0.01
C57BL/6J *ob/ob* chow diet	ND	12 ± 2*	0.11 ± 0.01**

3.2.3 Glucose tolerance test

In order to determine whether the animals had developed glucose intolerance, glucose tolerance tests were performed as described in section 2.3.4. The area under the curve for AKR/J mice fed a palatable diet was significantly increased by 14% compared to that of AKR/J mice on a normal chow diet indicating a small decrease in glucose tolerance ($P<0.05$). Figure 3.2 shows that the *ob/ob* mice were glucose intolerant, which is represented by the 36% increase in area under the hyperbolic curve compared to that of C57BL/6J +/? lean mice ($P<0.01$).

3.2.4 Changes in gene expression in the hypothalamus

TaqMan analysis of gene expression in the hypothalamus was adjusted for hypoxanthine phosphoribosyltransferase (HPRT) expression to reduce sample variability (see Appendix 1). The expression of OB-Ra and OB-Rb mRNA in AKR/J mice fed a palatable diet compared to chow and *ob/ob* compared to lean mice is shown in figure 3.3 (a). OB-Ra mRNA expression was significantly reduced by 40% ($P<0.05$) in AKR/J mice fed a palatable diet compared to chow-fed mice. There was no significant change in either OB-Ra or OB-Rb mRNA expression in the hypothalamus of *ob/ob* mice compared to lean control mice. However, comparison of the two lean mice strains showed that there was a 66% higher level in OB-Ra mRNA in the hypothalamus of AKR/J lean mice compared to C57BL/6 lean mice, fed on a chow diet for 14 weeks.

Figure 3.2 Glucose tolerance test

Blood was taken from the tail of mice at 45-minute intervals from 0 to 135 minutes after injection of glucose, in order to measure blood glucose concentrations.

(a) A plot of blood glucose concentrations at 0, 45, 90 and 135 minutes from the time of glucose injection, expressed as mean ± S.E.M. mmol/l.

■ AKR/J mice fed a normal diet

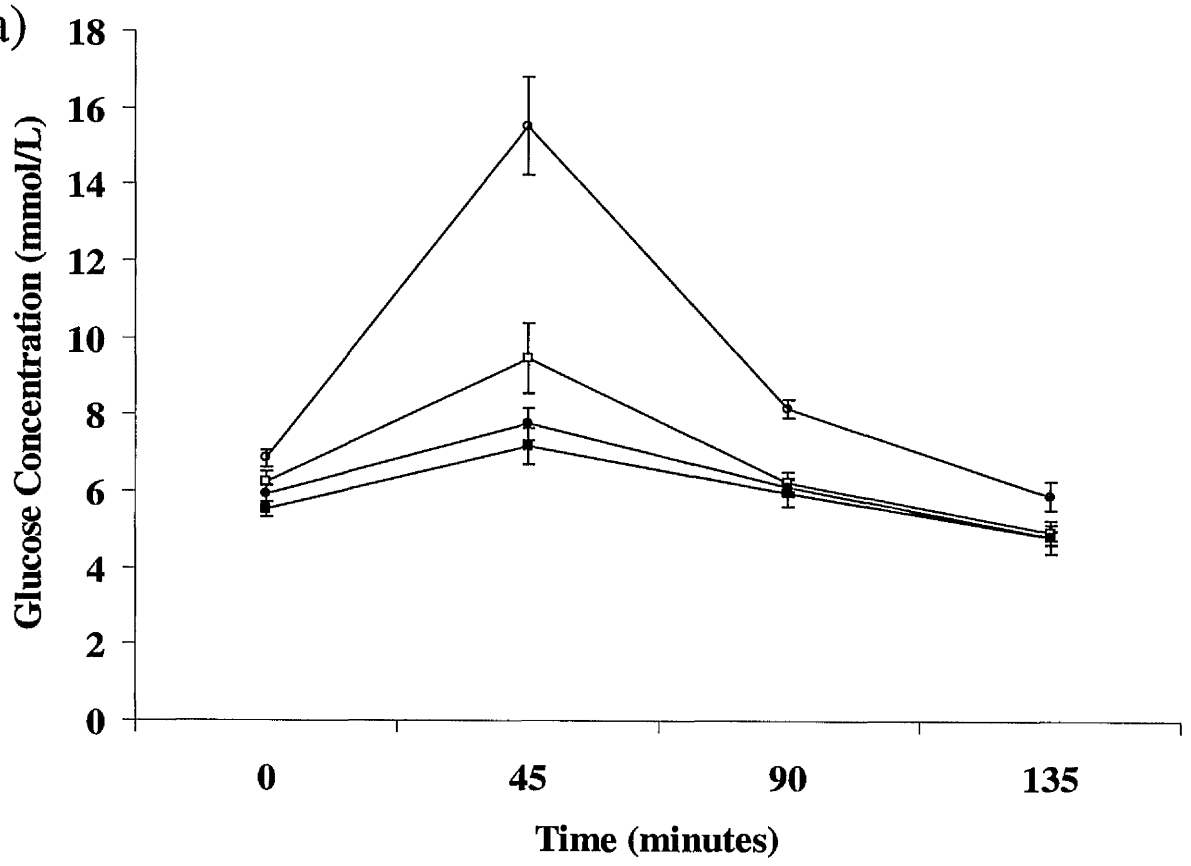
□ AKR/J mice fed a palatable diet

● C57BL/6 lean mice fed a normal diet

○ C57BL/6 *ob/ob* mice fed a normal diet

(b) The area under the curve of each group following the glucose tolerance test, expressed as mean ± S.E.M. mmol/l. * $P<0.05$ or ** $P<0.01$.

(a)


Glucose Concentration (mmol/L)
18
16
14
12
10
8
6
4
2
0
0
45
90
135
Time (minutes)

(b)

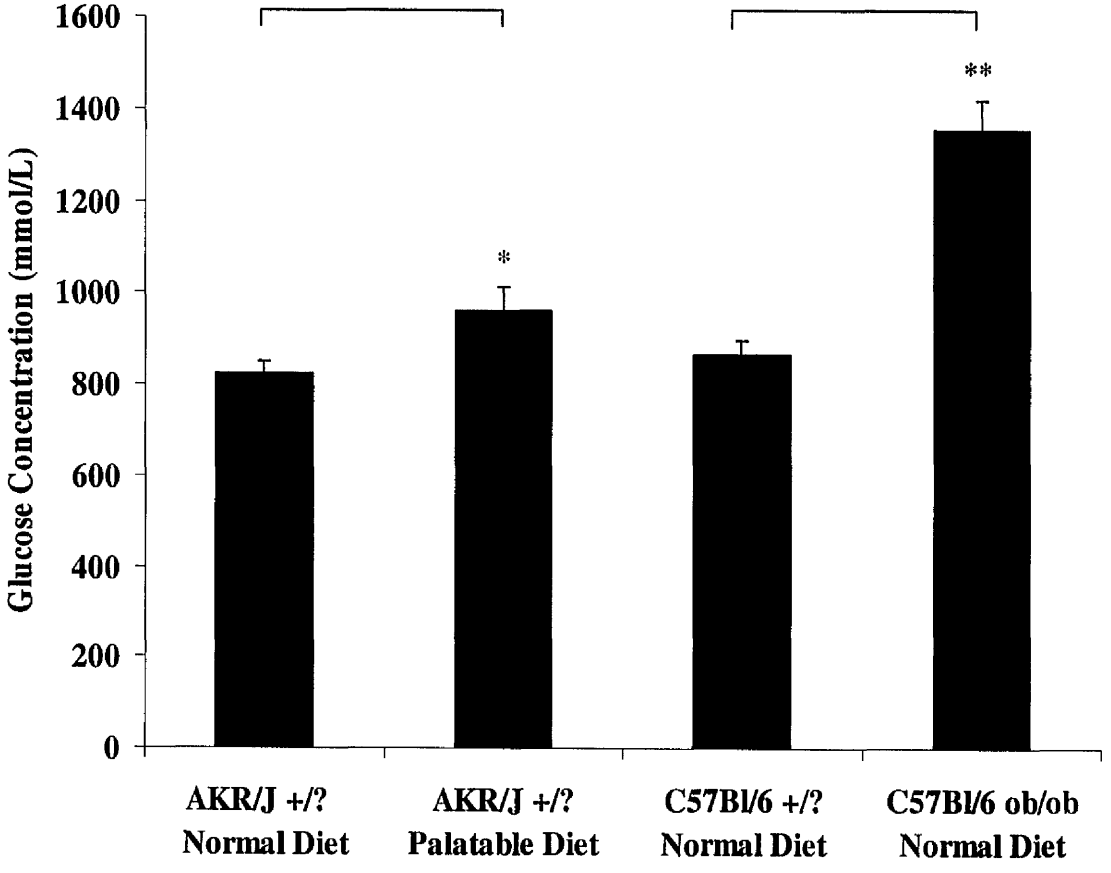

Glucose Concentration (mmol/L)
1600
1400
1200
1000
800
600
400
200
0
*
**
AKR/J +/?
Normal Diet
AKR/J +/?
Palatable Diet
C57Bl/6 +/?
Normal Diet
C57Bl/6 ob/ob
Normal Diet

Figure 3.3 Changes in gene expression of components of the leptin receptor-mediated signalling pathway in the hypothalamus of AKR/J mice fed either a chow or palatable diet, and C57BL/6 lean and *ob/ob* mice fed a chow diet

A comparison of gene expression changes are shown in the hypothalamus of AKR/J mice fed a palatable diet compared to those fed a chow diet, C57BL/6 *ob/ob* compared to leans on a chow diet and AKR/J lean mice compared to C57BL/6 lean mice. Following TaqMan analysis, the mRNA levels for the control animals have been ascribed a value of 1 and the symbols indicate relative changes from this baseline (as described in Appendix 1). Error bars indicate 95% confidence intervals and changes are shown as significant where * $P<0.05$ and ** $P<0.01$.

(a) ■ OB-Ra mRNA

□ OB-Rb mRNA

(b) ● STAT3 mRNA

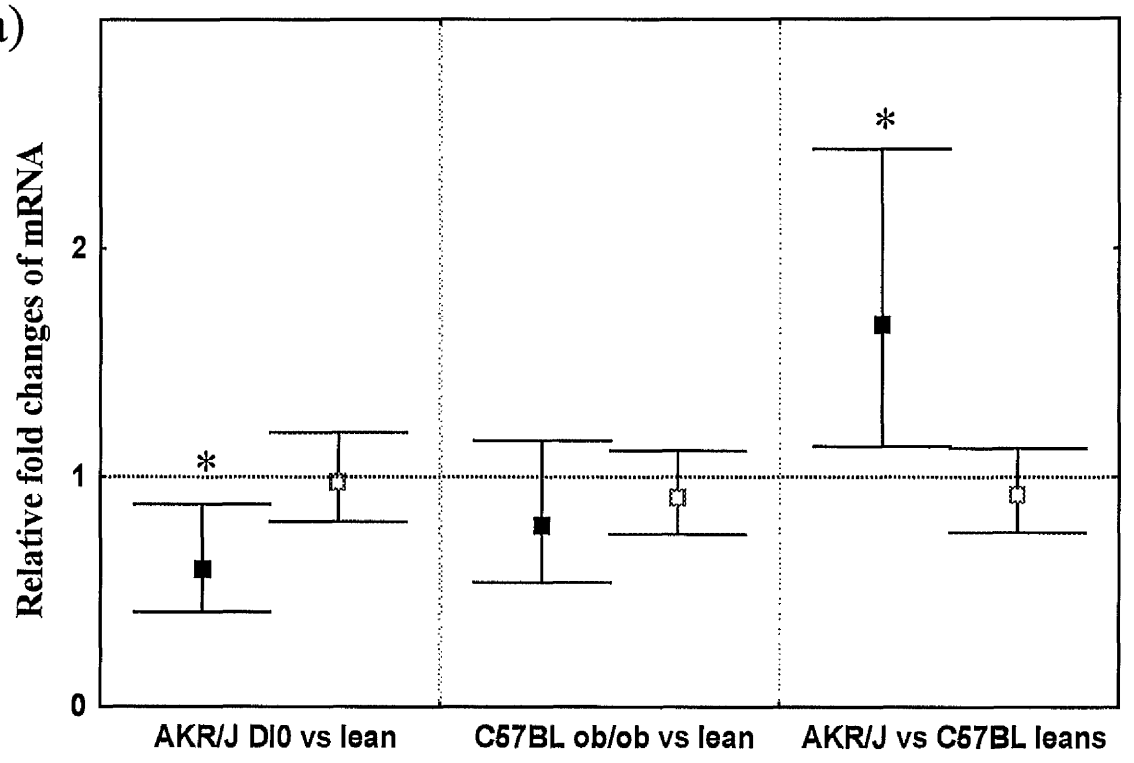
○ STAT5 mRNA

(c) ▲ SOCS-3 mRNA

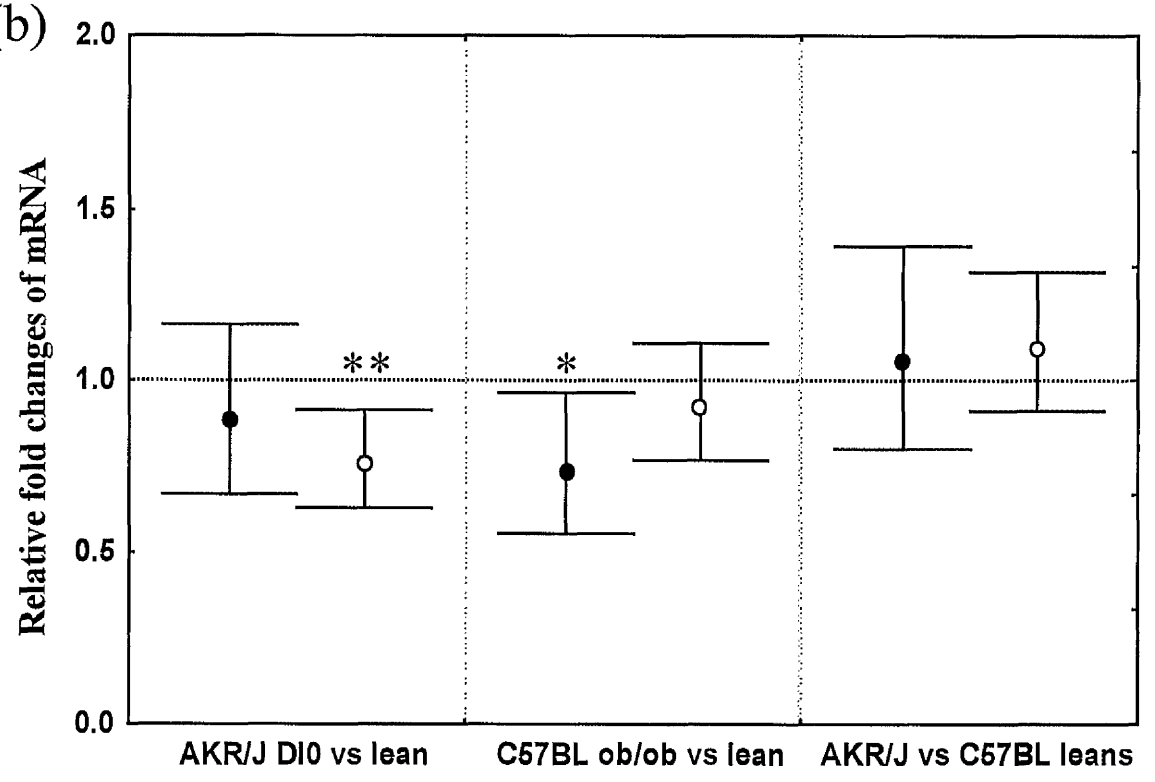
△ CIS mRNA

(d) ◆ NPY mRNA

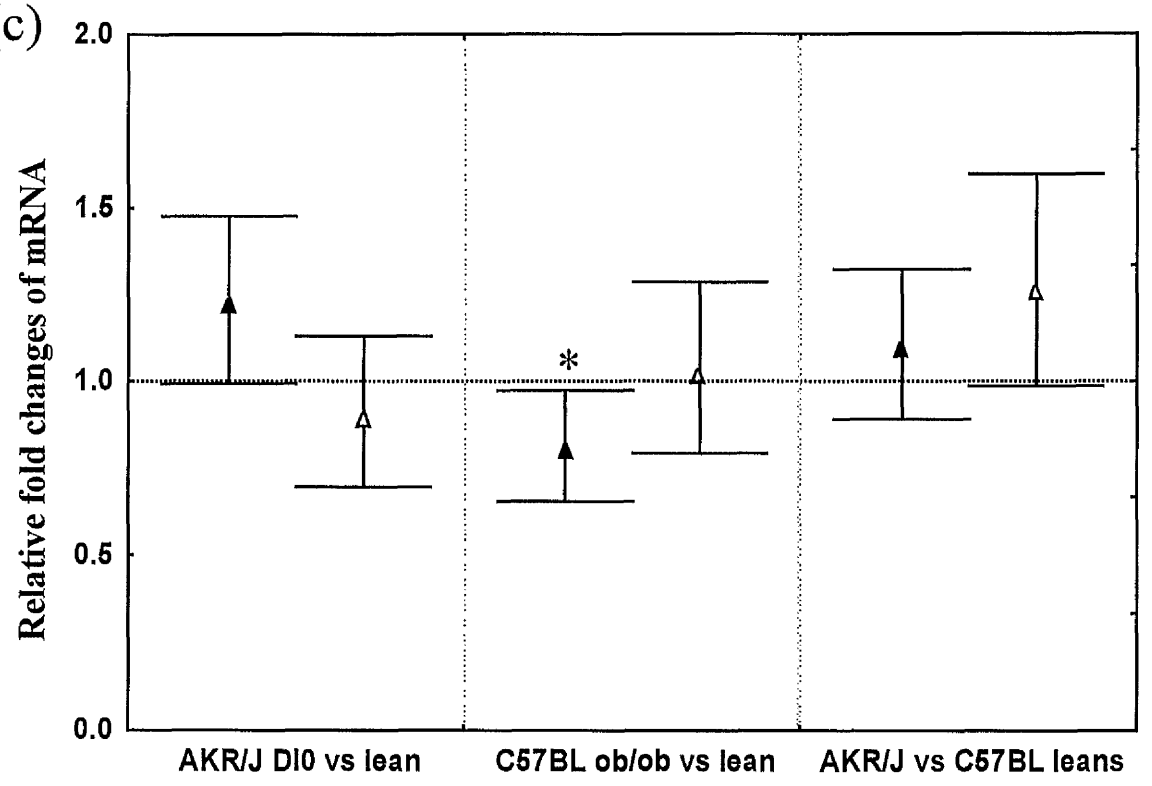
(a)


Relative fold changes of mRNA
0
1
2
*
*
AKR/J DI0 vs lean
C57BL ob/ob vs lean
AKR/J vs C57BL leans

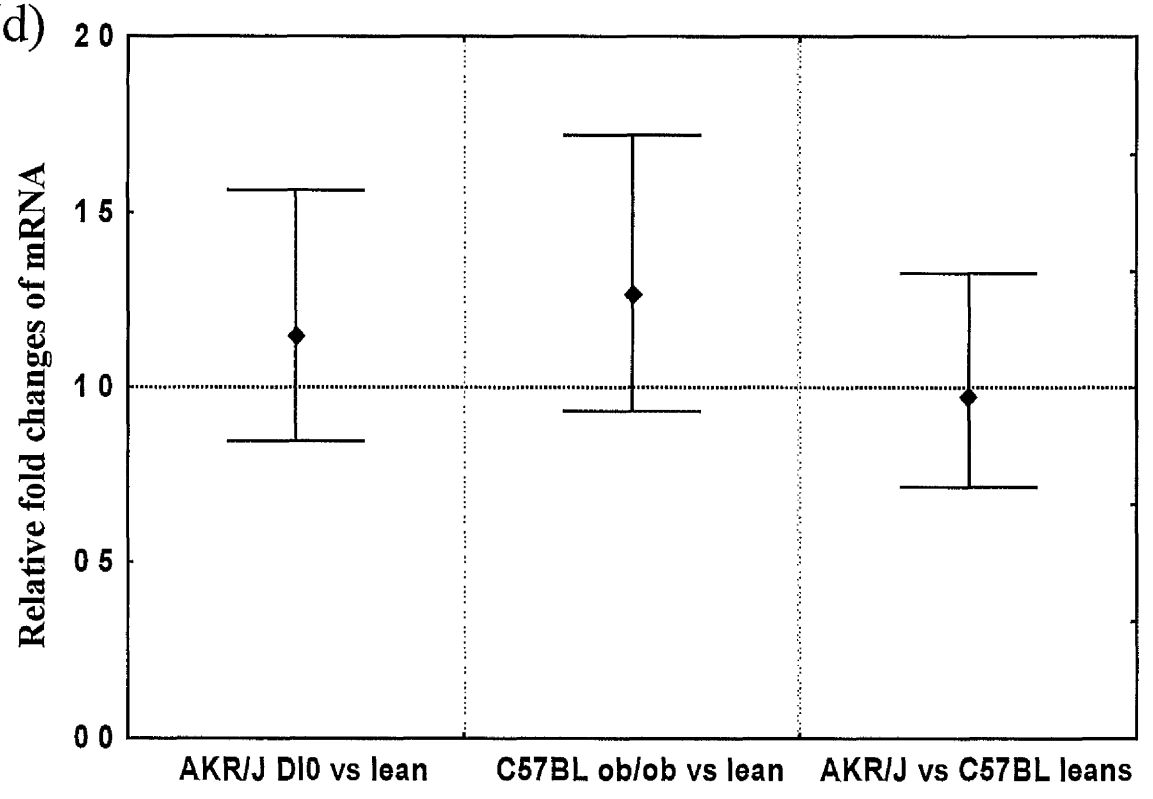
(b)


Relative fold changes of mRNA
0.0
0.5
1.0
1.5
2.0
**
*
AKR/J DI0 vs lean
C57BL ob/ob vs lean
AKR/J vs C57BL leans

(c)


2.0
1.5
1.0
0.5
0.0
Relative fold changes of mRNA
*
AKR/J DI0 vs lean
C57BL ob/ob vs lean
AKR/J vs C57BL leans

(d)


2 0
1 5
1 0
0 5
0 0
Relative fold changes of mRNA
AKR/J DI0 vs lean
C57BL ob/ob vs lean
AKR/J vs C57BL leans

The expression of STAT3 and STAT5 mRNA in AKR/J mice fed a palatable diet compared to chow and *ob/ob* compared to lean mice is shown in figure 3.3 (b). In the hypothalamus of *ob/ob* mice, STAT3 mRNA was reduced by 27% ($P<0.05$) compared to lean controls although no changes were observed in AKR/J mice. On the other hand, STAT5 mRNA expression was reduced by 24% ($P<0.01$) in the hypothalamus of AKR/J mice fed a palatable diet compared to those fed a chow diet, but there was no change in *ob/ob* mice compared to leans.

The expression of SOCS-3 and CIS mRNA in AKR/J mice fed a palatable diet compared to chow and *ob/ob* compared to lean mice is shown in figure 3.3 (c). The only significant difference was a 20% decrease of SOCS-3 expression in *ob/ob* mice compared to lean controls ($P<0.05$). There was no significant change in NPY expression in the hypothalamus of any of the group comparisons (Figure 3.3 (d)).

3.2.5 Changes in gene expression in the pituitary

TaqMan analysis of gene expression in the pituitary was adjusted for cyclophilin expression to reduce sample variability (see Appendix 1). The changes in expression of genes involved in leptin receptor-mediated signalling are shown in figure 3.4. In the pituitary of AKR/J mice fed a palatable diet compared to chow, there were no significant changes in OB-Ra, OB-Rb, STAT3, STAT5, SOCS-3 or CIS mRNA expression compared to AKR/J mice fed a chow diet. However, in the pituitary of *ob/ob* mice compared to leans, OB-Rb mRNA was reduced by 55% ($P<0.01$) whereas OB-Ra mRNA remained unchanged. A comparison of AKR/J and C57BL/6 lean mice demonstrated an increase in OB-Ra and OB-Rb mRNA expression of 110% ($P<0.01$) and 67% ($P<0.05$), respectively, in AKR/J lean mice (Figure 3.4a). The expression of STAT3 and STAT5 mRNA was reduced in the pituitary of *ob/ob* mice compared to leans by 41% ($P<0.05$) and 61% ($P<0.01$), respectively. Moreover, STAT5 mRNA was increased by 51% increase ($P<0.05$) in the pituitary of AKR/J lean mice compared to C57BL/6 lean mice (Figure 3.4b). The expression of SOCS-3 mRNA was reduced by 53% ($P<0.05$) in the pituitary of *ob/ob* mice compared to leans and increased by 140% ($P<0.05$) in AKR/J lean mice compared to C57BL/6 lean mice (Figure 3.4c). However, there was no change in the expression of CIS mRNA in comparing *ob/ob* and lean mice or the AKR/J and C57BL/6 lean mice.

Figure 3.4 Changes in gene expression of components of the leptin receptor-mediated signalling pathway in the pituitary of AKR/J mice fed either a chow or palatable diet, and C57BL/6 lean and *ob/ob* mice fed a chow diet

A comparison of gene expression changes are shown in the pituitary of AKR/J mice fed a palatable diet compared to those fed a chow diet, C57BL/6 *ob/ob* compared to leans on a chow diet and AKR/J lean mice compared to C57BL/6 lean mice. Following TaqMan analysis, the mRNA levels for the control animals have been ascribed a value of 1 and the symbols indicate relative changes from this baseline (as described in Appendix 1). Error bars indicate 95% confidence intervals and changes are shown as significant where * $P<0.05$ and ** $P<0.01$.

(a) ■ OB-Ra mRNA

□ OB-Rb mRNA

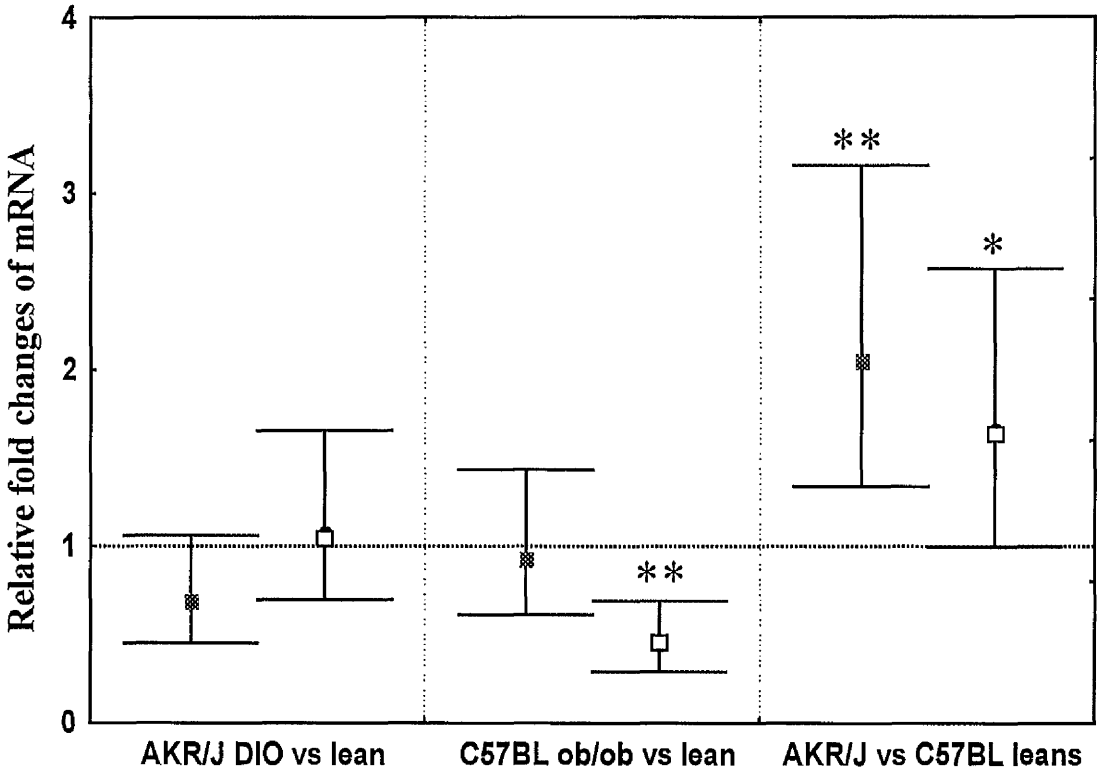
(b) ● STAT3 mRNA

○ STAT5 mRNA

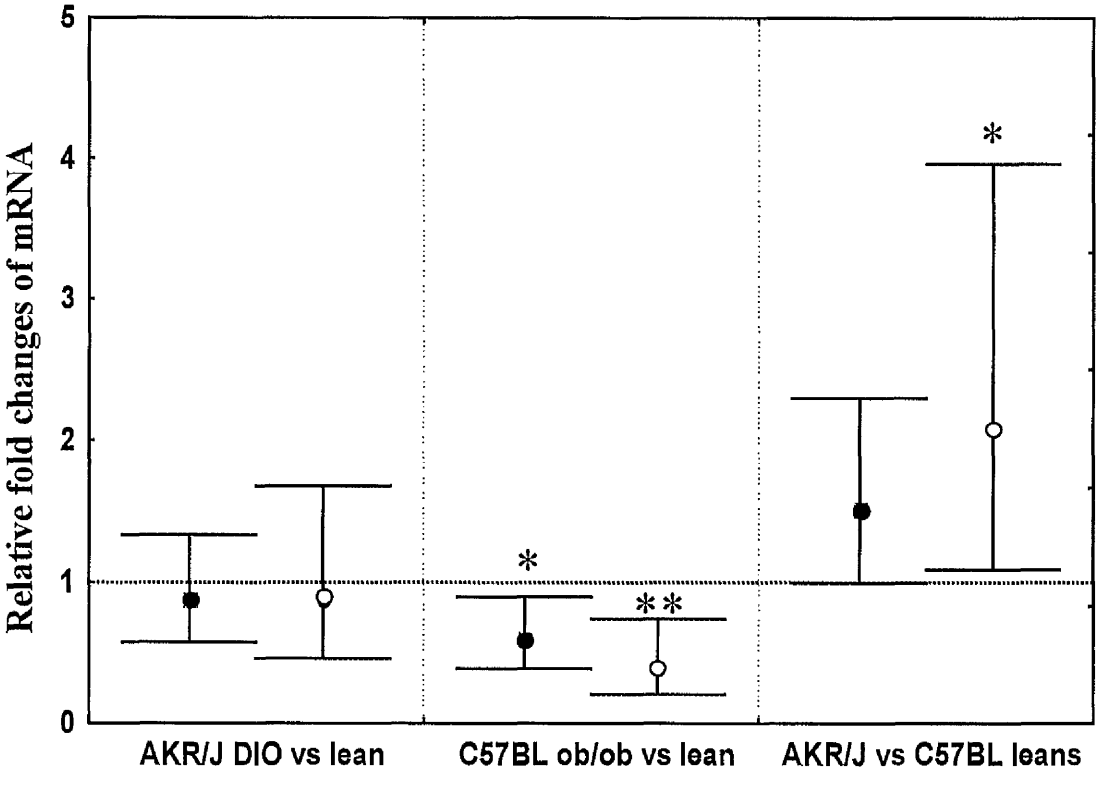
(c) ▲ SOCS-3 mRNA

△ CIS mRNA

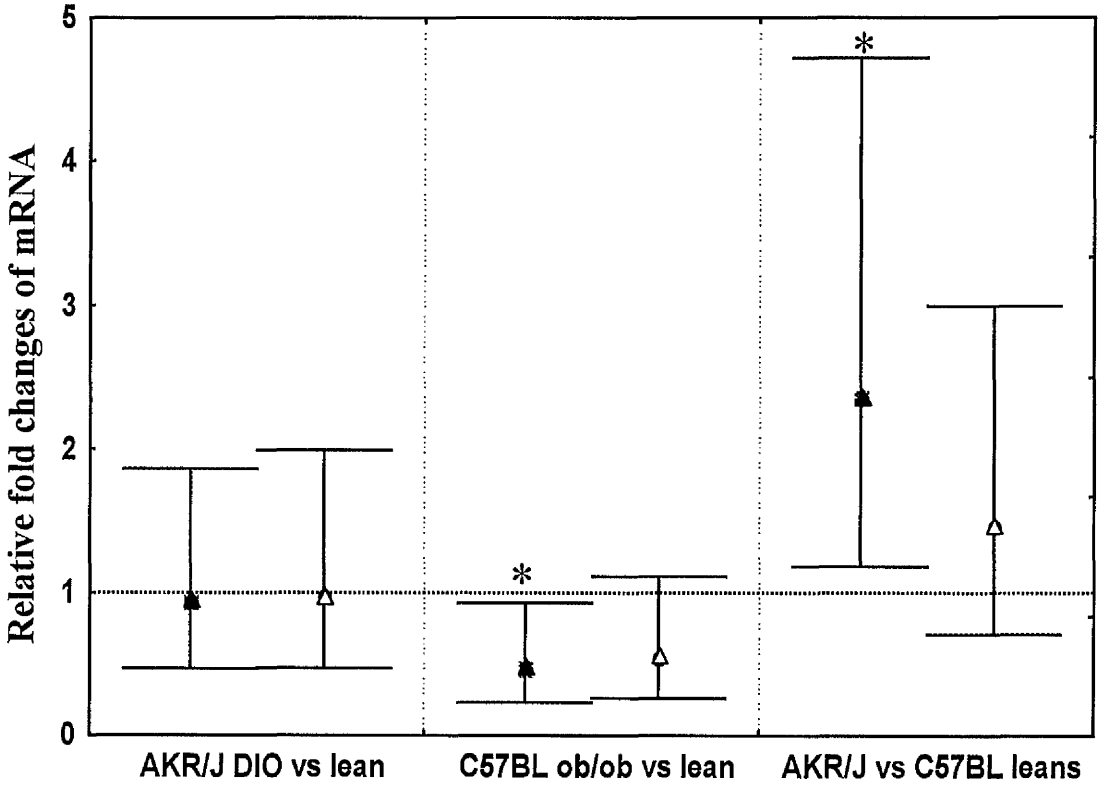
(a)


Relative fold changes of mRNA
0
1
2
3
4
**
*
**
AKR/J DIO vs lean
C57BL ob/ob vs lean
AKR/J vs C57BL leans

(b)


Relative fold changes of mRNA
0
1
2
3
4
5
*
**
*
AKR/J DIO vs lean
C57BL ob/ob vs lean
AKR/J vs C57BL leans

(c)


Relative fold changes of mRNA
5
4
3
2
1
0
*
*
AKR/J DIO vs lean
C57BL ob/ob vs lean
AKR/J vs C57BL leans

3.3 Discussion

3.3.1 Hypothalamus

Leptin is thought to act primarily on the hypothalamic nuclei that are associated with the control of feeding and energy expenditure (Baskin *et al.*, 1999; Schwartz *et al.*, 1996a). The aim of the study presented in this chapter was to investigate whether changes in central expression of the leptin receptor or leptin-mediated signalling components may alter with obesity, either following feeding of a palatable diet in which leptin levels are high, or in a genetically obese, leptin sensitive model, in which there is no leptin.

As a result of feeding AKR/J mice a palatable diet, there was an increase in body weight and food consumption, elevated serum levels of leptin and NEFA, unchanged plasma insulin levels and a small but significant decrease in glucose tolerance compared to AKR/J mice fed a normal chow diet. In the hypothalamus, OB-Ra mRNA was reduced in AKR/J mice fed a palatable diet compared to mice fed a chow diet but OB-Rb mRNA remained unchanged. Expression in the BBB was not measured in this study and as a result, conclusions regarding potential effects on leptin transport cannot be made. The leptin receptor is known to form homo-oligomers, however OB-Rb signalling may be reduced by the overexpression of OB-Ra, a homo-dimerising partner that does not signal via the JAK-STAT pathway (White *et al.*, 1997a). *In vitro* studies have observed that OB-Rb shows very weak dominant negative repression in the presence of excess OB-Ra (White *et al.*, 1997a). However, it is unclear if there is any effect exerted *in vivo* in the hypothalamus. Thus,

whether the reduction in hypothalamic OB-Ra observed in AKR/J mice has a functional effect on OB-Rb signalling remains to be determined.

A number of investigations have shown an increase in leptin receptor expression in the hypothalamus of *ob/ob* mice compared to lean controls (Baskin *et al.*, 1998; Emilsson *et al.*, 1999; Huang *et al.*, 1997). Huang *et al.* observed a 2-fold increase in all isoforms of the leptin receptor in the arcuate and ventromedial nuclei of *ob/ob* mice compared to lean controls by *in situ* hybridisation (Huang *et al.*, 1997). Also, using *in situ* hybridisation Baskin *et al.* reported a 2-fold increase in OB-Rb mRNA in the arcuate nucleus of *ob/ob* mice compared to lean controls by *in situ* hybridisation (Baskin *et al.*, 1998). Furthermore, Emilsson *et al.* showed a 2-fold increase of total leptin receptor mRNA expression relative to β-actin mRNA expression by RT-PCR in the whole hypothalamus of *ob/ob* mice compared to lean controls (Emilsson *et al.*, 1999). It was therefore surprising that we observed no increase in OB-Ra or OB-Rb mRNA expression in the hypothalamus of *ob/ob* mice compared to lean controls. The exact reason for this is unclear, but could be because these changes are not evident in the whole of the hypothalamus. Although Emilsson *et al.* observed changes in the whole hypothalamus using RT-PCR, their data was expressed as a ratio to β-actin mRNA expression (Emilsson *et al.*, 1999). Appendix 1 includes a discussion of the use of housekeeping in the analysis of gene expression.

Little is known about the mRNA expression profile of leptin receptor-mediated signalling components in different strains. There was a significant

increase in OB-Ra mRNA in the hypothalamus of AKR/J lean mice compared to C57BL/6 lean mice; however there was no change in OB-Rb mRNA. The only notable difference between these two strains is that the AKR/J mice gain more weight in comparison to the C57BL/6 as a result of being fed a high fat diet (communicated by Mohammad Tadayyon). Perhaps this is manifest in part by the different physiological characteristics of AKR/J lean mice compared to C57BL/6 lean mice, notably the increased body weight and leptin.

Reduced expression of STAT5 mRNA was observed in the hypothalamus of AKR/J mice fed a palatable diet compared to mice on a chow diet, but not in *ob/ob* mice compared to lean mice. However, as only leptin-mediated STAT3 activation in the hypothalamus has been demonstrated (Vaisse *et al.*, 1996), the effect of changes in STAT5 on the leptin signalling pathway *per se* is unclear. A decrease in STAT5 mRNA expression may reflect changes in the expression of as yet unidentified signals that are activated by STAT5 in the hypothalamus.

El-Haschimi *et al.* showed reduced STAT3 activation by electrophoretic mobility shift assays (EMSA) in the hypothalamus of mice fed a high-fat diet for 15 weeks (El Haschimi *et al.*, 2000). However, they were unable to detect a change in hypothalamic mRNA expression of OB-Rb, STAT3 or SOCS-3 in mice fed a low-fat or high-fat diet for 15 weeks (El Haschimi *et al.*, 2000). In the hypothalamus of *ob/ob* mice, we have observed reduced STAT3 mRNA expression compared to lean mice. The reduced STAT3 mRNA expression in the hypothalamus of *ob/ob* mice has previously been observed at comparable

levels by Hakansson-Ovesjo *et al.* (Hakansson-Ovesjo *et al.*, 2000), who also showed reduced STAT3 protein expression and localised the changes in expression to the arcuate nucleus. This suggests that the absence of functional leptin in *ob/ob* mice results in downregulation of STAT3 mRNA and consequently also of STAT3 protein levels.

The observation that SOCS-3 gene expression is STAT3-dependent and is negatively autoregulated by SOCS-3 protein (Auernhammer *et al.*, 1999), may explain the reduced SOCS-3 mRNA observed in the hypothalamus of *ob/ob* mice compared to lean controls, assuming the reduced levels of STAT3 and SOCS-3 mRNA is an indication of reduced levels of activated protein. However, Emilsson *et al.* showed an approximate 2-fold increase of SOCS-3 and CIS mRNA expression in the hypothalamus of leptin-sensitive *ob/ob* mice (Emilsson *et al.*, 1999), which is inconsistent with the proposed role of these genes as suppressors of cytokine signalling. The authors suggested that the increase in SOCS-3 and CIS may be counteracted by the observed 2-fold increase in OB-R mRNA in the *ob/ob* hypothalamus.

In agreement with the diet-induced obesity study of El-Haschimi *et al.* (El Haschimi *et al.*, 2000), no change in the suppressor of STAT signalling SOCS-3 was observed in AKR/J mice fed a palatable diet. Bjorbaek *et al.* demonstrated that acute (between 1-3 hour) peripheral leptin administration induced SOCS-3 mRNA expression in the hypothalamus of *ob/ob* mice, but had no effect on SOCS-1, SOCS-2 or CIS mRNA expression (Bjorbaek *et al.*, 1998b). Emilsson *et al.* showed that peripheral leptin administration of lean

mice, over a period of 48 hours, induces both SOCS-3 and CIS mRNA expression in the hypothalamus (Emilsson *et al.*, 1999). Our findings suggest that in AKR/J mice fed a palatable diet, the elevated leptin levels does not induce SOCS-3 and reduction in leptin sensitivity is not at the level of SOCS-3. As described, based on the time- and dose-dependent leptin treatment of mice, there are conflicting reports of whether CIS is involved in leptin signalling in the hypothalamus (Bjorbaek *et al.*, 1998b; Emilsson *et al.*, 1999). In my study, there appeared to be no effect on CIS mRNA expression in the hypothalamus as a result of high-fat feeding or in *ob/ob* mice compared to leans.

3.3.2 Pituitary

In the pituitary of AKR/J mice fed a palatable diet, there were no changes in leptin receptor-mediated signalling components, indicating that the elevated serum leptin levels and obesity does not affect leptin signalling in the pituitary. Conversely, in the pituitary of *ob/ob* mice, OB-Rb, STAT3, STAT5 and SOCS-3 were all reduced. There are no studies that compared leptin receptor-mediated signalling components in the pituitary of mouse models of obesity. In the pituitary of *ob/ob* mice, it is evident that the lack of circulating plasma leptin results in reduced leptin receptor-mediated signalling components, which may, in part, be responsible for the infertility and prepubertal gonadotropin secretion pattern observed in these mice. Furthermore, there is increasing evidence of the importance of leptin receptor-mediated signalling in the functioning of the pituitary. For example, the chronic treatment of *ob/ob*

mice with leptin can induce recovery in the reproductive system by promoting growth and function of the reproductive organs and fertility by increasing secretion of gonadotropins (Barash *et al.*, 1996; Chehab *et al.*, 1996). In addition, it has been observed that humans with a mutation in the leptin receptor gene have a dysfunctional pituitary (Clement *et al.*, 1998).

A comparison of leptin receptor-mediated signalling components in the pituitary of AKR/J lean compared to C57BL/6 lean mice shows that the mRNA expression of OB-Ra, OB-Rb, STAT5 and SOCS-3 are significantly increased. These changes may be a result of differences in circulating leptin, or perhaps other factors, which may affect expression of leptin receptor-mediated signalling components in the pituitary. For instance, GH and prolactin are known to activate STAT5, and SOCS-3 can inhibit GH-induced STAT5 dependent transcription in transfected COS cells (Gouilleux *et al.*, 1995; Ram & Waxman, 1999). The localisation of the changes in leptin receptor-mediated signalling components to pituitary cell types would give a further indication of the factors that may regulate these changes.

3.4 Conclusion

In summary, of all the leptin receptor-mediated signalling components analysed in AKR/J mice fed a palatable diet, only OB-Ra and STAT5 mRNA were reduced in the hypothalamus and no changes were observed in the pituitary. This suggests that leptin signalling in diet-induced obese mice are not primarily regulated at the level of leptin receptor signalling components investigated in the hypothalamus or pituitary. In *ob/ob* mice, STAT3 and SOCS-3 mRNA were reduced in both the hypothalamus and the pituitary, whereas OB-Rb and STAT5 mRNA were attenuated in the pituitary, indicating downregulation of key leptin receptor-mediated signalling components, which may contribute to the sensitivity of leptin in these mice. Finally, a comparison of AKR/J and C57BL/6 lean mice showed that some of the leptin receptor-mediated signalling components in the hypothalamus and pituitary were increased in AKR/J mice, suggesting that the regulation of leptin responses in the hypothalamus and pituitary may be controlled in a tissue and strain-specific manner.

Chapter 4

Quantitative Expression of Genes Involved in the Leptin Receptor-Mediated STAT Signalling Pathway in Peripheral Tissues of Diet-Induced and Genetically Obese Mice

4.1 Introduction

The short form of the leptin receptor was initially identified in the choroid plexus and hypothalamus, but several differentially expressed splice variants were subsequently detected in various other tissues (Chen *et al.*, 1996; Ghilardi *et al.*, 1996; Lee *et al.*, 1996; Tartaglia *et al.*, 1995). OB-Ra is expressed in many tissues and although the OB-Rb isoform is highly expressed in the hypothalamus and the lymphoid system, small amounts of OB-Rb mRNA have also been demonstrated in many other tissues, including pancreatic β-cells, WAT, BAT and the small intestine (Emilsson *et al.*, 1997; Ghilardi *et al.*, 1996; Kutoh *et al.*, 1997; Morton *et al.*, 1998). This suggests that leptin has direct physiological effect in these tissues, facilitating the regulation of feeding and maintaining energy balance.

4.1.1 Pancreas

4.1.1.1 Leptin receptor expression

The OB-Rb isoform of the leptin receptor is expressed in the pancreatic β-cells that produce insulin, raising the possibility that leptin directly regulates insulin release (Kieffer *et al.*, 1996). Using a probe common to all leptin receptor isoforms, Kieffer *et al.* (Kieffer *et al.*, 1996) showed that the leptin receptor mRNA was detected in rat islets in greater abundance than in total brain. Furthermore, of 21 tissues examined by sensitive RNase protection assay, the pancreas appeared to have the most abundant proportion of OB-Rb mRNA relative to OB-Ra mRNA of all the peripheral tissues examined. The

expression of OB-Ra and OB-Rb in pancreatic β-cells has since been confirmed by a number of groups (Briscoe *et al.*, 2001a; Emilsson *et al.*, 1997; Islam *et al.*, 1997; Kieffer *et al.*, 1997; Kulkarni *et al.*, 1997; Pallett *et al.*, 1997; Poitout *et al.*, 1998; Tanizawa *et al.*, 1997; Zhao *et al.*, 1998).

4.1.1.2 Effect of leptin on insulin synthesis and release

The first evidence of OB-Rb-mediated leptin action on pancreatic islets was the observation that leptin reduced plasma insulin levels in the leptin-deficient *ob/ob* mice but not in the leptin signalling defective *db/db* mice (Emilsson *et al.*, 1997; Kulkarni *et al.*, 1997). Since insulin is adipogenic and stimulates leptin secretion from WAT there appears to be a bidirectional adipoinsular axis (Kieffer *et al.*, 1996).

The *ob/ob* mice are hypersensitive to the feeding effects of leptin treatment due to the lack of circulating leptin and as a result have been instrumental in determining the effects of leptin on insulin secretion. In contrast, the β-cells in normal rodents are continually exposed to leptin and therefore may be expected to be less sensitive. Leptin treatment (6.25-100 nM) of pancreatic islets isolated from *ob/ob* mice for 1-2 hours reduced leptin secretion from between 13 to 29% (Emilsson *et al.*, 1997; Kieffer *et al.*, 1997). Furthermore, leptin reduced insulin release in the perfused pancreas of *ob/ob* mice, a model used to assess islet function (Emilsson *et al.*, 1997). It is important to note that increasing glucose level *in vivo* reduces the suppressive effect of leptin on insulin release in the islets of *ob/ob* mice. Overall, however,

there are conflicting reports regarding leptin effects on insulin secretion in normal rodents. Some investigators have found that leptin (0.625-100 nM) had no effect on insulin release in isolated rat or mouse islets (Karlsson *et al.*, 1998; Poitout *et al.*, 1998), or in the perfused rat pancreas (Leclercq-Meyer *et al.*, 1996; Leclercq-Meyer & Malaisse, 1997; Leclercq-Meyer & Malaisse, 1998). One study found that leptin (1 nM) stimulates insulin release from isolated rat islets (Tanizawa *et al.*, 1997). However, in most studies, insulin was reduced by leptin (1-20 nM) in the perfused rat pancreas and isolated rat or mouse islets in the presence of low, normal or high glucose (Emilsson *et al.*, 1997; Fehmann *et al.*, 1997b; Ishida *et al.*, 1997; Kulkarni *et al.*, 1997; Pallett *et al.*, 1997).

Leptin may also exert its effect at the level of insulin mRNA expression. However, again there are conflicting reports. Leptin suppresses preproinsulin mRNA expression in isolated rat islets (Kulkarni *et al.*, 1997; Pallett *et al.*, 1997), in mouse βTC6 cells (Kulkarni *et al.*, 1997), in *ob/ob* mouse islets, in the rat pancreatic β-cell line INS-1 (Seufert *et al.*, 1999b), and in human islets (Seufert *et al.*, 1999a). However, some studies showed leptin had no effect on insulin biosynthesis (Karlsson *et al.*, 1998; Roduit & Thorens, 1997), whilst another study showed leptin increased insulin preproinsulin mRNA in HIT-T15 cells (Shimizu *et al.*, 1997).

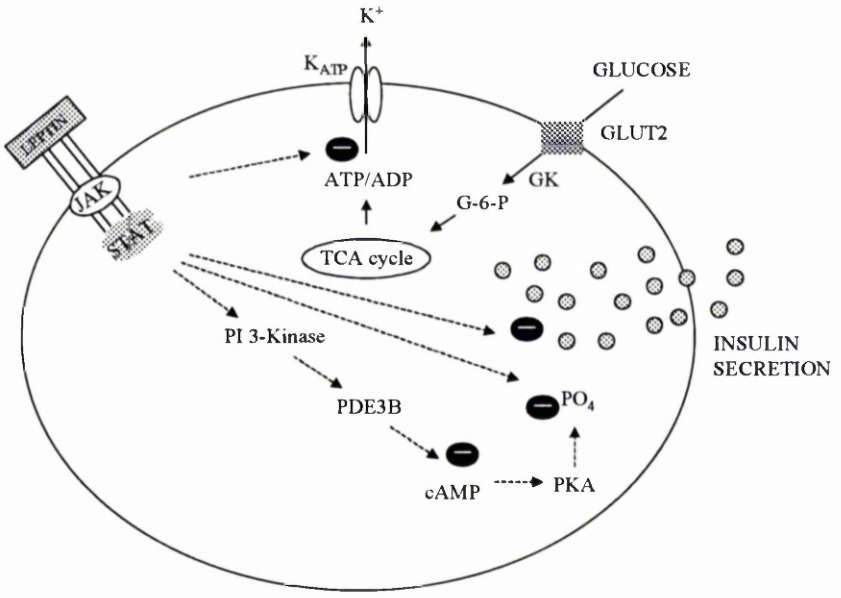
4.1.1.3 Signalling pathways

To investigate the effects of leptin in the pancreatic β-cells, it is necessary to outline the mechanism by which insulin is released from β-cells.

Insulin secretion from pancreatic β-cells is controlled in part by the activity of K_{ATP} channels. Closure (or inactivation) of K_{ATP} channels in response to metabolism of glucose depolarises β-cells, resulting in the activation of voltage-dependent Ca^{2+} channels, a rise in cytosolic calcium concentration ($[Ca^{2+}]_i$), and exocytosis of insulin granules (Ashcroft & Rorsman, 1989). Glucose metabolism within β-cells also results in an elevation of fatty acyl-CoA esters, which accompanied by increased $[Ca^{2+}]_i$ causes induction of insulin release (Prentki *et al.*, 1997). Glucose-induced insulin secretion is further regulated by hormone-mediated elevation of the intracellular second messengers cAMP/PKA (Holz & Habener, 1992) and phospholipase C/PKC (Zawalich *et al.*, 1997).

In pancreatic β-cells, leptin reduces insulin secretion by the opening of ATP-sensitive K^+ (K_{ATP}) channels (Kieffer *et al.*, 1997). Harvey *et al.* investigated the mechanism by which leptin activates K_{ATP} channels and found that inhibitors of PI 3-kinase (e.g. wortmannin) prevented opening of K_{ATP} channels by leptin (Harvey *et al.*, 2000a). Consistent with an involvement of PI 3-kinase, leptin suppressed any increase in cAMP, and as a result insulin secretion, after treatment with agents known to increase cAMP (e.g. GLP-1), through the activation of a PI 3-kinase-dependent PDE3B (Zhao *et al.*, 1998). Furthermore, leptin reduced insulin secretion induced by PMA (a PKC activator) in mouse *ob/ob* islets and rat islets (Chen *et al.*, 1997; Ookuma *et al.*, 1998). These observations suggest that the leptin receptor-mediated signalling pathway interacts with both cAMP-dependent PKA and PKC pathways.

Leptin signalling in the β-cell



DNA-binding assays suggest that STAT proteins may mediate the inhibition of insulin gene expression after leptin treatment. Sequences between -307 and -410 bp of the rat insulin I gene promoter bind multiple protein complexes contained in nuclear extracts from leptin-treated *ob/ob* islets (Seufert *et al.*, 1999b). One of the complexes contained STAT5b, which formed on a previously described consensus STAT binding site (Seufert *et al.*, 1999b). Leptin was shown to activate STAT3 in BRIN-BD11 cells, RINm5F cells and isolated rats islets, though STAT5 activation was detected in BRIN-BD11 cells (Briscoe *et al.*, 2001a; Morton *et al.*, 1999). The treatment of rat pancreatic islets with leptin produced an increase in the tyrosine phosphorylation of STAT3, but not STAT5 (Tanabe *et al.*, 1997). These observations implicate STAT3 and STAT5 activation as a potential mechanism by which leptin

regulates gene expression in β-cells. Although STAT proteins are generally considered to be activators of gene transcription, they may also be inhibitory, depending on the promoter context and cell type. For example, STAT5b stimulated by prolactin induces the β-casein promoter but inhibits the interferon regulatory factor-1 promoter (Luo & Yu-Lee, 1997).

There are no publications to date of leptin-mediated induction of SOCS-3 or CIS mRNA in the pancreas *in vivo*, but SOCS-3 and CIS have been implicated as negative regulators of leptin signalling in the hypothalamus, liver and small intestine of lean mice (Bjorbaek *et al.*, 1998b; Emilsson *et al.*, 1999). An *in vitro* study has shown that the forced expression of CIS in RINm5F cells, a leptin-responsive β-cell line, prevented leptin-mediated STAT3 transcriptional activation of STAT3-dependent reporter constructs in a dose dependent manner (Emilsson *et al.*, 1999). To date, there are no studies demonstrating reduced leptin sensitivity in the pancreas after feeding of a high-fat diet.

Glucose-induced insulin secretion occurs through a sensing pathway that requires glucose metabolism, a process which is initiated by the uptake of the glucose transporter GLUT2 (Orci *et al.*, 1989; Thorens *et al.*, 1988). The transcriptional control of GLUT2 in β-cells involves PDX-1 (Waeber *et al.*, 1996), a transcription factor that controls the expression of the insulin and glucokinase genes (Ohlsson *et al.*, 1993; Watada *et al.*, 1996). Expression of PDX-1 is regulated by glucose, fatty acids and glucocorticoids (Gremlich *et al.*, 1997; Shinozuka *et al.*, 2001). The expression of GLUT2, glucokinase and

PDX-1 mRNA has been shown to be altered in type II diabetes (Leibowitz *et al.*, 2001; Weir *et al.*, 1997).

In order to identify gene changes that may be involved in obesity and leptin sensitivity, a number of genes involved in leptin receptor-mediated signalling and glucose sensing have been analysed in the pancreas of dietary obese and genetically obese mice.

4.1.2 Adipose Tissue

There are two distinct types of adipose tissue, white and brown, which have different physiological functions. WAT is widely distributed throughout the body and serves as a major depot of stored energy in the form of triglycerides. The triglycerides are stored during periods of excess energy availability and are mobilised during periods of energy deprivation. Therefore, WAT has enormous potential for changes in cell mass in accordance to the energy state. The adipose mass reflects the net balance between energy intake and energy expenditure and is stable over a long period of time.

BAT resides in the central axis of the body and functions to dissipate energy and provide heat. BAT plays a major role in the regulation of body temperature in small animals such as mice and rats, which have a high surface area to body volume and require hormonal regulation of thermogenesis to maintain body temperature. Although BAT is well developed in human infants, where it contributes to maintaining body temperature, it atrophies in adults.

Several studies have shown the presence of OB-Rb mRNA in WAT and BAT (Fei *et al.*, 1997; Ghilardi *et al.*, 1996; Kutoh *et al.*, 1997; Siegrist-Kaiser *et al.*, 1997), suggesting leptin has direct effects on these tissues. Evidence for a direct effect of leptin on WAT came from the observation that leptin administration of lean Zucker +/? rats after 2 hours increased the rate of lipolysis in WAT (Siegrist-Kaiser *et al.*, 1997). Commins *et al.* observed reduced WAT mass in wild-type (+/+) mice treated with leptin (i.p.), but not in UCP1-deficient (-/-) mice, suggesting UCP-1 is required for leptin to decrease WAT mass (Commins *et al.*, 2001). Furthermore, there was a 3-fold increase in UCP1 mRNA and protein in BAT of leptin-treated wild-type mice (Commins *et al.*, 2001). The chronic i.p. administration of leptin *in vivo* for 4 days had direct effects on BAT, resulting in increased glucose utilisation, whereas i.c.v. leptin administration had no significant effect (Siegrist-Kaiser *et al.*, 1997).

Further investigations have shown that *in vivo* i.p. leptin administration can activate STAT1 and STAT3 in WAT of C57BL/6J wild-type and *ob/ob* mice (Bendinelli *et al.*, 2000). The peripheral, but not central, administration of leptin *in vivo* activates STAT1 in BAT (Siegrist-Kaiser *et al.*, 1997). Moreover, in terms of negative regulators of leptin signalling, SOCS-1 and SOCS-3 have been shown to be increased in WAT of DIO rats suggesting that increased expression of these genes may play a role in leptin sensitivity in WAT (Wang *et al.*, 2000). Furthermore, the ability of A/J mice (obesity-resistant) to resist diet-induced obesity is associated with increased UCP1 and UCP2 mRNA

expression in BAT and WAT, respectively, which is not observed in obesity-prone C57Bl/6J mice (Watson *et al.*, 2000).

In summary, leptin appears to have differential effects in WAT and BAT. The studies to date in WAT, where leptin regulates lipolysis, suggest regulation of leptin sensitivity may be at the level of STAT-1 and 3, and/or SOCS-1 and SOCS-3, whereas leptin directly affects glucose utilisation and the expression of UCP mRNA. In BAT, there are no studies to date of leptin affecting the expression of components of the leptin receptor-mediated signalling pathway. To identify some of the genes which may be involved in leptin sensitivity, the expression of leptin receptor-mediated signalling components were analysed in WAT of dietary obese mice and in BAT of dietary and genetically obese mice.

4.1.3 Small Intestine

Multiple short forms of the leptin receptor are expressed throughout the gastrointestinal tract suggesting a potential role of leptin in nutrient absorption (Morton *et al.*, 1998). However, the functional OB-Rb is predominantly expressed in the jejunum and with weaker expression in the ileum, the two major sites that are involved in nutrient handling (Morton *et al.*, 1998). In addition, OB-Rb expression was readily detected in the human intestinal epithelial Caco-2 cells, implying a role for leptin in enterocyte function (Morton *et al.*, 1998). In this context, it has been shown that leptin produced a rapid inhibitory effect on sugar absorption in rat intestinal rings (Lostao *et al.*, 1998).

Western blot analysis of STAT proteins in lean and *ob/ob* jejunum readily detects STAT5 but more weakly detects STAT1 and STAT3 (Morton *et al.*, 1998). The intravenous injection of leptin induced STAT5, but not STAT3, DNA binding activity in the jejunum of lean and *ob/ob* mice but had no effect in the db/db mouse (Morton *et al.*, 1998). Furthermore, the leptin-induced activation of STAT5 in the jejunum is associated with a reduction of the apolipoprotein-AIV mRNA levels 90 minutes after a pure fat load, suggesting leptin might be involved in lipid handling (Morton *et al.*, 1998). The increased STAT5 activation in the jejunum contrasts with the effect of leptin in the hypothalamus, where only STAT3 is activated (Vaisse *et al.*, 1996). The possible importance of STAT5 in mediating the effect of leptin on body weight homeostasis is suggested by *in vitro* evidence from the obese (*fa/fa*) Zucker rat, which contain a missense mutation (Gln→Pro) in the extracellular domain of the leptin receptor Streamson *et al.*, 1996). OB-R(*fa*) mediates a leptin-independent (constitutive) activation of STAT1 and STAT3, whereas the activation of STAT5 is completely abolished (White *et al.*, 1997b) suggesting a lack of leptin effect through STAT5 in the small intestine in addition to other tissues could in part contribute to the obesity phenotype.

The expression of SOCS-3 and CIS mRNA has been analysed in the small intestine of leptin treated (48 hours) lean and *ob/ob* mice. Emilsson *et al.* demonstrated that leptin treatment of lean and *ob/ob* mice induced expression of CIS mRNA, whereas SOCS-3 mRNA was only induced upon leptin treatment of lean mice (Emilsson *et al.*, 1999). Furthermore, CIS mRNA, but not SOCS-3

mRNA, was elevated 2-fold in the small intestine of *ob/ob* mice compared to leans. The authors suggest increased expression of CIS mRNA dampens the response to leptin and that some tissues, such as the small intestine in *ob/ob* mice, might be less sensitive to leptin compared to the same tissues in leans. As the CIS promoter contains multiple STAT5 binding sites, the increased expression of CIS mRNA upon leptin treatment further suggests that STAT5 is the STAT protein predominantly activated by leptin in the small intestine (Matsumoto, A., *et al.*, 1997).

In this study, therefore, the expression of components of leptin receptor-mediated signalling pathway was analysed in the small intestine of dietary obese and genetically obese mice to identify genes that may be involved in obesity and leptin sensitivity.

4.2 Results

The tissues analysed in this chapter are from the study described in Chapter 3. The physiological characteristics of the dietary-induced obese AKR/J mice and the C57BL/6 *ob/ob* mice are detailed in sections 3.2.1-3.2.3. This section details pancreatic insulin-content and changes in gene expression in the pancreas, WAT, BAT and small intestine of palatable diet-fed AKR/J and C57BL/6 *ob/ob* mice.

4.2.1 Pancreatic Insulin

Pancreatic insulin content was determined by ELISA, as described in section 2.3.1 (c). Table 4.1 shows the insulin levels in the pancreas of AKR/J mice fed either a chow or palatable diet and C57BL/6 lean and *ob/ob* mice fed on chow diets. Pancreatic insulin levels were increased 4.5-fold ($P<0.01$) in palatable diet-fed AKR/J mice compared to chow-fed mice and 23-fold ($P<0.01$) in C57BL/6 *ob/ob* mice compared to lean controls. There was no significant difference in pancreatic insulin content of AKR/J lean mice compared to C57BL/6 lean mice.

Table 4.1 Insulin levels in the pancreas of AKR/J mice fed either a chow or palatable diet, and C57BL/6 lean and *ob/ob* mice fed a chow diet

AKR/J mice were fed either a chow or palatable diet, and C57BL/6 lean and *ob/ob* mice were fed a chow diet for 14 weeks. All values are represented as mean ± S.E.M. ng/ml, where ** P<0.01.

Group	Pancreatic Insulin (ng/mg)
AKR/J +/? mice on chow diet	**22 ± 4**
AKR/J +/? mice on palatable diet	**99 ± 17 ****
C57BL/6J +/? mice on chow diet	**9 ± 2**
C57BL/6J *ob/ob* mice on chow diet	**227 ± 34 ****

4.2.2 Changes in gene expression in the pancreas

In the whole pancreas, the mRNA expression of cyclophilin was found to be the most suitable housekeeper (see Appendix 1) and was used as a covariate for ANCOVA analysis. In addition to the genes OB-Ra, OB-Rb, STAT3, STAT5, SOCS-3 and CIS, a number of other genes important for β-cell function were also analysed, namely insulin, PDX-1, glucokinase and GLUT2. Unfortunately, whilst analysing the expression of OB-Ra or OB-Rb in the pancreas, the amplification plots were inconsistent and there was too much

variation of the data to make any accurate comparisons of leptin receptor expression in the pancreas.

Figure 4.1 shows that there was no significant change in STAT3, STAT5, SOCS-3 or CIS mRNA in palatable diet-fed AKR/J mice compared to those fed a chow diet, although there was a trend towards a decrease in CIS mRNA expression (28% decrease). In the pancreas of *ob/ob* mice compared to leans, STAT5 and SOCS-3 mRNA expression was increased by 26% and 36% ($P<0.05$), respectively. The expression of STAT3 mRNA tended to increase (30%), whilst CIS mRNA showed little change. A comparison of the lean AKR/J and C57BL/6 strains showed that SOCS-3 mRNA was unchanged but STAT3, STAT5 and CIS were all increased in the pancreas of AKR/J lean mice by 31% ($P<0.05$), 35% ($P<0.01$) and 120% ($P<0.01$), respectively.

Figure 4.2 shows that in the pancreas of AKR/J mice fed a palatable compared to a chow diet, there was no change in the mRNA expression of insulin, PDX-1 or GLUT2, whereas glucokinase was reduced by 40% ($P<0.01$). In the pancreas of *ob/ob* mice compared to leans, insulin mRNA was increased by 11.7-fold ($P<0.01$), PDX-1 was increased by 25% ($P<0.05$) and GLUT2 was reduced by 53% ($P<0.01$), but there was no difference in expression of glucokinase. Figure 4.2 also shows that compared to C57BL/6 lean mice, the AKR/J lean mice have a 52% increase in insulin mRNA expression ($P<0.05$), a 45% increase in PDX-1 expression ($P<0.01$) and a 50% reduction in glucokinase expression ($P<0.01$), but no significant change in GLUT2 expression.

Figure 4.1 Changes in STAT3, STAT5, SOCS-3 and CIS mRNA in the pancreas of AKR/J mice fed either a chow or palatable diet, and C57BL/6 lean and *ob/ob* mice fed a chow diet

Comparisons of STAT3, STAT5, SOCS-3 and CIS mRNA are shown in the pancreas of AKR/J mice fed a palatable diet compared to those fed a chow diet, C57BL/6 *ob/ob* compared to leans fed on chow and AKR/J lean mice compared to C57BL/6 lean mice. Following TaqMan analysis and adjustment for cyclophilin, the mRNA levels for the control animals have been ascribed a value of 1 and the symbols indicate relative changes from this baseline (as described in Appendix 1). Error bars indicate 95% confidence intervals and changes are shown as significant where * $P<0.05$, ** $P<0.01$.

■ STAT3 mRNA

□ STAT5 mRNA

● SOCS-3 mRNA

○ CIS mRNA

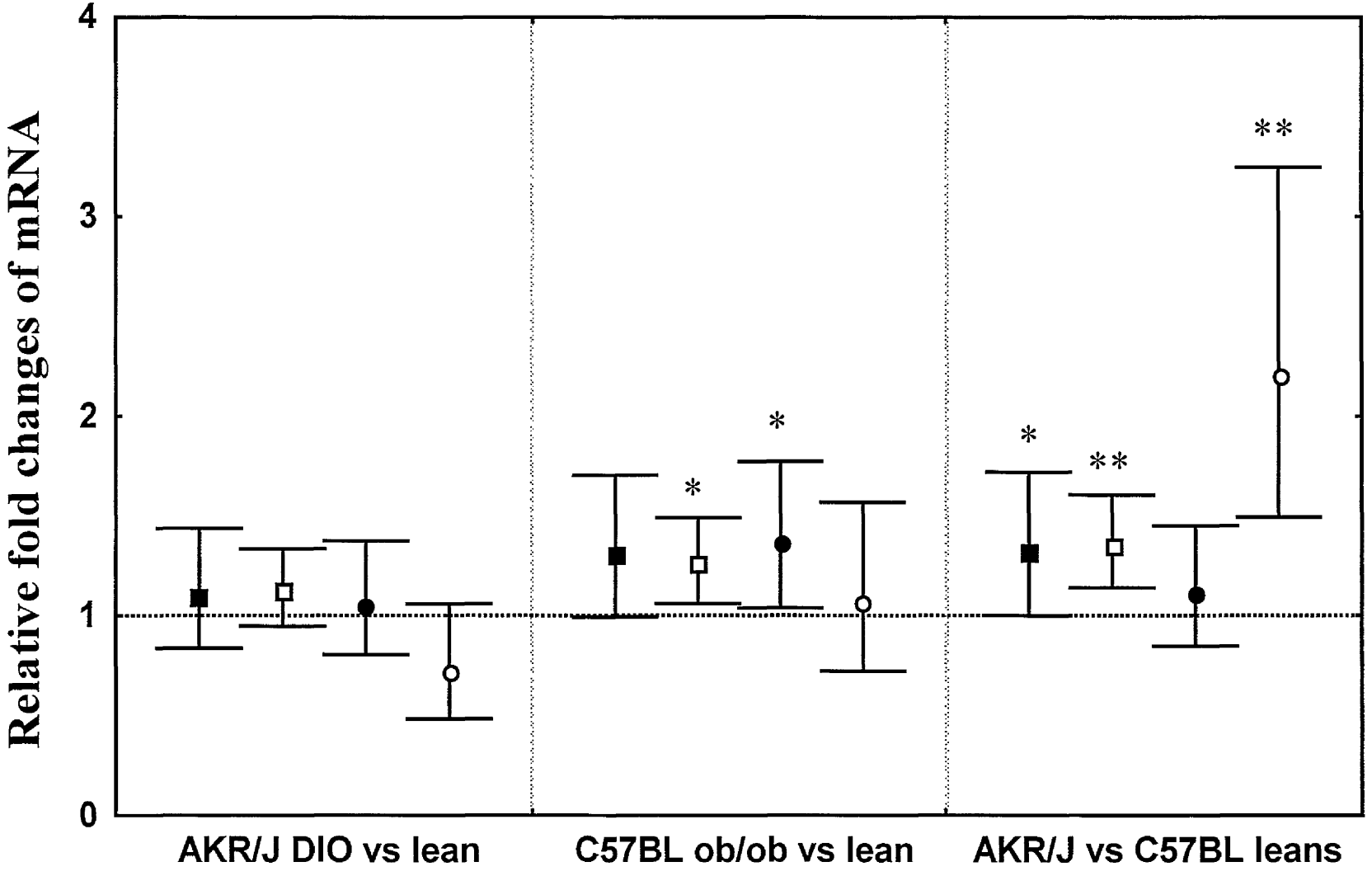

Relative fold changes of mRNA
4
3
2
1
0
*
*
*
**
**
AKR/J DIO vs lean
C57BL ob/ob vs lean
AKR/J vs C57BL leans

Figure 4.2 Changes in insulin, PDX-1, glucokinase and GLUT2 mRNA in the pancreas of AKR/J mice fed either a chow or palatable diet, and C57BL/6 lean and *ob/ob* mice fed a chow diet

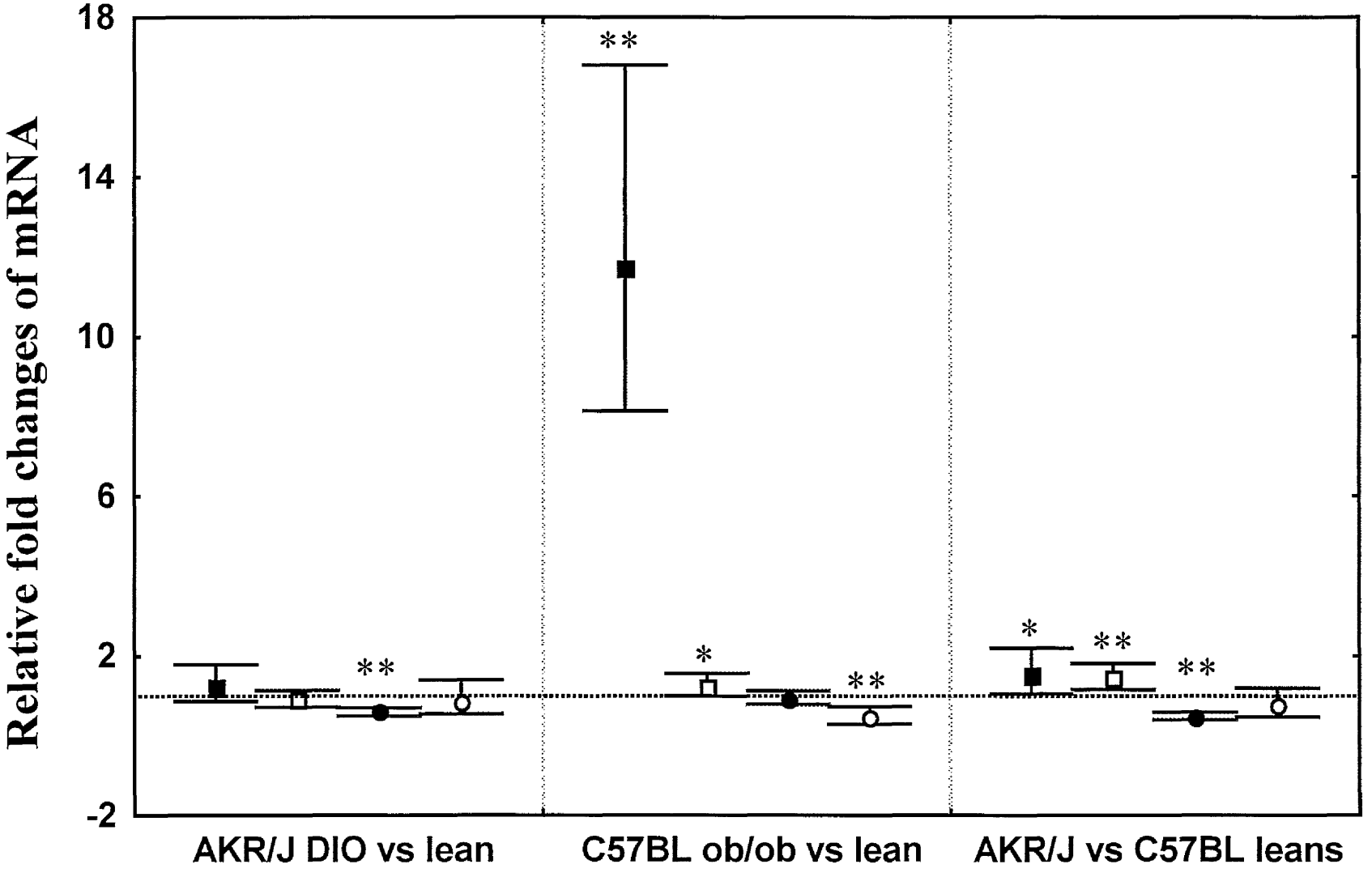
Comparisons of insulin, PDX-1, glucokinase and GLUT2 mRNA are shown in the pancreas of AKR/J mice fed a palatable diet compared to those fed a chow diet, C57BL/6 *ob/ob* compared to leans fed a chow diet and AKR/J lean mice compared to C57BL/6 lean mice. Following TaqMan analysis and adjustment for cyclophilin, the mRNA levels for the control animals have been ascribed a value of 1 and the symbols indicate relative changes from this baseline (as described in Appendix 1). Error bars indicate 95% confidence intervals and changes are shown as significant where * $P<0.05$, ** $P<0.01$

■ Insulin mRNA

□ PDX-1 mRNA

● Glucokinase mRNA

○ GLUT2 mRNA


Relative fold changes of mRNA
18
14
10
6
2
-2
**
*
**
*
**
**
AKR/J DIO vs lean
C57BL ob/ob vs lean
AKR/J vs C57BL leans

4.2.3 Changes in gene expression in WAT

For WAT, the mRNA expression of cyclophilin was found to be the most suitable as a housekeeper (see Appendix 1) and was used as a covariate for ANCOVA analysis. Only a comparison of leptin receptor-mediated signalling components were made in WAT of AKR/J mice fed palatable diet compared to mice on chow, since some of the WAT RNA samples from the *ob/ob* mice were lost whilst extracting the RNA. In palatable diet-fed AKR/J mice compared to chow fed mice, the change in expression of OB-Ra and OB-Rb mRNA tended to increase, altering by 72% and 40% (Figure 4.3), respectively, but these changes were not significant. There was little change in STAT3 mRNA but the expression of STAT5 mRNA was increased by 61% ($P>0.01$). Furthermore, expression of SOCS-3 mRNA was reduced by 60% whereas CIS mRNA expression was increased by 241% ($P<0.01$).

4.2.4 Changes in gene expression in BAT

In BAT, none of the housekeeping genes tested were suitable to use as a covariate for ANCOVA analysis, hence the data was represented as unadjusted. The changes in gene expression of OB-Ra and OB-Rb mRNA are shown in figure 4.4 (a). In AKR/J mice fed a palatable diet compared to mice on chow, there was no change in OB-Ra mRNA. OB-Rb mRNA was reduced by 34%, though the change was not significant. Both OB-Ra and OB-Rb mRNA expression was increased in *ob/ob* mice compared to leans by 62% and 53% ($P<0.01$), respectively. Furthermore, OB-Ra and OB-Rb were increased in

AKR/J lean mice compared to C57BL/6 lean mice by 125% and 224% ($P<0.01$), respectively.

The changes in gene expression of STAT3 and STAT5 mRNA are shown in figure 4.4 (b). The expression of STAT3 and STAT5 mRNA was increased, but not significantly, in AKR/J mice fed a palatable diet compared to chow by 15% and 20%, respectively. In *ob/ob* mice compared to leans, only STAT3 mRNA was increased by 145% ($P<0.01$), whereas STAT5 mRNA remained unchanged. No changes of STAT3 or STAT5 mRNA expression were observed in lean AKR/J mice compared to lean C57BL/6 mice.

The changes in gene expression of SOCS-3 and CIS mRNA are shown in figure 4.4 (c). In AKR/J mice fed a palatable diet compared to chow, SOCS-3 mRNA was increased by 24% and CIS was reduced by 29%, but the changes were not significant. The expression of SOCS-3 mRNA was substantially increased in *ob/ob* mice compared to leans by 406% ($P<0.01$), but CIS mRNA showed a non-significant 39% reduction. A comparison of AKR/J lean and C57BL/6 lean mice showed that CIS mRNA was increased by 280% in the BAT of AKR/J lean mice, but SOCS-3 mRNA remained unchanged.

Figure 4.3 Changes in gene expression of components of the leptin receptor-mediated signalling pathway in WAT of AKR/J mice fed either a chow or palatable diet

Comparisons of OB-Ra, OB-Rb, STAT3, STAT5, SOCS-3 and CIS mRNA are shown in WAT of AKR/J mice fed a palatable diet compared to those fed a chow diet. Following TaqMan analysis and adjustment for cyclophilin, the mRNA levels for the control animals have been ascribed a value of 1 and the symbols indicate relative changes from this baseline (as described in Appendix 1). Error bars indicate 95% confidence intervals and changes are shown as significant where * $P<0.05$, ** $P<0.01$.

■ OB-Ra mRNA

□ OB-Rb mRNA

● STAT3 mRNA

○ STAT5 mRNA

▲ SOCS-3 mRNA

△ CIS mRNA

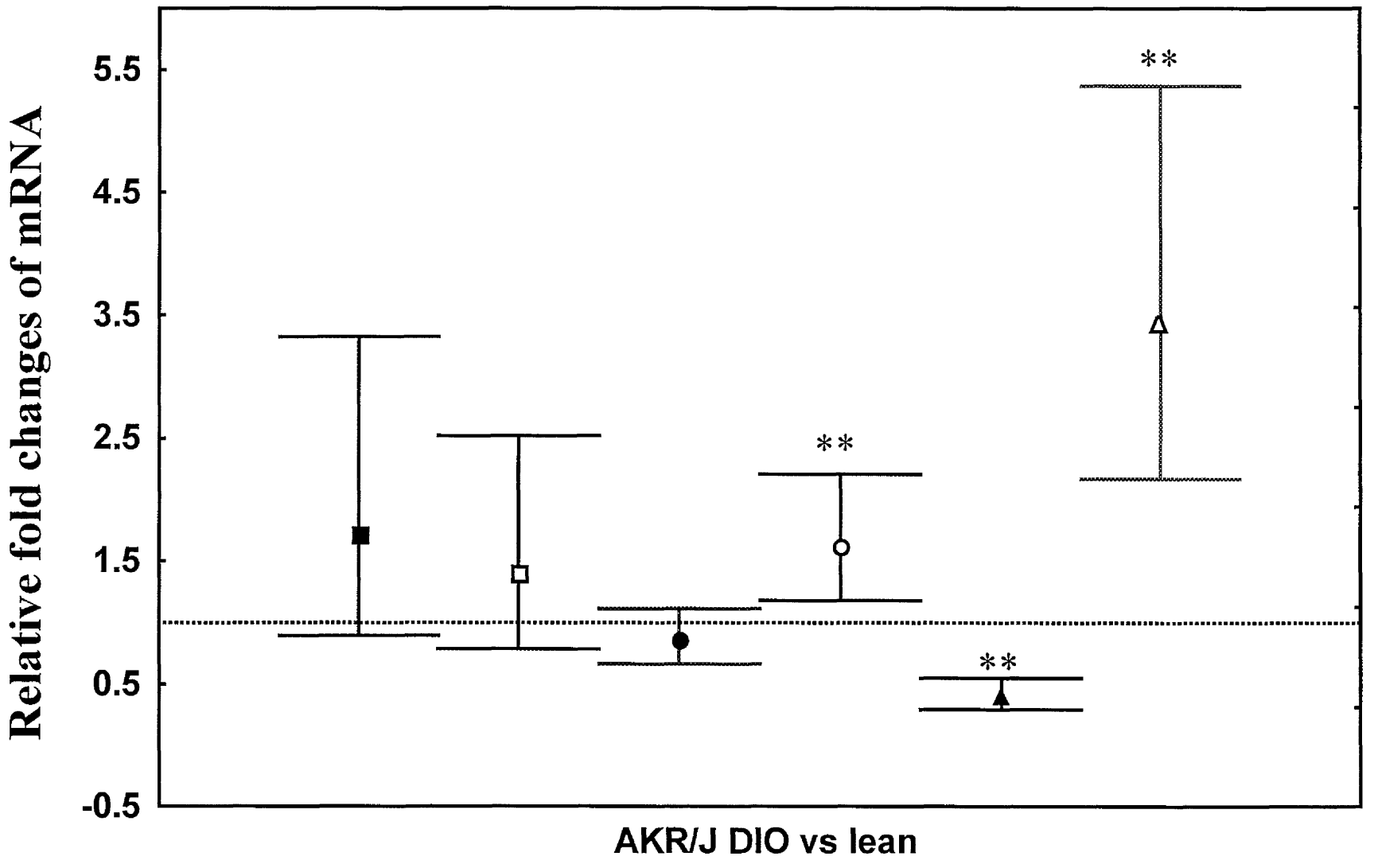

Relative fold changes of mRNA
5.5
4.5
3.5
2.5
1.5
0.5
-0.5
**
**
**
AKR/J DIO vs lean

Figure 4.4 Changes in gene expression of components of the leptin receptor-mediated signalling pathway in BAT of AKR/J mice fed either a chow or palatable diet, and C57BL/6 lean and *ob/ob* mice fed a chow diet

A comparison of gene expression changes are shown in BAT of AKR/J mice fed a palatable diet compared to those fed a chow diet, C57BL/6 *ob/ob* compared to leans on a chow diet and AKR/J lean mice compared to C57BL/6 lean mice. Following TaqMan analysis, the mRNA levels for the control animals have been ascribed a value of 1 and the symbols indicate relative changes from this baseline (as described in Appendix 1). Error bars indicate 95% confidence intervals and changes are shown as significant where * $P<0.05$ and ** $P<0.01$.

(a) ■ OB-Ra mRNA

□ OB-Rb mRNA

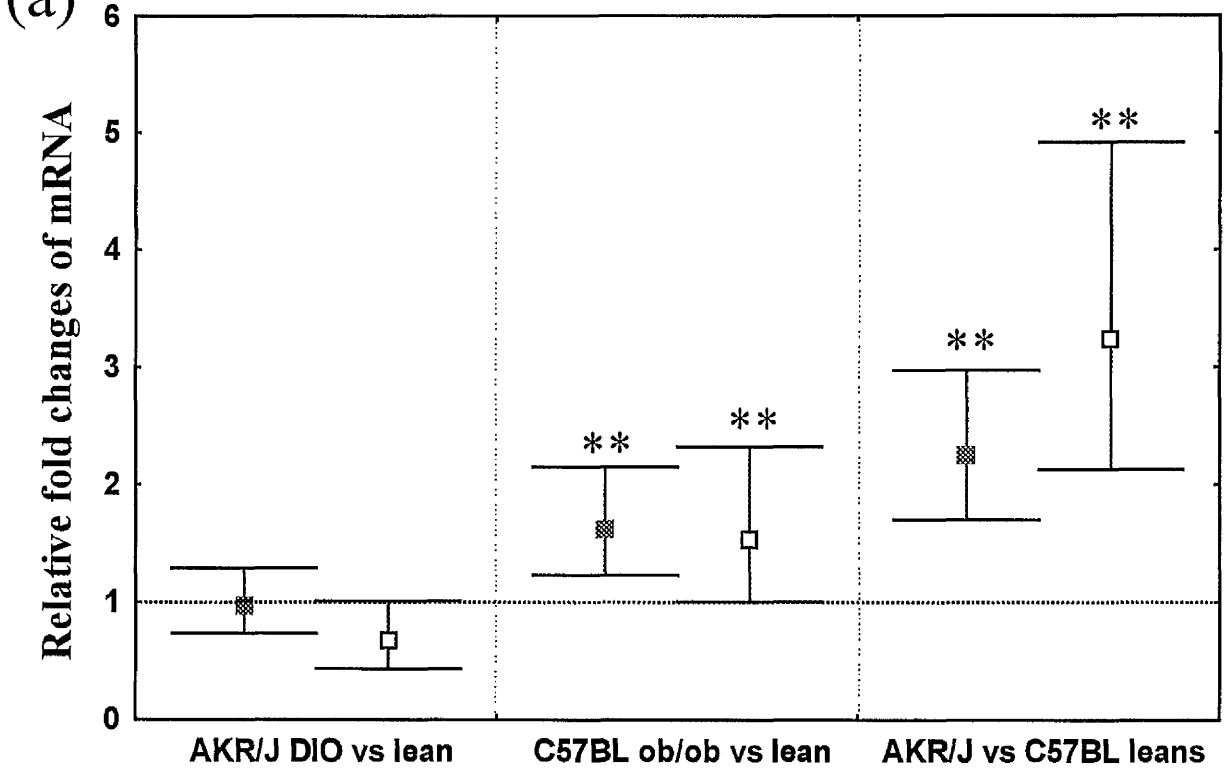
(b) ● STAT3 mRNA

○ STAT5 mRNA

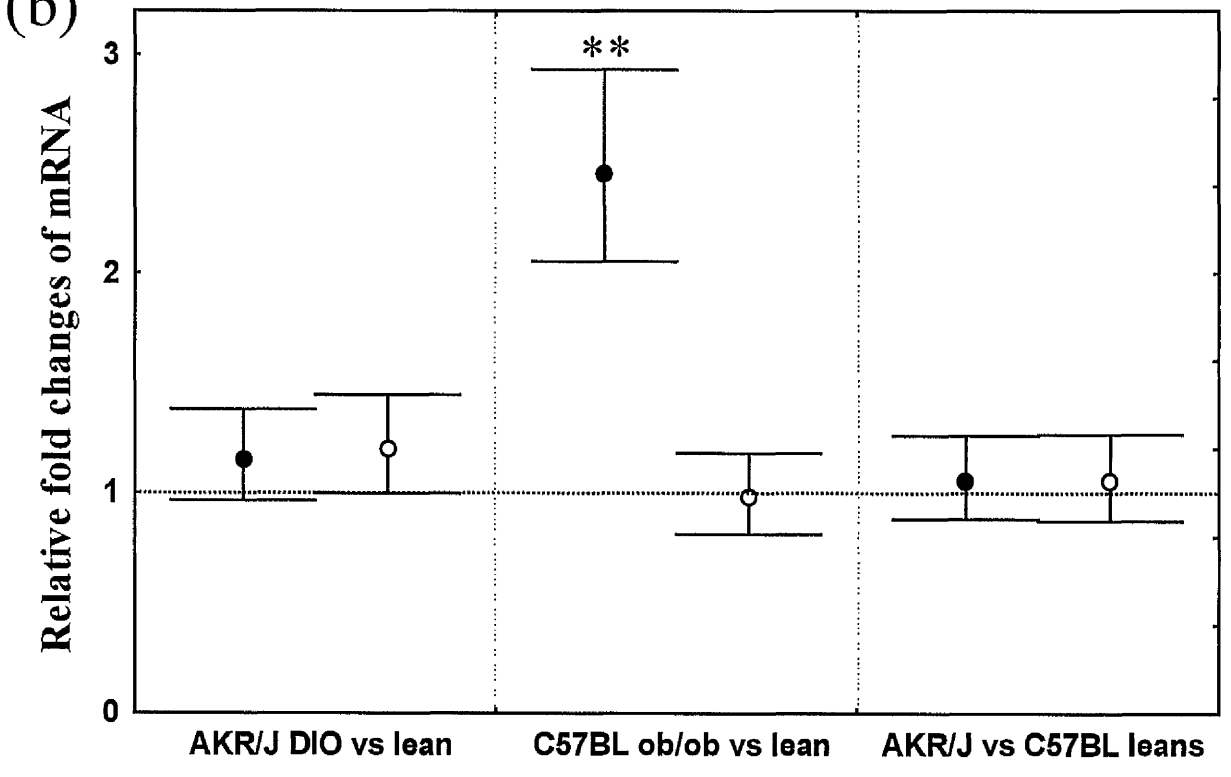
(c) ▲ SOCS-3 mRNA

△ CIS mRNA

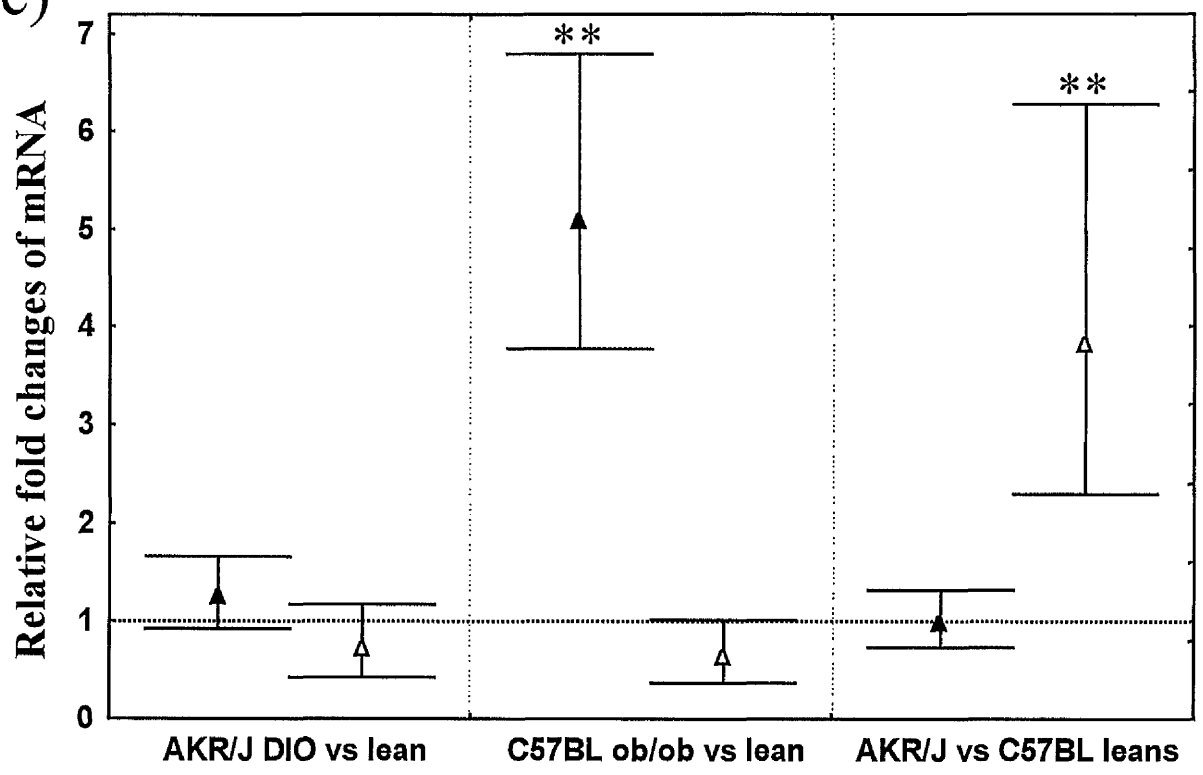
(a)


Relative fold changes of mRNA
0
1
2
3
4
5
6
**
**
**
**
AKR/J DIO vs lean
C57BL ob/ob vs lean
AKR/J vs C57BL leans

(b)


Relative fold changes of mRNA
0
1
2
3
**
AKR/J DIO vs lean
C57BL ob/ob vs lean
AKR/J vs C57BL leans

(c)


Relative fold changes of mRNA
0
1
2
3
4
5
6
7
**
**
AKR/J DIO vs lean
C57BL ob/ob vs lean
AKR/J vs C57BL leans

4.2.5 Changes in gene expression in small intestine

For the small intestine, the mRNA expression of HPRT was found to be the most suitable as a housekeeper (see Appendix 1) and was used as a covariate for ANCOVA analysis. The changes in gene expression of OB-Ra and OB-Rb mRNA are shown in figure 4.5 (a). OB-Ra and OB-Rb mRNA was reduced by 44% and 50% ($P<0.01$), respectively, in AKR/J mice fed a palatable diet compared to chow. Whilst OB-Ra mRNA was unchanged in *ob/ob* mice compared to lean, OB-Rb mRNA was reduced by 30% ($P<0.01$). In AKR/J lean mice compared to C57BL/6 lean mice, OB-Ra tended to increase but not significantly whereas OB-Rb was significantly increased by 46% ($P<0.01$).

The changes in gene expression of STAT3 and STAT5 mRNA are shown in figure 4.5 (b). In palatable diet-fed compared to chow-fed AKR/J mice, both STAT3 and STAT5 were reduced by 25% ($P<0.01$). However, only STAT3 mRNA, but not STAT5 mRNA, was increased in *ob/ob* mice compared to lean mice by 12% ($P<0.05$) and in AKR/J lean mice compared C57BL/6 lean mice by 32% ($P<0.01$). The expression of STAT5 mRNA was increased by 13% in AKR/J lean mice compared to C57BL/6 lean mice, but the change was not significant.

The changes in gene expression of SOCS-3 and CIS mRNA are shown in figure 4.4 (c). In AKR/J mice fed a palatable diet compared to chow fed mice, SOCS-3 mRNA was reduced by 45% ($P<0.05$) but CIS mRNA was unchanged. However, in *ob/ob* mice compared to leans, CIS mRNA was reduced by 47% ($P<0.01$) and SOCS-3 mRNA was unchanged. SOC-3 mRNA

was increased by 55% in AKR/J lean mice compared to C57BL/6 lean mice, but the change did not reach significance. Whilst CIS mRNA was also increased in AKR/J lean mice, the change was not as large as that in SOCS-3 mRNA and was not significant.

Figure 4.5 Changes in gene expression of components of the leptin receptor-mediated signalling pathway in the small intestine of AKR/J mice fed either a chow or palatable diet, and C57BL/6 lean and *ob/ob* mice fed a chow diet

A comparison of gene expression changes are shown in the small intestine of AKR/J mice fed a palatable diet compared to those fed a chow diet, C57BL/6 *ob/ob* compared to leans on a chow diet and AKR/J lean mice compared to C57BL/6 lean mice. Following TaqMan analysis, the mRNA levels for the control animals have been ascribed a value of 1 and the symbols indicate relative changes from this baseline (as described in Appendix 1). Error bars indicate 95% confidence intervals and changes are shown as significant where * $P<0.05$ and ** $P<0.01$.

(a) ■ OB-Ra mRNA

□ OB-Rb mRNA

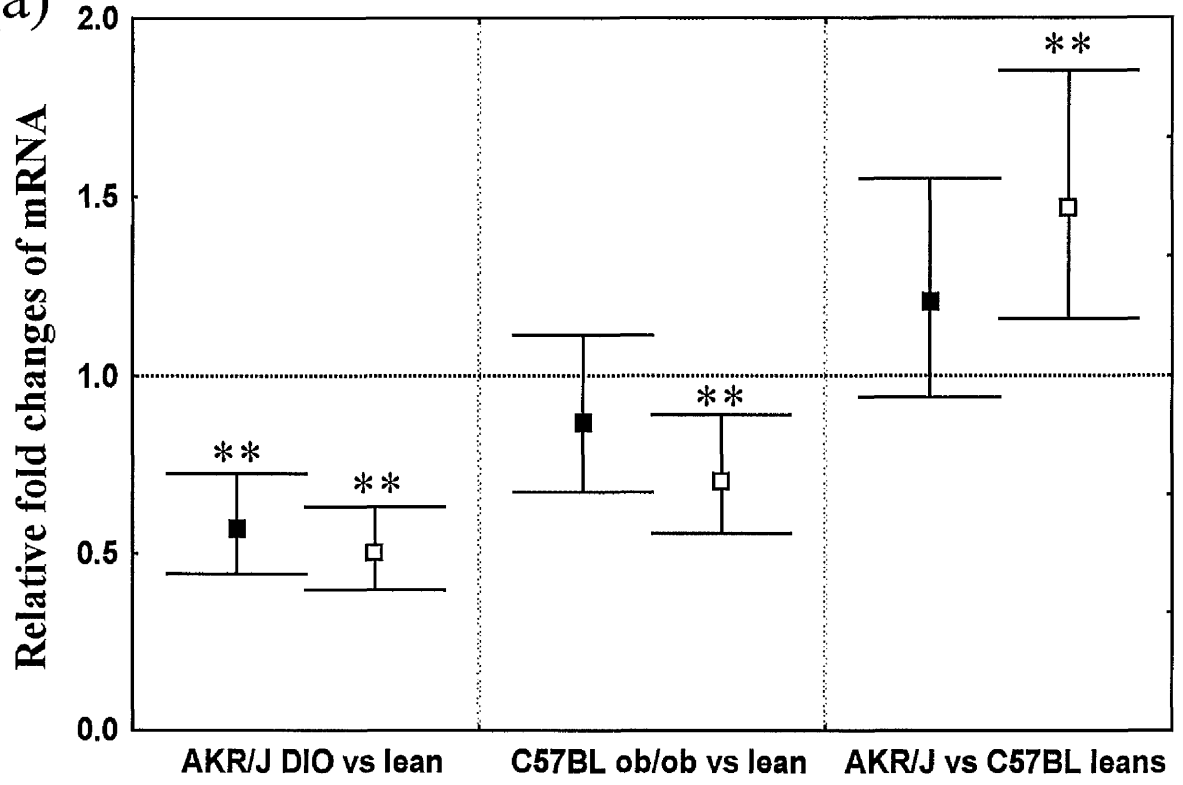
(b) ● STAT3 mRNA

○ STAT5 mRNA

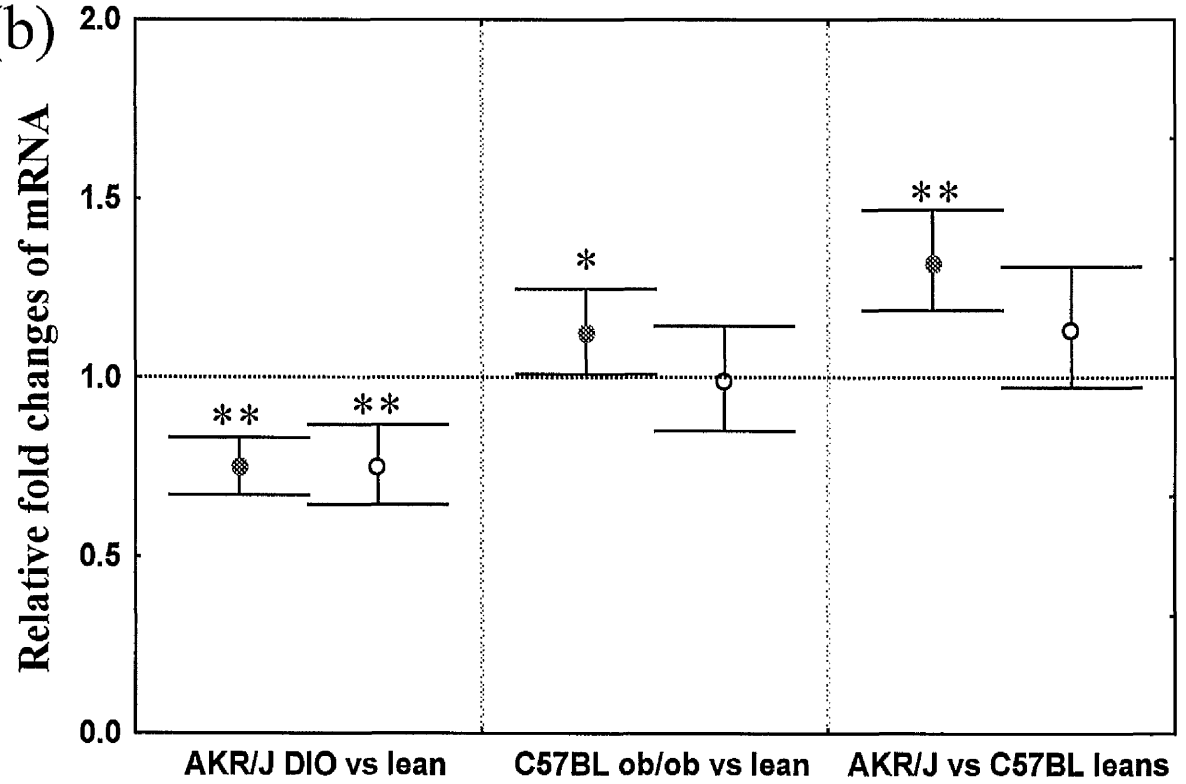
(c) ▲ SOCS-3 mRNA

△ CIS mRNA

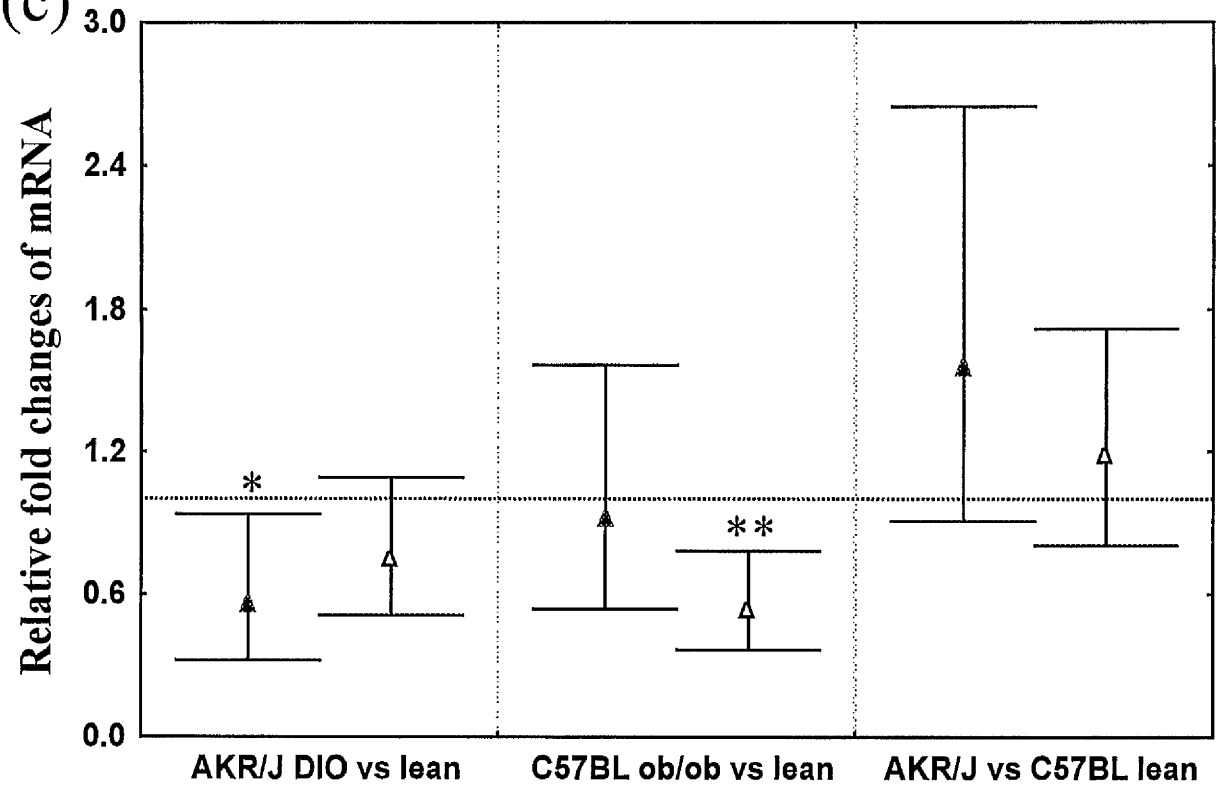
(a)


Relative fold changes of mRNA
2.0
1.5
1.0
0.5
0.0
**
**
**
**
**
AKR/J DIO vs lean
C57BL ob/ob vs lean
AKR/J vs C57BL leans

(b)


Relative fold changes of mRNA
2.0
1.5
1.0
0.5
0.0
**
**
*
**
AKR/J DIO vs lean
C57BL ob/ob vs lean
AKR/J vs C57BL leans

(c)


3.0
2.4
1.8
1.2
0.6
0.0
Relative fold changes of mRNA
*
**
AKR/J DIO vs lean
C57BL ob/ob vs lean
AKR/J vs C57BL lean

4.3 Discussion

The identification of leptin receptors in the hypothalamus implied that leptin action in the hypothalamus was responsible for the regulation of food intake and energy expenditure. However, the subsequent detection of leptin receptors in many peripheral tissues suggested leptin action was not restricted to the hypothalamus. The present chapter investigates the changes in expression of components of the leptin receptor-mediated signalling pathway in some of the peripheral tissues where leptin is known to exert its effects. The tissues examined were the pancreas, WAT, BAT and small intestine of AKR/J mice fed either a chow diet or a palatable diet and of C57BL/6 lean and genetically obese *ob/ob* mice fed on chow.

4.3.1 Pancreas

Of all the peripheral tissues examined by Ghilardi *et al.* (Ghilardi *et al.*, 1996), the pancreas was the most abundant in expression of OB-Rb mRNA, suggesting that it may play an important role in peripheral leptin action. Insulin is adipogenic, increases body fat mass, and stimulates the production and secretion of leptin (Barr *et al.*, 1997; Malmstrom *et al.*, 1996; Patel *et al.*, 1998), which acts centrally to reduce food intake and increase energy expenditure. Leptin in turn suppresses insulin secretion both centrally and through direct actions on β-cells (Ahren & Havel, 1999; Fehmann *et al.*, 1997b; Ishida *et al.*, 1997; Kulkarni *et al.*, 1997; Pallett *et al.*, 1997; Poitout *et al.*, 1998; Seufert *et al.*, 1999a; Shi *et al.*, 1998b; Shimizu *et al.*, 1997; Tanizawa *et al.*, 1997). Since

plasma leptin levels are directly proportional to body fat mass, an increase in adiposity increases plasma leptin, thereby curtailing insulin production and a further increase in fat mass. This bidirectional feedback loop may maintain nutrient balance and a dysregulation of this adipoinsular axis may contribute to obesity and the development of hyperinsulinaemia associated with diabetes. Analysis of changes in expression of components of the leptin receptor-mediated signalling pathway in leptin insensitive dietary obese and leptin-sensitive *ob/ob* mice compared to lean mice may indicate whether dysregulation of the adipoinsular axis is at the level of leptin signalling in the pancreas and adipose tissue.

In the pancreas, quantitative TaqMan RT-PCR amplified all the transcripts investigated efficiently except for OB-Ra and OB-Rb. Therefore, no conclusions regarding the expression of OB-Ra and OB-Rb in this tissue of obesity could be made. The exact reason for this is unclear, but it may be due to the reduced expression of leptin receptors over the whole pancreas compared to the islets (Emilsson *et al.*, 1997) or problem with amplification in the pancreas using the primer and probe set although this set worked well in other tissues.

Analysis of TaqMan data showed that there were no changes in the expression of STAT3 mRNA in the pancreas of AKR/J mice fed a palatable diet compared to chow-fed mice and chow-fed C57BL/6 *ob/ob* mice compared to leans. STAT3 activation has been reported in RINm5F cells, isolated rat islets, MIN6 cells and BRIN-BD11 cells (Briscoe *et al.*, 2001a; Morton *et al.*, 1999; Tanabe *et al.*, 1997) upon leptin treatment. This suggests that leptin sensitivity

in the pancreas of *ob/ob* mice or palatable diet-fed AKR/J mice is not regulated at the level of STAT3 mRNA. However, levels of STAT3 protein were not determined in this series of experiments to corroborate this suggestion. This observation is in contrast to the 27% reduction in hypothalamic STAT3 mRNA in *ob/ob* mice compared to leans suggesting leptin sensitivity in different tissues may be regulated in different ways. A comparison of STAT3 mRNA expression in the pancreas of AKR/J lean and C57BL/6 lean mice showed an increase in STAT3 mRNA in AKR/J lean mice.

Table 4.2 Summary of gene expression changes involved in leptin receptor-mediated signalling in the pancreas

	STAT3	STAT5	SOCS-3	CIS
Palatable vs Chow	NC	NC	NC	↓ 28% †
***ob/ob* vs lean**	↑ 30% †	↑ 26% *	↑ 36% *	NC
AKR/J vs C57BL/6	↑ 31% *	↑ 35% **	NC	↑ 120% **

Key: NC – no change, † - non-significant change, * $P<0.05$, ** $P<0.01$

The increased STAT3 and STAT5 mRNA in *ob/ob* mice compared to leans may be related to the increased insulin signalling through STAT3 (Ceresa *et al.*, 1997; Ceresa & Pessin, 1996; Coffer *et al.*, 1997) and STAT5 (Chen *et al.*, 1997; Storz *et al.*, 1999). Interestingly, it has been shown that leptin reduces the transcriptional activity of the rat insulin I gene promoter in *ob/ob* mice and alters binding of distinct proteins, including STAT5b complexes to upstream

sequences within the 5' promoter region of the rat insulin I gene (Seufert *et al.*, 1999b). The expression of STAT3 mRNA, though not significant, was increased by a similar magnitude in *ob/*ob mice to that of STAT5 mRNA suggesting the increased STAT3 and STAT5 mRNA may be due to increased leptin sensitivity or perhaps a result of increased insulin signalling. Furthermore, in the pancreas of *ob/ob* mice compared to leans, SOCS-3 mRNA was increased by 36%. It has recently been shown that triglyceride lowering in pancreatic islets, a major biologic action of leptin, was largely prevented by the overexpression of SOCS-3 (Wang *et al.*, 2000). The recent observations that insulin can induce SOCS-3 expression (Emanuelli *et al.*, 2000) and tyrosine phosphorylation (Peraldi *et al.*, 2001) suggest that the increase in SOCS-3 mRNA may be insulin-mediated and due to the hyperinsulinaemia in *ob/ob* mice. A comparison of AKR/J and C57BL/6 lean mice showed that STAT3 and STAT5 mRNA was increased by 31% and 35%, respectively, in AKR/J lean mice. The elevated STAT3 and STAT5 mRNA in the pancreas may be intrinsic to the AKR/J strain. In the pancreas of AKR/J mice fed a palatable diet and C57BL/6 *ob/ob* mice compared to their relative lean mice, there was no change in the expression of CIS mRNA. However, a comparison of CIS mRNA expression in the pancreas of AKR/J lean and C57BL/6 lean mice showed a 120% increase in AKR/J lean mice. It has been observed that the forced expression of CIS in RINm5F cells prevented leptin-mediated STAT3 transactivation (Emilsson *et al.*, 1999), suggesting the increased CIS mRNA in AKR/J lean mice may indicate reduced leptin sensitivity in the pancreas.

Insulin mRNA and pancreatic insulin was increased by 12-fold and 23-fold (see Table 4.1), respectively, in the pancreas of *ob/ob* mice with plasma insulin being increased 4.8-fold (see section 3.2.2), which is not surprising considering the *ob/ob* mice have an increased number of pancreatic β-cells and are insulin resistant. However, insulin mRNA was not significantly changed in the pancreas of AKR/J mice fed a palatable diet compared to chow, nor was plasma insulin significantly changed (see section 3.2.2). However, the pancreatic insulin content in these mice was significantly increased by over 4-fold compared to chow-fed mice suggesting the increased pancreatic insulin content in AKR/J mice fed a palatable diet may be due to increased proinsulin synthesis. A comparison of insulin mRNA between AKR/J lean and C57BL/6 lean mice showed that insulin mRNA was increased by 52% in the pancreas of AKR/J lean mice. A comparison of plasma insulin and pancreatic insulin content in AKR/J and C57BL/6 lean mice showed that there was no statistically significant difference between the two strains.

Table 4.3 Summary of changes in glucose-responsive genes in the pancreas

	Insulin	**PDX-1**	**Glucokinase**	**GLUT2**
Palatable vs Chow	NC	NC	↓ 40% **	NC
***ob/ob* vs lean**	↑1070% **	↑25% *	NC	↓ 53% *
AKR/J vs C57BL/6	↑ 52% *	↑ 45% **	↓ 50% **	NC

Key: NC – no change, * $P<0.05$, ** $P<0.01$

PDX-1 mRNA was increased in the pancreas of *ob/ob* mice compared to leans but remained unchanged in AKR/J mice fed a palatable diet compared to mice on chow. Since PDX-1 is a β-cell specific transcription factor, which binds to promoters of several β-cell specific transcripts, including insulin and glucokinase, the observed increase along with that of insulin is probably related to the β-cell hyperplasia observed in *ob/ob* mice. The increase in PDX-1 corresponds with the increase in insulin mRNA expression also observed in AKR/J lean mice compared to C57BL/6 lean mice. Reduced glucose tolerance is usually associated with reduced glucokinase and/or GLUT2 (Ishihara *et al.*, 1995; Valera *et al.*, 1994). In the pancreas of AKR/J mice fed a palatable diet compared to chow, glucokinase mRNA was reduced by 40% though GLUT2 mRNA was unchanged. GLUT2 mRNA was reduced by 53% in the pancreas of *ob/ob* mice compared to leans, but glucokinase mRNA was unchanged, suggesting glucose sensitivity may be regulated more through glucose transport than through glucokinase expression in *ob/ob* mice. The reduced expression of glucokinase mRNA in AKR/J compared to C57BL/6 lean mice suggests the AKR/J strain are more susceptible to reduced glucose sensitivity.

4.3.2 WAT

The components of the leptin receptor-mediated signalling pathway were also analysed in WAT, but only in AKR/J mice fed a palatable diet because the RNA was not extracted appropriately from the *ob/ob* mice. The expression of OB-Ra and OB-Rb mRNA was increased by 72% and 40%, respectively, in

AKR/J mice fed a palatable diet, but the changes were not significant. The variability in these measurements are large, however if there is an actual increase of OB-R mRNA, then it may be that regulation of leptin sensitivity is at a post-transcriptional level.

Table 4.4 Summary of gene expression changes involved in leptin receptor-mediated signalling in WAT

	OB-Ra	OB-Rb	STAT3	STAT5	SOCS-3	CIS
Palatable vs Chow	↑ 72% †	↑ 40% †	NC	↑ 61% **	↓ 60% **	↑ 241% **

Key: NC – no change, † - non-significant change, ** P <0.01

Investigations of leptin-mediated STAT activation in BAT and WAT have implicated the involvement of STAT1 and STAT3. Bendinelli *et al.* (Bendinelli *et al.*, 2000) showed that intraperitoneal leptin administration to C57BL/6 lean and *ob/ob* mice activates STAT3 and STAT1 in epididymal adipose tissue after 10 minutes. Siegrist-Kaiser *et al.* (Siegrist-Kaiser *et al.*, 1997) showed leptin-inducible binding of STATs to the M67-SIF element that binds STAT1 and STAT3 in nuclear extracts from brown and white adipocytes. Despite evidence of STAT1 as well as STAT3 involvement in leptin signalling in WAT and BAT, the present study investigated changes in STAT3 and STAT5 mRNA in these tissues as a comparison with changes in these genes with other tissues. There were no changes in STAT3 mRNA in AKR/J mice fed

a palatable diet compared to chow, but STAT5 mRNA was increased. There have been no indications of STAT5 responsiveness to leptin action in WAT and so the increase in STAT5 mRNA may be leptin-independent.

Wang *et al.* have demonstrated that a negative feedback loop links circulating leptin to its own biosynthesis in adipose tissue (Wang *et al.*, 1999b). The authors demonstrated that the protein expression of SOCS-3 was increased in adipocytes of diet-induced obese rats, which were hyperleptinaemic, and consistent with a role of SOCS-3 in the resistance of the adipocyte to autosuppression by leptin (Wang *et al.*, 2000). Analysis of SOCS-3 mRNA in WAT of AKR/J mice fed a palatable diet showed a 60% reduction compared to mice on chow. Therefore, the reduced SOCS-3 mRNA does not support the hypothesis of Wang *et al*, although it is conceivable that the decrease is not reflected at the protein level. The expression of CIS mRNA was increased by 240% in WAT of AKR/J mice fed a palatable diet. Although CIS has not been implicated in any leptin-mediated effects in WAT, it is conceivable that it is the increased expression of CIS mRNA rather than SOCS-3 that mediates the reduced sensitivity to leptin in this study. Alternatively, the increased CIS mRNA may be a result of increased STAT5 mRNA expression and activity (assuming STAT5 basal activity is increased if STAT5 protein is increased) and may also be leptin independent.

4.3.4 BAT

In BAT, leptin increases the expression of UCP-1 (Commins *et al.*, 2000), which is consistent with the role of BAT in regulating the body heat in small animals, and shifting fuel selection by reducing carbohydrate oxidation and increasing fat oxidation (Hwa *et al.*, 1996; Hwa *et al.*, 1997). This change in fuel consumption is thought to involve changes in gene expression including both centrally mediated effects and direct effects through the OB-Rb isoform. Therefore, it is possible, that there may be changes in expression of components of the leptin receptor-mediated signalling pathway in BAT of dietary obese and genetically obese mice.

Table 4.5 Summary of gene expression changes involved in leptin receptor-mediated signalling in BAT

	OB-Ra	OB-Rb	STAT3	STAT5	SOCS-3	CIS
Palatable vs Chow	NC	↓ 34% †	↑ 15% †	↑ 20% †	NC	NC
***ob/ob* vs lean**	↑ 62% **	↑ 53% *	↑ 145% **	NC	↑406% **	↓ 39% †
AKR/J vs C57BL/6	↑ 125% **	↑ 224% **	NC	NC	NC	↑ 279% **

Key: NC – no change, † - non-significant change, * $P<0.05$, ** $P<0.01$

In BAT of AKR/J mice fed a palatable diet compared to chow, the expression of OB-Rb was reduced by 34% but not significantly suggesting leptin sensitivity in BAT may be low in these mice. However, in *ob/ob* mice, both OB-Ra and OB-Rb mRNA was increased by 62% and 53%, respectively, which is in line with the high levels of leptin sensitivity in these mice due to reduced circulating leptin. The expression of OB-Ra and OB-Rb mRNA was also increased in AKR/J compared to C57BL/6 lean mice by 125% and 224%, respectively. It is not clear why there is such a large difference in leptin receptor mRNA expression between AKR/J and C57BL/6 lean mice, but may be due to strain-specific differences.

The expression of STAT3 and STAT5 mRNA was marginally increased, but not significantly, in AKR/J mice fed a palatable diet compared to chow by 15% and 20%, respectively. The increased fat oxidation in BAT may result in increased expression of STAT mRNA amongst other genes. The expression of STAT3 mRNA was also increased by 145% in BAT of *ob/ob* mice, again in line with an increase in leptin sensitivity in this tissue. There was no difference in STAT5 mRNA expression in BAT of *ob/ob* mice compared to lean animals or in either of the STATs in BAT of AKR/J compared to C57BL/6 lean mice. The lack of change in STAT5 mRNA expression in BAT of *ob/ob* mice may suggest that changes in STAT5 are not leptin-mediated or that STAT3 is more important in leptin signalling in this tissue.

In AKR/J mice fed a palatable diet compared to chow, the expression of SOCS-3 mRNA was increased by 24% and CIS was reduced by 29%, however

these changes were not significant. The expression of SOCS-3 mRNA was considerably increased in BAT of *ob/ob* mice by about 400%, which would be expected to lead to reductions in leptin sensitivity. It is possible that the change in SOCS-3 mRNA may be due to compensation for the increased STAT3 or due to other obesity related factors. However, CIS mRNA was reduced by 39%, but not significantly, in *ob/ob* mice compared to leans, suggesting it is not involved in increased leptin sensitivity of *ob/ob* mice if it is involved in leptin-mediated signalling in BAT. A comparison of AKR/J and C57BL/6 lean mice showed no change in SOCS-3 mRNA, but CIS mRNA was increased in AKR/J lean mice by about 280% compared to C57BL/6 lean mice. It is not clear why there are such large changes of CIS mRNA expression in AKR/J lean mice but this may play a role in the phenotype of this mouse possibly in decreasing leptin sensitivity or as a compensatory mechanism for increases in OB-Rb.

4.3.4 Small Intestine

Several studies have reported the expression of leptin receptors in the small intestine (Lostao *et al.*, 1998; Morton *et al.*, 1998). STAT5 was activated by leptin in the small intestine with STAT3 and STAT5 being activated by leptin in CACO-2 cells, a human model of small intestine epithelium (Emilsson *et al.*, 1999). In particular, Morton *et al.* have demonstrated that the multiple short isoforms are expressed throughout the gastrointestinal tract, whereas the functional OB-Rb is predominantly expressed in the jejunum and more weakly in the ileum, the two major sites that are involved with nutritional absorption

and lipid uptake (Morton *et al.*, 1998). Furthermore, Emilsson *et al.* showed CIS mRNA was increased 2-fold in the small intestine of *ob/ob* mice compared to leans, but no changes in expression of OB-R, STAT3, STAT5 or SOCS-3 mRNA were observed (Emilsson *et al.*, 1999). Leptin has an inhibitory effect on intestinal sugar uptake (Lostao *et al.*, 1998), consistent with leptin's effect on appetite suppression and increases in basal metabolism in order to maintain body weight.

The expression of OB-Ra and OB-Rb mRNA was reduced by 44% and 50%, respectively, in the small intestine of AKR/J mice fed a palatable diet compared to chow. It may be that the increased circulating leptin levels may have had an inhibitory effect on OB-Ra and OB-Rb mRNA expression. However, in the small intestine of *ob/*ob mice compared to leans, OB-Rb mRNA was reduced although these mice are hypersensitive to leptin. Perhaps OB-Rb mRNA may be regulated in the small intestine of *ob/ob* mice by obesity-related factors other than leptin. A comparison of AKR/J lean and C57BL/6 lean mice showed that OB-Ra and OB-Rb mRNA was increased in AKR/J lean mice by 21 and 46%, respectively, however, the increased OB-Ra mRNA was not statistically significant. The increased OB-R expression may be related to the AKR/J strain being more prone to dietary obesity compared to the C57BL/6 strain.

Investigations published to date in the small intestine, have only looked at the difference in expression of components of the leptin receptor-mediated signalling pathway in C57BL/6 lean and *ob/ob* mice, but not in a diet-induced

model of obesity. Emilsson *et al.* reported no change in the expression of OB-R mRNA (common to all isoforms) in the small intestine of *ob/ob* mice compared to lean (Emilsson *et al.*, 1999). Similarly, Morton *et al.* observed no change of OB-R mRNA (common to all isoforms) in the duodenum, jejunum or ileum of the small intestine in *ob/ob* mice (Morton *et al.*, 1998). However, both of the above studies have quantified the expression of OB-R mRNA as a ratio of β-actin mRNA assuming the expression of β-actin remains unchanged in all the tissue they have tested. It may be that changes in the level of housekeeping genes may influence the expression of leptin receptors in any given tissue. In this study, four different housekeeping genes were analysed and the most suitable housekeeping gene (HPRT) was selected that changed the least and is independent of the test gene in the tissues analysed. These differences in housekeeping genes may account for the differences observed in OB-R expression. It is also important to consider that the small changes observed in this study may not have a significant effect on leptin signalling. Alternatively, these mRNA changes may not be reflected at the protein level.

Table 4.6 Summary of gene expression changes involved in leptin receptor-mediated signalling in the small intestine

	OB-Ra	OB-Rb	STAT3	STAT5	SOCS-3	CIS
Palatable vs Chow	↓ 44% **	↓ 50% **	↓ 25% **	↓ 25% **	↓ 45% *	↓ 25% †
***ob/ob* vs lean**	NC	↓ 30% **	↑ 12% *	NC	NC	↓ 47% **
AKR/J vs C57BL/6	↑ 21% †	↑ 46% **	↑ 32% **	↑ 13% †	↑ 55% †	NC

Key: NC – no change, † - non-significant change, * $P<0.05$, ** $P<0.01$

The analysis of STAT3 and STAT5 mRNA expression showed that both genes were reduced by 25% in the small intestine of AKR/J mice fed a palatable diet compared to chow-fed mice. This reduction, albeit small is consistent with the reductions observed in Ob-Ra and Ob-Rb suggesting a general decrease in leptin signalling capacity. In *ob/ob* mice, STAT3 mRNA was increased by 12% compared to lean mice, yet STAT5 mRNA remained unchanged. Emilsson *et al.* observed no changes in the expression of STAT3 or STAT5 in *ob/ob* mice compared to lean mice (Emilsson *et al.*, 1999). Therefore, it is unclear whether such a small increase in STAT3 mRNA is biologically significant. A comparison of AKR/J and C57BL/6 lean mice showed that both STAT3 and STAT5 mRNA expression was increased in AKR/J mice by 32% and 13%, respectively, but the change in STAT5 mRNA was small and not significant.

The expression of SOCS-3 and CIS mRNA in the small intestine of AKR/J mice fed a palatable diet compared to chow-fed mice was reduced by 45% and 25%, respectively, but the reduced CIS mRNA was not significant. The reduced expression of SOCS-3 and CIS mRNA in AKR/J mice fed a palatable diet may be due to the reduced expression of STATs, or by a general downregulation of leptin signalling as indicated by the reduced expression of leptin receptor and STATs in these mice. As mentioned previously, Emilsson *et al.* observed no change in SOCS-3 mRNA, but CIS mRNA was increased 2-fold in *ob/ob* mice compared to leans Emilsson *et al.* (1999). In this study, SOCS-3 mRNA was unchanged, however, CIS mRNA was reduced by 47% in *ob/ob* mice compared to leans. Contrary to the reduced CIS mRNA observed here, Emilsson *et al.* observed a 2-fold increase of CIS mRNA in *ob/ob* mice compared to leans (Emilsson *et al.*, 1999). The reduced expression of CIS mRNA may compensate for the reduced OB-Rb in the small intestine of *ob/ob* mice and is consistent with elevated leptin sensitivity in *ob/ob* mice. In AKR/J compared to C57BL/6 lean mice, the expression of SOCS-3 mRNA was increased by 55%, but not significantly, and CIS mRNA remained unchanged, suggesting the increased SOCS-3 mRNA may be involved in AKR/J lean mice being more prone to dietary obesity.

4.3.5 Summary

In summary, there were no changes in STAT3, STAT5 or SOCS-3 mRNA in the pancreas of AKR/J mice fed a palatable diet compared to those fed chow, suggesting leptin sensitivity is not regulated at the level of these components of leptin receptor-mediated signalling pathway. The increased STAT3, STAT5 and SOCS-3 mRNA in the pancreas of *ob/ob* mice could perhaps be due to increased insulin signalling. In WAT of AKR/J mice fed a palatable diet, the increased OB-Ra and OB-Rb mRNA, and reduced SOCS-3 mRNA are inconsistent with low leptin sensitivity in this tissue, however instead this could be controlled at the level of CIS.

In BAT of AKR/J mice fed a palatable diet, OB-Rb mRNA was reduced, which is consistent with low leptin sensitivity in this tissue. Furthermore, the increased expression of STAT3 and STAT5 mRNA may be indicative of increased fat oxidation or UCP expression in BAT. The increased OB-Ra and OB-Rb mRNA in BAT of *ob/ob* mice is consistent with increased leptin sensitivity. However, the increased STAT3 and SOCS-3 mRNA may be due to other obesity related factors. In the small intestine of AKR/J mice fed a palatable diet, Ob-Ra and OB-Rb mRNA may be downregulated due to elevated plasma leptin levels and the reduced STAT3, STAT5, SOCS-3 and CIS mRNA may be a result of reduced leptin signalling. The reduced OB-Rb and increased STAT3 mRNA in the small intestine of *ob/ob* mice may be regulated by obesity-related factors other than leptin deficiency as an increase in OB-Rb may be expected. In AKR/J lean mice, the increased expression of components of

the leptin receptor-mediated signalling pathway in the pancreas (STAT3, STAT5 and CIS), BAT (OB-Ra, OB-Rb and CIS) and small intestine (OB-Ra, OB-Rb, STAT3, STAT5 and SOCS-3) may contribute to the phenotype and susceptibility to diet-induced obesity of AKR/J mice.

The observation that diet-induced obese mice respond to central but not peripheral leptin administration with respect to reduced food intake (Lin *et al.*, 2000; Van Heek *et al.*, 1997), suggests that peripheral tissues may exhibit changes in leptin sensitivity. However, changes in leptin sensitivity in peripheral tissues have not been demonstrated. The changes in expression of genes involved in leptin receptor-mediated signalling in peripheral tissues may be better understood knowing the effects of leptin sensitivity on other metabolic effects in these models of obesity.

Chapter 5

Quantitative Expression of Genes Involved in the Leptin Receptor-Mediated STAT Signalling Pathway in *ob/ob* mice treated with Intraperitoneal Leptin

5.1 Introduction

The homology of the leptin receptor to the class I cytokine family of receptors suggested that leptin signals via the activation of JAKs and STAT transcription factors. This hypothesis was verified in a number of *in vitro* and *in vivo* studies that investigated leptin signalling by the long form of the leptin receptor. OB-Rb can activate JAK2 upon leptin binding and JAK2 can be co-immunoprecipitated with OB-Rb both in the presence or absence of leptin in BaF3 cells, a factor-dependent haematopoietic cell line (Ghilardi & Skoda1997). *In vitro* studies showed that leptin can activate STATs 1, 3, 5 and 6 in COS cells transfected with leptin receptor (Baumann *et al.*, 1996; Ghilardi *et al.*, 1996), but *in vivo* leptin activated only STAT3 in the hypothalamus of *ob/ob* mice and normal rats with maximal activation achieved after 30 minutes (Mccowen *et al.*, 1998; Vaisse *et al.*, 1996). Leptin was also shown to activate STAT5 in the jejunum of wild-type and *ob/ob* mice but not *db/db* mice (Morton *et al.*, 1998). Peripheral leptin administration of lean mice can induce the transcription of cytokine-inducible inhibitors of signalling genes, SOCS-3 and CIS, in the hypothalamus, liver and small intestine (Emilsson *et al.*, 1999). These proteins can in turn negatively regulate leptin signalling, either through inhibition of JAK kinases or by directly binding to STATs (Starr *et al.*, 1997; Starr & Hilton, 1998; Yoshimura *et al.*, 1995).

As discussed in chapter 3, there are a number of possible reasons for changes in leptin sensitivity, including changes in expression of leptin receptor isoforms or signalling components. Therefore, the expression of components of

leptin receptor-mediated signal transduction (namely OB-Ra, OB-Rb, STAT3, STAT5, SOCS-3 and CIS) was examined in the hypothalamus of *ob/ob* mice, which are highly sensitive to the leptin effects on feeding, treated intraperitoneally with leptin.

Most of the *in vivo* studies to date have looked at the expression of STATs and SOCS a short period after leptin treatment (Bjorbaek *et al.*, 1998b; Vaisse *et al.*, 1996). However, a study by Emilsson *et al.* has investigated changes in expression of components of leptin receptor-mediated signalling in *ob/ob* mice treated with leptin for 48 hours (Emilsson *et al.*, 1999). Leptin treatment of lean and *ob/ob* mice for 48 hours showed no change in leptin receptor expression, although there was a 2-fold increase in leptin receptor expression (all isoforms) in the hypothalamus of *ob/ob* mice (Emilsson *et al.*, 1999). The increased leptin receptor expression has also been observed in the arcuate nucleus in two other studies (Baskin *et al.*, 1998; Huang *et al.*, 1997). No change in STAT3 or STAT5 mRNA was observed in the hypothalamus after 48 hours of leptin treatment (Emilsson *et al.*, 1999). In the study by Emilsson *et al.*, the 48 hour leptin treatment resulted in a 2-fold increase in CIS, but not SOCS-3, mRNA in the hypothalamus of *ob/ob* mice, but a 5-fold increase in both SOCS-3 and CIS in the hypothalamus of lean mice (Emilsson *et al.*, 1999). Furthermore, in comparing lean and *ob/ob* mice, the expression of SOCS-3 and CIS was increased 2-fold in the hypothalamus of *ob/ob* mice treated with vehicle. A 2-fold increase of SOCS-3, but not CIS, mRNA in the hypothalamus

of *ob/ob* mice was maximal between 1-3 hours after leptin treatment (Bjorbaek *et al.*, 1998b).

In this study, I have examined the mRNA expression of leptin receptor-mediated signalling components by quantitative TaqMan RT-PCR in the hypothalamus of *ob/ob* mice treated with leptin for 24-hours and 2-weeks. The aim was to compare short- and long-term leptin treatment of *ob/ob* mice to changes in expression of components of the leptin receptor-mediated signalling pathway that may contribute to leptin sensitivity. Furthermore, changes in components of leptin receptor-mediated signalling were compared between lean and *ob/ob* mice. The expression of hypothalamic NPY mRNA expression was also examined, since the effect of leptin on food intake and energy balance involves altered hypothalamic NPY gene expression.

5.2 Results

The groups of animals are as described in the table below. Groups B and C were sacrificed after 24 hours of vehicle/leptin treatment and the remaining groups were sacrificed after 2 weeks from the beginning of the study.

Table 5.1 Group descriptions of leptin-treated C57BL/6 *ob/ob* mice

Group	Description
A	C57BL/6 lean (+/?) mice
B	C57BL/6 *ob/ob* mice treated with vehicle for 24 hours
C	C57BL/6 *ob/ob* mice treated with leptin for 24 hours
D	C57BL/6 *ob/ob* mice treated with vehicle for 2 weeks
E	C57BL/6 *ob/ob* mice treated with leptin for 2 weeks
F	C57BL/6 *ob/ob* mice pair-fed for 2 weeks

5.2.1 Body Weight

The body weight of the mice was measured daily before leptin treatment (Figure 5.1). The body weight of the C57Bl/6 lean mice changed little during the two weeks being 19.6 ± 0.5 g at the beginning and 20.7 ± 0.5 g at the end of the study. In the C57Bl/6 *ob/ob* mice treated for 24-hours with vehicle, the body weight remained similar at 35.5 ± 0.9 g before vehicle treatment and 35.7 ± 1.0 g after vehicle treatment. After 24-hour leptin treatment (1 mg/kg/day), the body weight of C57Bl/6 *ob/ob* mice was reduced from 36.5 ± 0.7 g to 35.1± 0.7 g but the change was not statistically significant. In the C57Bl/6 *ob/ob* mice treated with vehicle for 2-weeks, the body weight steadily increased from 35.8 ± 1.1 g to 40.6 ± 1.0 g. However, the body weight of leptin-treated C57Bl/6 *ob/ob* mice was significantly reduced over the 2-week period from 37.1 ± 0.5 g to 30.8 ± 0.7 g ($P<0.01$). A comparison of C57Bl/6 *ob/ob* mice treated with leptin for 2-weeks compared to vehicle-treated mice showed that changes in body weight were statistically significant after 4 days of treatment ($P<0.01$). The body weight of the pair-fed group was intermediate between the vehicle and leptin-treated groups and after 2-weeks changed from 36.2 ± 2.0 g to 36.3 ± 1.0 g. In comparing vehicle-treated *ob/ob* mice to pair-fed *ob/ob* mice, the body weight of the pair-fed group was significantly reduced from day 9 of leptin treatment ($P<0.05$). A comparison of body weight in the leptin-treated *ob/ob* mice and pair-fed group showed the weight of the pair-fed group was significantly higher 2 days after the start of the study, then at 4 days and from day 6 onwards of the study ($P<0.05$).

Figure 5.1 Changes in body weight of leptin-treated C57BL/6 *ob/ob* mice

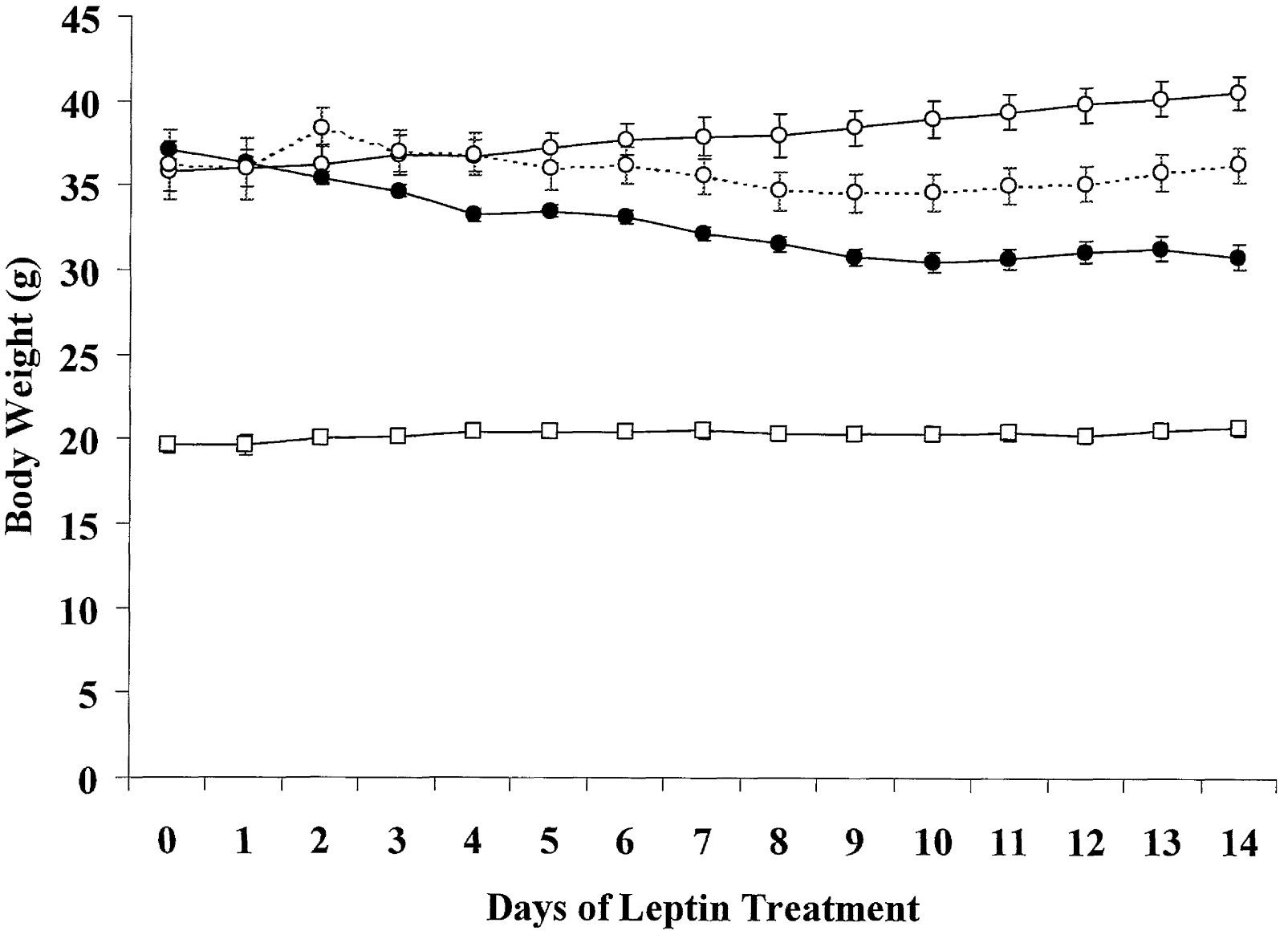
C57BL/6 *ob/ob* mice (n=8) were treated with leptin (1 mg/kg/day) for a 24-hour-period and 2-week-period. The body weight of the mice was measured daily and is represented here in g ± SEM.

C57Bl/6 lean 2-weeks vehicle —□—

C57Bl/6 *ob/ob* 2-weeks vehicle —○—

C57Bl/6 *ob/ob* 2-weeks leptin —●—

C57Bl/6 *ob/ob* 2-weeks pair-fed --○--


45
40
35
30
25
20
15
10
5
0
Body Weight (g)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days of Leptin Treatment

5.2.2 Plasma Insulin

Insulin levels (see Figure 5.2) were significantly increased from 1 ng/ml in C57Bl/6 lean mice to 75 ± 6 ng/ml ($P<0.01$) in C57Bl/6 *ob/ob* 24-hour vehicle-treated mice and 33 ± 5 ng/ml ($P<0.01$) in C57Bl/6 *ob/ob* 2-week vehicle-treated mice. There was a significant decrease in insulin levels after 24-hours of leptin treatment from 75 ± 6 ng/ml in vehicle-treated *ob/ob* mice compared to 33 ± 7 ng/ml in leptin-treated *ob/ob* mice. In comparison to C57Bl/6 *ob/ob* 24-hour vehicle-treated mice, insulin in C57Bl/6 *ob/ob* 2-week vehicle-treated mice was significantly decreased by 2.6-fold from 75 ± 6 ng/ml to 33 ± 5 ng/ml ($P<0.01$). Insulin levels in C57Bl/6 *ob/ob* mice treated with leptin for 2-weeks were significantly reduced compared to 2-week vehicle-treated *ob/ob* mice by 15-fold from 33 ± 5 ng/ml to 5 ± 1 ng/ml ($P<0.01$). However, compared to 2-week vehicle-treated *ob/ob* mice, insulin levels were not significantly reduced in the pair-fed group (44 ± 7 ng/ml in pair-fed group compared to 33 ± 5 ng/ml in 2-week vehicle-treated group). Insulin levels were, however, significantly reduced in 2-week leptin-treated *ob/ob* mice compared to pair-fed *ob/ob* mice (5 ± 1 ng/ml compared to 44 ± 7 ng/ml; $P<0.01$).

Figure 5.2 Plasma insulin concentrations in leptin-treated C57Bl/6 *ob/ob* mice

Plasma insulin levels in C57Bl/6 *ob/ob* mice (n=8) treated with vehicle or leptin for either a 24-hour or a 2-week period or pair-fed for 2 weeks as described in section 2.3.1. Plasma insulin levels were measured in ng/ml ± SEM. The groups comparisons are listed below and the bars above the graph show significant changes between the groups ($P<0.05$).

A vs B lean vs *ob/ob* 24-hour vehicle

A vs D lean vs *ob/ob* 2-week vehicle

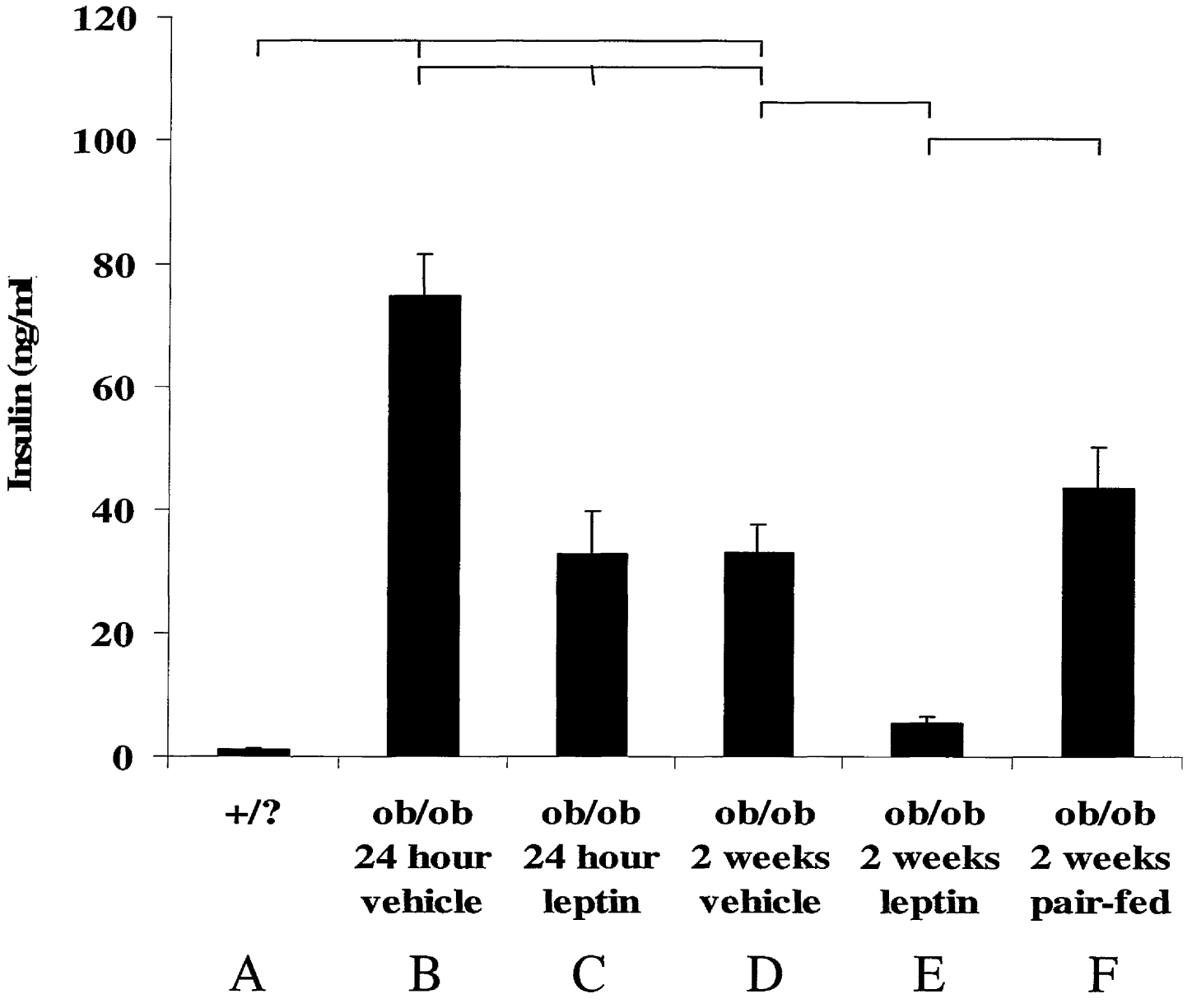
B vs C *ob/ob* 24-hour vehicle vs *ob/ob* 24-hour leptin

B vs D *ob/ob* 24-hour vehicle vs *ob/ob* 2-week vehicle

D vs E *ob/ob* 2-week vehicle vs *ob/ob* 2-week leptin

D vs F *ob/ob* 2-week vehicle vs *ob/ob* 2-week pair-fed

E vs F *ob/ob* 2-week leptin vs *ob/ob* 2-week pair-fed


120
100
80
60
40
20
0
Insulin (ng/ml)
+/?
ob/ob 24 hour vehicle
ob/ob 24 hour leptin
ob/ob 2 weeks vehicle
ob/ob 2 weeks leptin
ob/ob 2 weeks pair-fed
A
B
C
D
E
F

5.2.3 Gene Expression

The quantitative expression of components of the leptin receptor-mediated signalling pathway was examined by TaqMan RT-PCR in vehicle- and leptin-treated *ob/ob* mice. In this study, a number of housekeeping genes were analysed to determine which would be best to use as a covariate in ANCOVA analysis. The genes examined were glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin, 36B4 and HPRT. Of these genes, 36B4 was the most consistent and suitable to be used as a covariate (see appendix 1).

5.2.3.1 OB-Ra and OB-Rb

The changes in expression of OB-Ra and OB-Rb in the hypothalamus of leptin-treated *ob/ob* mice are shown in Figure 5.3. The expression of OB-Ra mRNA was unchanged in any of the treatment groups. There was a non-significant increase of 17% in OB-Rb mRNA expression in the hypothalamus of lean mice compared to 24-hour vehicle-treated *ob/ob* mice (A vs B). However, there was a significant increase in the expression of OB-Rb mRNA in 2-week vehicle-treated (A vs D), leptin-treated (A vs E) and pair-fed (A vs F) *ob/ob* mice compared to leans by 31% ($P<0.01$), 23% ($P<0.05$) and 39% ($P<0.01$), respectively. There was no significant change in OB-Rb mRNA expression in the remaining group comparisons of the study, namely, after leptin treatment of *ob/ob* mice for 24-hours compared to vehicle-treated *ob/ob* mice; in 24-hour vs 2-week vehicle-treated *ob/ob* mice; in 2-week leptin-treated compared to vehicle-treated *ob/ob* mice; in pair-fed *ob/ob* mice vs the 2-week vehicle-treated

ob/ob mice and in the 2-week leptin-treated *ob/ob* mice vs the 2-week pair-fed *ob/ob* mice. In summary, the expression of OB-Rb, but not OB-Ra, is higher in the hypothalamus of *ob/ob* mice compared to leans. Furthermore, there is no effect on hypothalamic leptin receptor expression after *ob/ob* mice have been treated with leptin for either 24-hours or 2-weeks.

5.2.3.2 STAT3 and STAT5

The changes in expression of the STAT3 and STAT5 genes in the hypothalamus of leptin-treated *ob/ob* mice are shown in Figure 5.4. In comparing lean mice and 24-hour vehicle-treated *ob/ob* mice (A vs B), STAT3 and STAT5 mRNA expression were increased in *ob/ob* mice by 32% ($P<0.01$) and 23% ($P<0.05$), respectively. Similarly, the expression of STAT3 and STAT5 mRNA was increased in 2-week vehicle-treated *ob/ob* mice compared to lean mice (A vs D) by 47% and 49% ($P<0.01$), respectively. The expression of both STAT3 and STAT5 was increased in 2-week leptin-treated *ob/ob* mice compared to leans (A vs E) by 43% and 50% ($P<0.01$), respectively. Whereas, STAT3 was increased by 20% ($P<0.01$) in 2-week pair-fed *ob/ob* mice compared to leans (A vs F), however STAT5 remained unchanged. There was no significant change in STAT3 or STAT5 in *ob/ob* mice treated with leptin compared to vehicle for 24-hours (B vs C). The expression of STAT3 and STAT5 was unchanged in *ob/ob* mice treated with leptin for 2-weeks compared to vehicle-treated *ob/ob* mice (D vs E), but both STAT3 and STAT5 were significantly reduced by 19% and 27% ($P<0.01$), respectively, in the pair-fed

group when compared to vehicle-treated *ob/ob* mice (D vs F). However, a comparison of 24-hour and 2-week vehicle-treated *ob/ob* mice (B vs D) showed STAT5 mRNA was significantly increased by 20% ($P<0.05$) and STAT3 was increased by 11%, but not statistically significant, in 2-week vehicle-treated *ob/ob* mice. Reduced STAT3 and STAT5 mRNA (16% and 28%, respectively; $P<0.01$) was also observed in the pair-fed *ob/ob* mice in comparison to the 2-week leptin-treated *ob/ob* mice (E vs F). In summary, the expression of STAT3 and STAT5 was higher in *ob/ob* mice compared to leans. Furthermore, the expression of STAT3 and STAT5 was lower in pair-fed *ob/ob* mice compared to *ob/ob* mice treated with vehicle or leptin for 2-weeks. Although leptin treatment did not change STAT3 or STAT5 mRNA expression in *ob/ob* mice, pair-feeding did reduce STAT3 and STAT5 mRNA expression.

Figure 5.3 Changes in OB-Ra and OB-Rb mRNA expression in the hypothalamus of C57Bl/6 *ob/ob* mice treated with leptin for a 24-hour and 2-week period

The mRNA levels for the reference group (e.g. A in A vs B) has been ascribed a value of 1 and the symbols indicate relative changes from this baseline. Comparisons are shown of (a) OB-Ra and (b) OB-Rb mRNA expression in the hypothalamus of:

A vs B lean vs *ob/ob* 24-hour vehicle

A vs D lean vs *ob/ob* 2-week vehicle

A vs E lean vs *ob/ob* 2-week leptin

A vs F lean vs *ob/ob* 2-week pair-fed

B vs C *ob/ob* 24-hour vehicle vs *ob/ob* 24-hour leptin

B vs D *ob/ob* 24-hour vehicle vs *ob/ob* 2-week vehicle

D vs E *ob/ob* 2-week vehicle vs *ob/ob* 2-week leptin

D vs F *ob/ob* 2-week vehicle vs *ob/ob* 2-week pair-fed

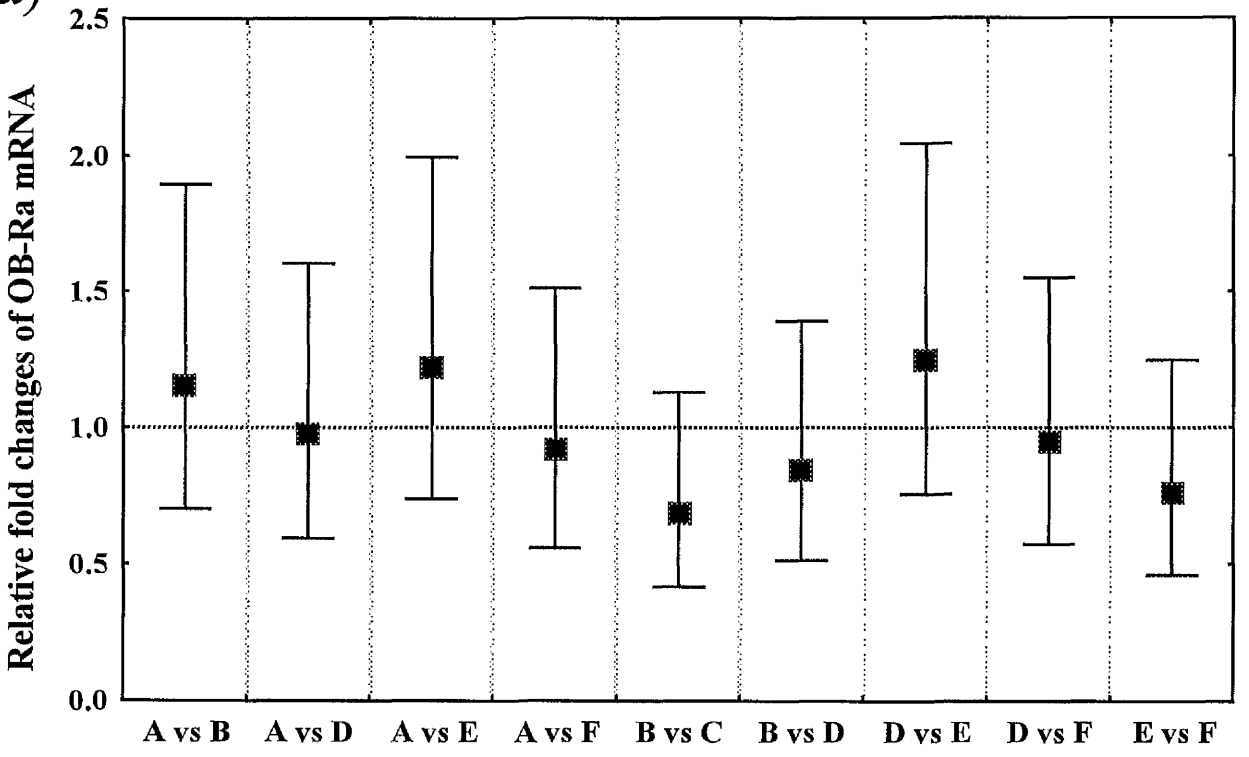
E vs F *ob/ob* 2-week leptin vs *ob/ob* 2-week pair-fed

Error bars indicate 95% confidence intervals and changes are shown as significant where * $P<0.05$ and ** $P<0.01$.

■ OB-Ra mRNA

● OB-Rb mRNA

(a)


Relative fold changes of OB-Ra mRNA
2.5
2.0
1.5
1.0
0.5
0.0
A vs B
A vs D
A vs E
A vs F
B vs C
B vs D
D vs E
D vs F
E vs F

(b)

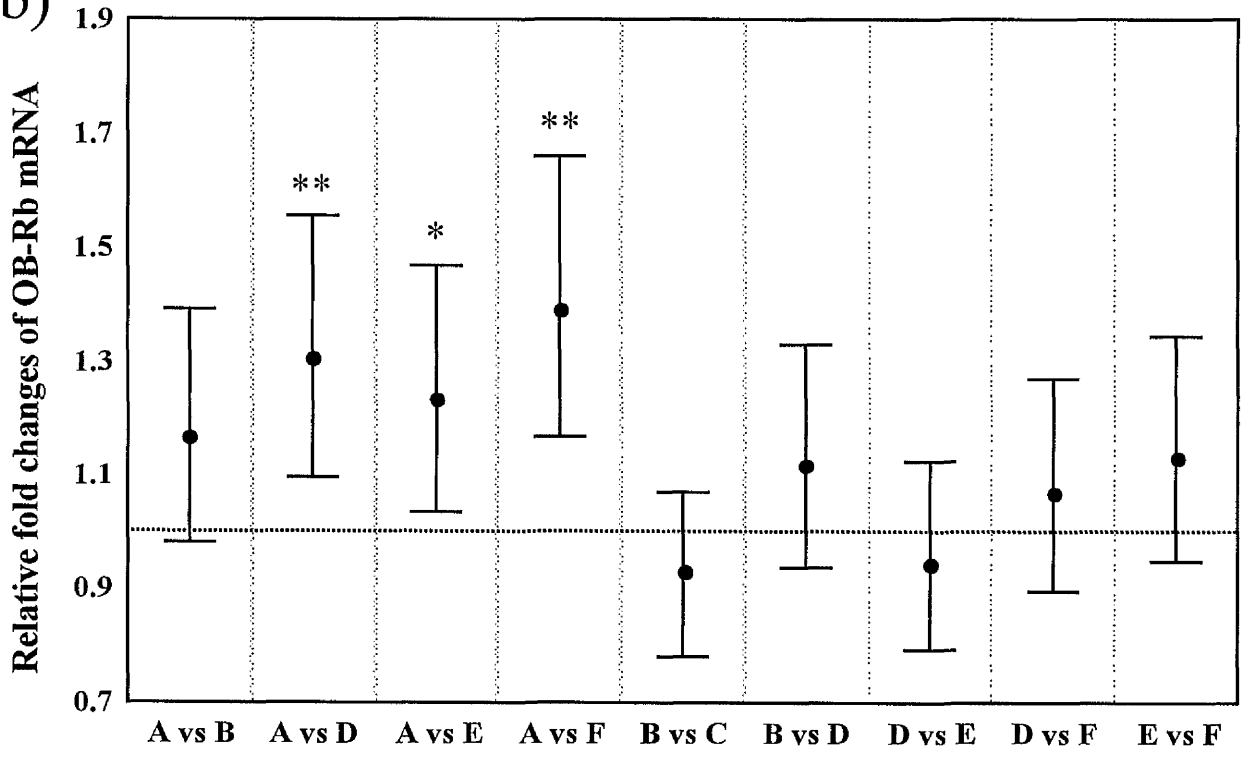

Relative fold changes of OB-Rb mRNA
1.9
1.7
1.5
1.3
1.1
0.9
0.7
**
*
**
A vs B
A vs D
A vs E
A vs F
B vs C
B vs D
D vs E
D vs F
E vs F

Figure 5.4 Changes in STAT3 and STAT5 mRNA expression in the hypothalamus of C57Bl/6 *ob/ob* mice treated with leptin for a 24-hour and 2-week period

The mRNA levels for the reference group (e.g. A in A vs B) has been ascribed a value of 1 and the symbols indicate relative changes from this baseline. Comparisons are shown of (a) STAT3 and (a) STAT5 mRNA in the hypothalamus of:

A vs B lean vs *ob/ob* 24-hour vehicle

A vs D lean vs *ob/ob* 2-week vehicle

A vs E lean vs *ob/ob* 2-week leptin

A vs F lean vs *ob/ob* 2-week pair-fed

B vs C *ob/ob* 24-hour vehicle vs *ob/ob* 24-hour leptin

B vs D *ob/ob* 24-hour vehicle vs *ob/ob* 2-week vehicle

D vs E *ob/ob* 2-week vehicle vs *ob/ob* 2-week leptin

D vs F *ob/ob* 2-week vehicle vs *ob/ob* 2-week pair-fed

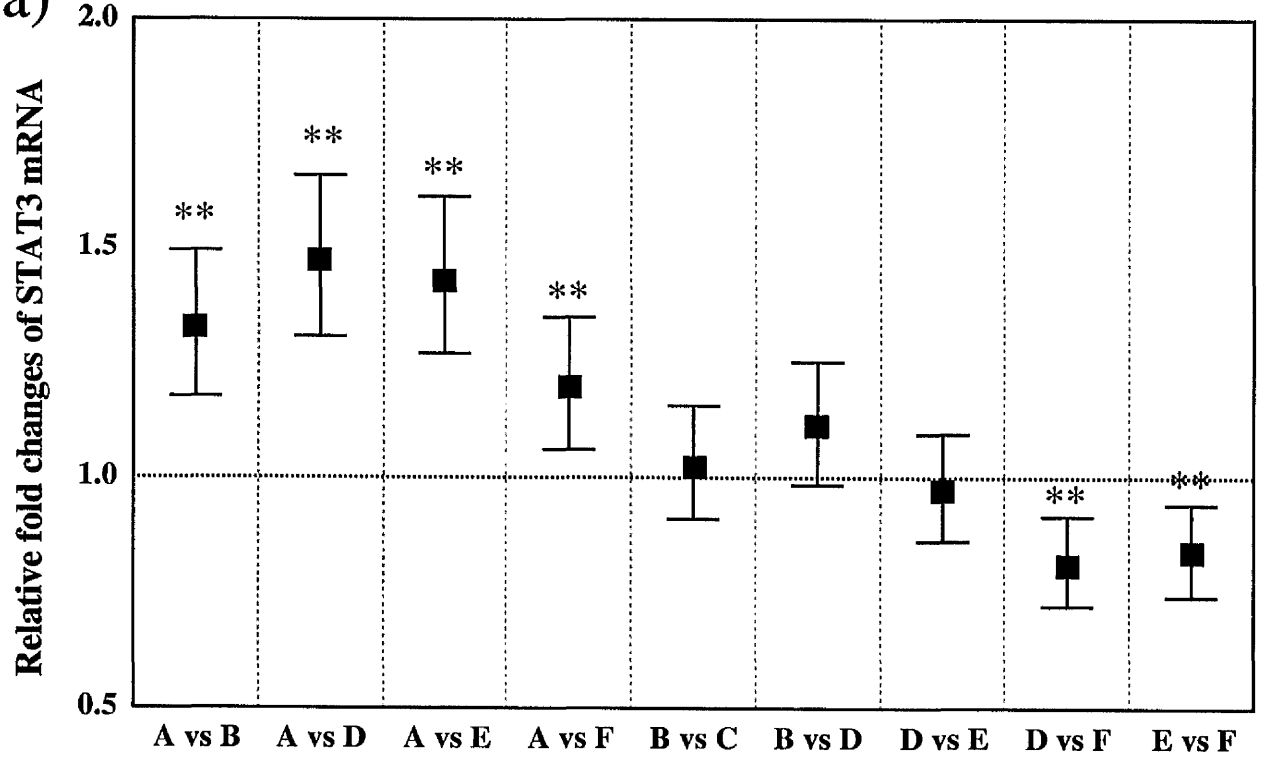
E vs F *ob/ob* 2-week leptin vs *ob/ob* 2-week pair-fed

Error bars indicate 95% confidence intervals and changes are shown as significant where * $P<0.05$ and ** $P<0.01$.

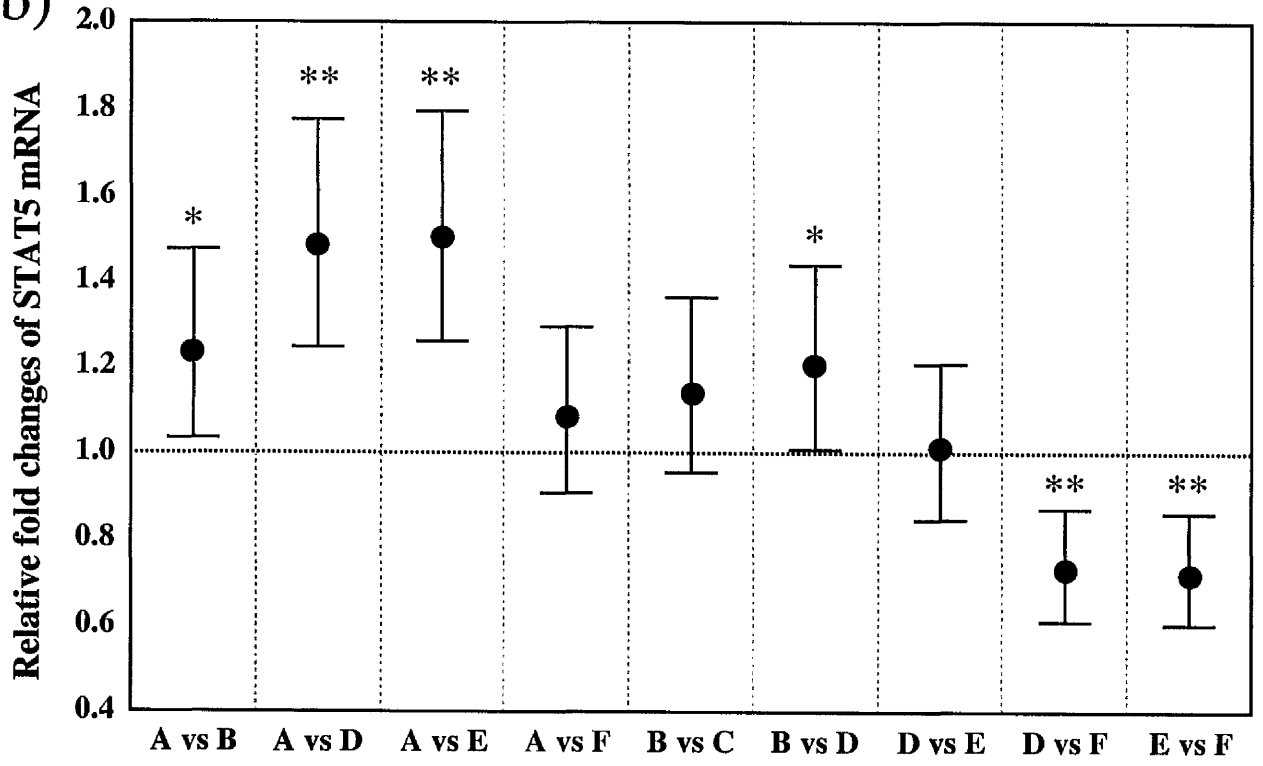
■ STAT3 mRNA

● STAT5 mRNA

(a)


Relative fold changes of STAT3 mRNA
2.0
1.5
1.0
0.5
**
**
**
**
**
**
A vs B
A vs D
A vs E
A vs F
B vs C
B vs D
D vs E
D vs F
E vs F

(b)


Relative fold changes of STAT5 mRNA
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
*
**
**
*
**
**
A vs B
A vs D
A vs E
A vs F
B vs C
B vs D
D vs E
D vs F
E vs F

5.2.3.3 SOCS-3 and CIS

The changes in expression of SOCS-3 and CIS in the hypothalamus of leptin-treated *ob/ob* mice are shown in Figure 5.5. The expression of CIS mRNA did not change in any of the group comparisons made. The expression of SOCS-3 mRNA in the hypothalamus of 24-hour vehicle-treated *ob/ob* mice compared to lean mice (A vs B) was reduced by 14% but not significantly. However, in the hypothalamus of 2-week vehicle-treated *ob/ob* mice compared to lean mice (A vs D), the expression of SOCS-3 was significantly reduced by 28% ($P<0.01$). The expression of SOCS-3 mRNA remained unchanged in 2-week leptin-treated compared to leans (A vs E), however pair-fed *ob/ob* mice compared to leans (A vs F) showed a reduction of 13% that was not significant. After *ob/ob* mice were treated with leptin for 24-hours (B vs C), SOCS-3 mRNA expression was reduced by 17% ($P<0.05$). However, SOCS-3 mRNA was reduced by 16% ($P<0.05$) in the hypothalamus of 2-week vehicle-treated *ob/ob* mice compared to 24-hour vehicle-treated *ob/ob* mice (B vs D). In comparing 2-week leptin-treated *ob/ob* mice and the pair-fed group to vehicle-treated *ob/ob* mice (D vs E and D vs F), the expression of SOCS-3 was increased by 29% ($P<0.01$) and 20% ($P<0.05$), respectively. However, when the *ob/ob* mice treated with leptin for 2-weeks were compared with the pair-fed group (E vs F), there was no significant change in SOCS-3 mRNA expression. In summary, the expression of hypothalamic SOCS-3 mRNA tends to be lower in *ob/ob* mice compared to leans. Although 24-hour leptin treatment reduced SOCS-3 mRNA expression, the chronic 2-week leptin treatment increased

SOCS-3 mRNA expression though pair-feeding had a similar effect. The analysis of hypothalamic CIS mRNA expression showed no significant changes in *ob/ob* mice compared to leans, or after leptin treatment for either 24-hours or 2-weeks.

5.2.3.4 NPY

The changes in hypothalamic NPY expression of leptin-treated *ob/ob* mice are shown in Figure 5.6. The expression of NPY in the hypothalamus of vehicle-treated *ob/ob* mice for 24-hours and 2-weeks compared to lean mice (A vs B and A vs D) was significantly increased by 89% and 148% ($P<0.01$), respectively. The expression of NPY was increased in 2-week leptin-treated and pair-fed *ob/ob* mice compared to leans (A vs E and A vs F) by 97% and 252% ($P<0.01$), respectively. When *ob/ob* mice were treated with leptin for 24-hours (B vs C), there was a 17% reduction in NPY mRNA expression but this was not significant. The expression of NPY was increased by 31% ($P<0.05$) in 2-week vehicle-treated *ob/ob* mice compared to 24-hour vehicle-treated *ob/ob* mice (B vs D). When *ob/ob* mice were treated with leptin for 2-weeks, NPY mRNA expression was reduced by 20% ($P<0.05$) compared to vehicle (D vs E). In comparison to the 2-week vehicle-treated *ob/ob* mice, the expression of NPY in the pair-fed mice (D vs F) was significantly increased by 42% ($P<0.01$). Furthermore, when compared to *ob/ob* mice treated with leptin for 2-weeks, the expression of NPY in the pair-fed mice (E vs F) was significantly increased by 79% ($P<0.01$). In summary, the expression of hypothalamic NPY mRNA was

higher in *ob/ob* mice compared to leans. Moreover, leptin treatment of *ob/ob* mice reduces hypothalamic NPY mRNA after 2-weeks, whereas diet restriction increased hypothalamic NPY mRNA.

Figure 5.5 Changes in SOCS-3 and CIS mRNA expression in the hypothalamus of C57Bl/6 *ob/ob* mice treated with leptin for a 24-hour and 2-week period

The mRNA levels for the reference group (e.g. A in A vs B) has been ascribed a value of 1 and the symbols indicate relative changes from this baseline. Comparisons are shown of (a) SOCS-3 and (b) CIS mRNA in the hypothalamus of:

A vs B lean vs *ob/ob* 24-hour vehicle

A vs D lean vs *ob/ob* 2-week vehicle

A vs E lean vs *ob/ob* 2-week leptin

A vs F lean vs *ob/ob* 2-week pair-fed

B vs C *ob/ob* 24-hour vehicle vs *ob/ob* 24-hour leptin

B vs D *ob/ob* 24-hour vehicle vs *ob/ob* 2-week vehicle

D vs E *ob/ob* 2-week vehicle vs *ob/ob* 2-week leptin

D vs F *ob/ob* 2-week vehicle vs *ob/ob* 2-week pair-fed

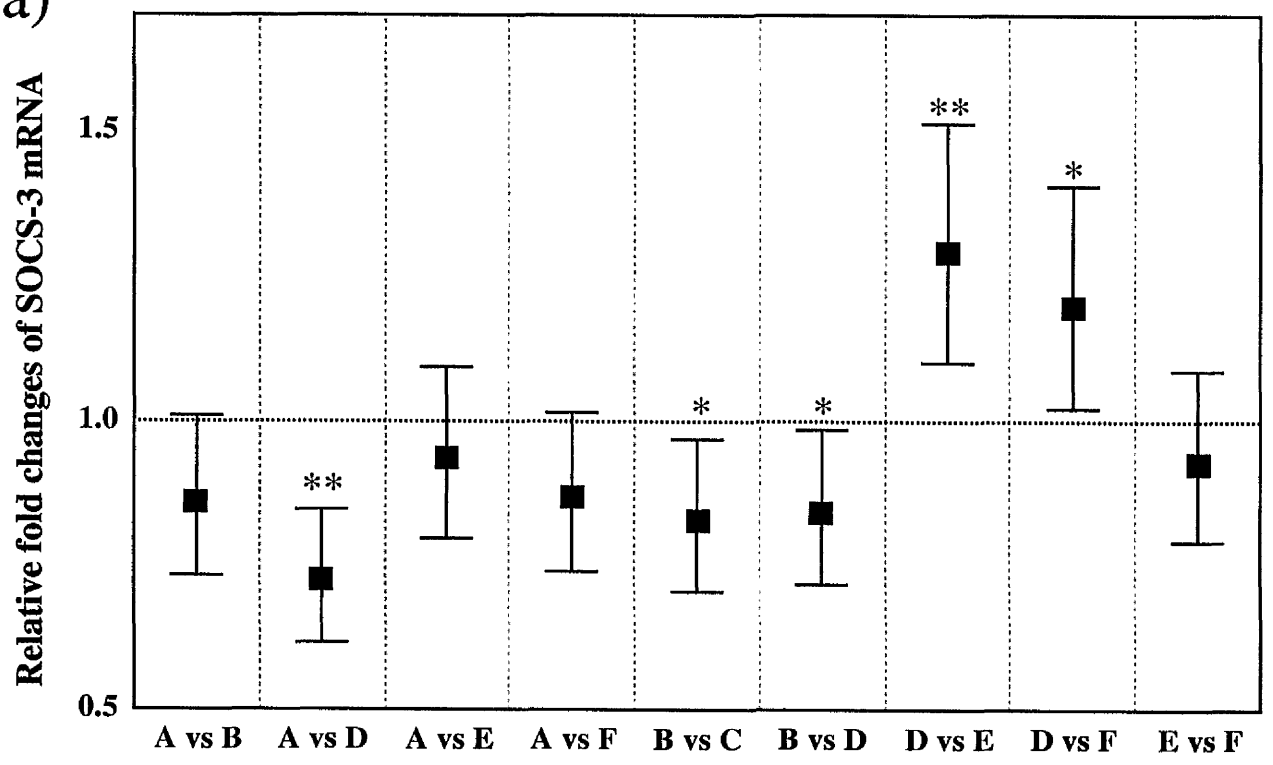
E vs F *ob/ob* 2-week leptin vs *ob/ob* 2-week pair-fed

Error bars indicate 95% confidence intervals and changes are shown as significant where * $P<0.05$ and ** $P<0.01$.

■ SOCS-3 mRNA

● CIS mRNA

(a)


Relative fold changes of SOCS-3 mRNA
1.5
1.0
0.5
**
*
*
**
*
A vs B
A vs D
A vs E
A vs F
B vs C
B vs D
D vs E
D vs F
E vs F

(b)

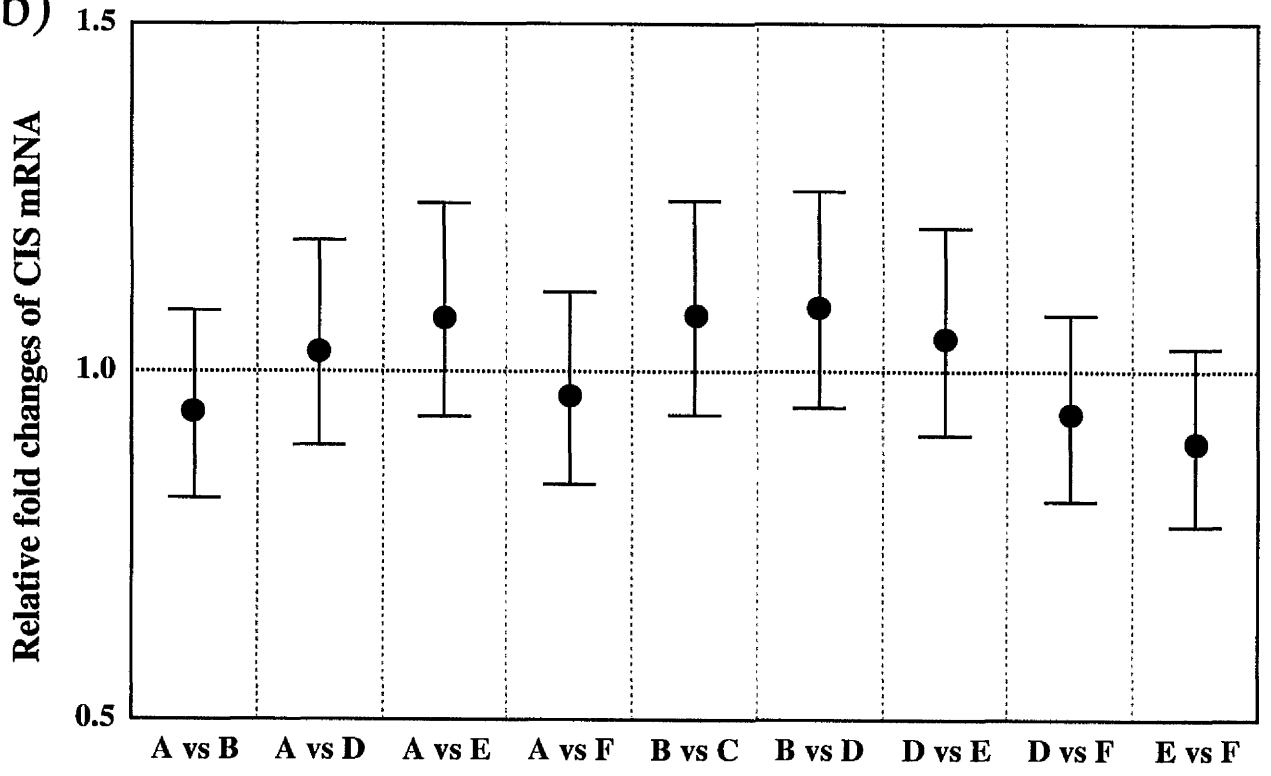

Relative fold changes of CIS mRNA
1.5
1.0
0.5
A vs B
A vs D
A vs E
A vs F
B vs C
B vs D
D vs E
D vs F
E vs F

Figure 5.6 Changes in NPY mRNA expression in the hypothalamus of C57Bl/6 *ob/ob* mice treated with leptin for a 24-hour and 2-week period

The mRNA levels for the group (e.g. A in A vs B) has been ascribed a value of 1 and the symbols indicate relative changes from this baseline. Comparisons are shown of NPY mRNA in the hypothalamus of:

A vs B lean vs *ob/ob* 24-hour vehicle

A vs D lean vs *ob/ob* 2-week vehicle

A vs E lean vs *ob/ob* 2-week leptin

A vs F lean vs *ob/ob* 2-week pair-fed

B vs C *ob/ob* 24-hour vehicle vs *ob/ob* 24-hour leptin

B vs D *ob/ob* 24-hour vehicle vs *ob/ob* 2-week vehicle

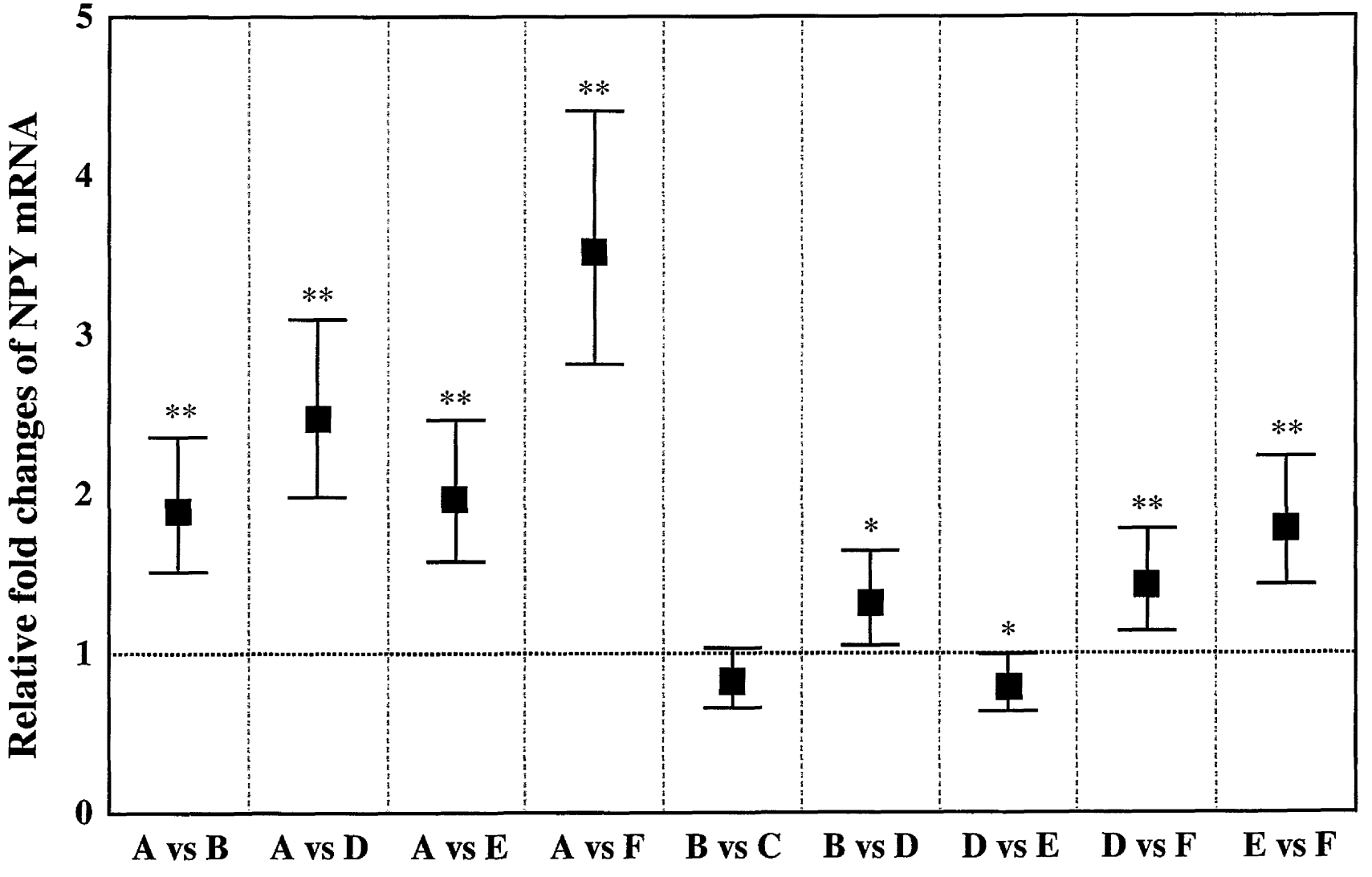
D vs E *ob/ob* 2-week vehicle vs *ob/ob* 2-week leptin

D vs F *ob/ob* 2-week vehicle vs *ob/ob* 2-week pair-fed

E vs F *ob/ob* 2-week leptin vs *ob/ob* 2-week pair-fed

Error bars indicate 95% confidence intervals and changes are shown as significant where * $P<0.05$ and ** $P<0.01$.

■ NPY mRNA


Relative fold changes of NPY mRNA
5
4
3
2
1
0
**
**
**
**
*
*
**
**
A vs B
A vs D
A vs E
A vs F
B vs C
B vs D
D vs E
D vs F
E vs F

5.3 Discussion

This set of investigations are similar in design to a previous study by Emilsson *et al.*, who looked at the effect of 48 hours of leptin treatment (1.5 mg/kg/day) on the expression of components of the leptin receptor-mediated signalling pathway in a number of tissues in lean and obese (*ob/ob*) mice by RT-PCR (Emilsson *et al.*, 1999). In this study, the expression of components of the leptin receptor-mediated signalling pathway was examined in the hypothalamus of *ob/ob* mice treated with leptin for 24-hours and 2-weeks by quantitative TaqMan RT-PCR. The dose of i.p. leptin used was 1.0 mg/kg/day, a sub-maximal leptin concentration within the dose response range originally reported to inhibit food intake and activation of the JAK/STAT pathway (Morton *et al.*, 1998; Pelleymounter *et al.*, 1995; Vaisse *et al.*, 1996).

Leptin signalling in both peripheral and central tissues (Vaisse *et al.*, 1996) is primarily mediated through the OB-Rb receptor isoform by the activation of the JAK/STAT pathway. Investigation of leptin receptor expression showed a 17% increase of OB-Rb mRNA in the hypothalamus of 24-hour vehicle-treated *ob/ob* mice compared to leans, but the change was not significant. However, the expression of OB-Rb mRNA in the hypothalamus of 2-week vehicle-treated *ob/ob* compared to lean mice was significantly increased by 31% increase and the increase was maintained after leptin treatment or pair-feeding. This increase is consistent with previous reports; although the authors report a greater (2-fold) increase of OB-R expression in the hypothalamus of

ob/ob mice compared to leans by *in situ* hybridisation and RT-PCR (Baskin *et al.*, 1998; Emilsson *et al.*, 1999; Huang *et al.*, 1997). Perhaps the lack of circulating leptin results in increased hypothalamic OB-R expression. The observations in this set of experiments are inconsistent with the study described in Chapter 3 (see 3.3.1 Discussion) where no change in OB-Ra or OB-Rb mRNA was observed in *ob/ob* mice compared to leans. Furthermore, there was no change in hypothalamic leptin receptor expression after *ob/ob* mice were treated with leptin for 24-hours and 2-weeks, which is in agreement with the observations by Emilsson *et al.* where *ob/ob* mice were treated with leptin for 48 hours (Emilsson *et al.*, 1999).

The expression of STAT3 and STAT5 mRNA was increased in the hypothalamus of *ob/ob* mice compared to leans, which contrasts with the results by Emilsson *et al.* where no change was observed (Emilsson *et al.*, 1999). The increase in STAT3 and STAT5 mRNA expression is consistent with the increased OB-Rb mRNA in *ob/ob* mice suggesting an increase in the potential for leptin signalling in these animals. There was no change in STAT3 or STAT5 mRNA expression after leptin treatment of *ob/ob* mice for 24-hours or 2-weeks compared to vehicle-treated *ob/ob* mice, which is in agreement with the observations by Emilsson *et al.* after 48 hours of leptin treatment (Emilsson *et al.*, 1999). However, both STAT3 and STAT5 mRNA expression were slightly increased in 2-week vehicle-treated *ob/ob* mice compared to 24-hour vehicle-treated *ob/ob* mice, although the STAT3 mRNA increase was not significant. It is not clear why STAT3 and STAT5 are increased but it may be related to the

weight gain that occurs during the 2-week period. In addition, the expression of STAT3 and STAT5 was reduced by similar levels in pair-fed *ob/ob* mice compared to 2-week vehicle or leptin-treated *ob/ob* mice. This suggests the reduced STAT3 and STAT5 expression in pair-fed *ob/ob* mice may be due to the effects of reduced food intake, and suggests that the lack of effect of leptin on STATs involves the interplay of other factors. Although the expression of STAT3 and STAT5 was reduced in 2-week pair-fed compared to vehicle-treated and leptin-treated *ob/ob* mice, there was still a significant elevation in expression compared to leans.

Table 5.2 Summary of gene expression changes involved in leptin receptor-mediated signalling in the hypothalamus

	OB-Ra	OB-Rb	STAT3	STAT5	SOCS-3	CIS	NPY
A vs B	NC	↑ 17% †	↑ 32% *	↑ 23% *	↓ 14% †	NC	↑ 89% **
A vs D	NC	↑ 31% **	↑ 47% **	↑ 49% **	↓ 28% **	NC	↑ 148% **
A vs E	NC	↑ 23% *	↑ 43% **	↑ 50% **	NC	NC	↑ 97% **
A vs F	NC	↑ 39% **	↑ 20% **	NC	↓ 13% †	NC	↑ 252% **
B vs C	NC	NC	NC	NC	↓ 17% *	NC	↓ 17% †
B vs D	NC	NC	↑ 11% †	↑ 20% *	↓ 16% *	NC	↑ 31% *
D vs E	NC	NC	NC	NC	↑ 29% **	NC	↓ 20% *
D vs F	NC	NC	↓ 19% **	↓ 27% **	↑ 20% *	NC	↑ 42% **
E vs F	NC	NC	↓ 16% **	↓ 28% **	NC	NC	↑ 79% **

Key: NC – no change, † - non-significant change, * $P<0.05$, ** $P<0.01$

There was a trend towards reduced SOCS-3 mRNA expression in 24-hour vehicle-treated *ob/ob* mice and a significant reduction in 2-week vehicle-treated *ob/ob* mice when compared to lean mice. Emilsson *et al.* have observed a 2-fold increase in hypothalamic SOCS-3 mRNA expression in *ob/ob* compared to lean mice (Emilsson *et al.*, 1999), the effects of which they propose could be counteracted by the observed 2-fold increase in OB-R mRNA in the hypothalamus of *ob/ob* mice. Since *ob/ob* mice are more sensitive to the effects of leptin on feeding than lean mice, the reduced expression of SOCS-3 mRNA in leptin deficient *ob/ob* mice observed here is consistent with its role as a

negative regulator of leptin signalling. Interestingly, SOCS-3 mRNA expression was reduced after 24-hours of leptin treatment but increased after 2-weeks of leptin treatment compared to vehicle-treated *ob/ob* mice. Whilst there have been reports of increased SOCS-3 mRNA expression after leptin treatment implying initiation of a negative feedback loop, the expression has been measured from between 0.5-2 hours after leptin treatment. In a study by Emilsson *et al.*, there was no change in SOCS-3 mRNA expression after 48 hours of leptin treatment at 1.5 mg/kg/day (Emilsson *et al.*, 1999). Surprisingly therefore, in our study, SOCS-3 mRNA was significantly reduced by 17% after 24-hours of leptin treatment of *ob/ob* mice, which is inconsistent with previous *in vitro* and *in vivo* studies and its role as a suppressor of leptin signalling. The differences in SOCS-3 mRNA expression after 24-hours and 2-weeks of leptin treatment suggest that leptin may have different acute and chronic effects. The increased expression of SOCS-3 mRNA in 2-week leptin-treated and pair-fed *ob/ob* mice compared to vehicle could be explained by the reduced levels of SOCS-3 mRNA in 2-week vehicle-treated *ob/ob* mice compared to 24-hour vehicle-treated *ob/ob* mice or due to reduced food intake rather than leptin *per se*. Emilsson *et al.* observed a 2-fold increase in hypothalamic CIS mRNA expression in *ob/ob* mice compared to leans and after 48 hours of leptin treatment (Emilsson *et al.*, 1999). However, no change in hypothalamic CIS mRNA was observed in this study in comparing *ob/ob* mice to leans or after leptin treatment for 24-hours or 2-weeks (Emilsson *et al.*, 1999). An *in vitro*

study in CHO cells expressing OB-Rb, showed no change in CIS mRNA expression up to 4 hours after leptin treatment (Bjorbaek *et al.*, 1999).

Hypothalamic NPY expression was increased in 24-hour and 2-week vehicle-treated *ob/ob* mice compared to lean mice by 89% and 148%, respectively. This is consistent with previous observations, where it has been observed that NPY is expressed at high levels in the hypothalamus of *ob/ob* mice compared to leans. After 24-hours leptin treatment, there was a small reduction, though not significant, in hypothalamic NPY mRNA expression in *ob/ob* mice. There may be a number of factors why NPY levels are higher in 2-week compared to 24-hour vehicle-treated *ob/ob* mice, such as the reduced insulin levels in 2-week vehicle *ob/ob* mice (Davies & Marks, 1994). After 2-weeks of leptin treatment there was a significant reduction in NPY mRNA expression compared to vehicle-treated *ob/ob* mice. Furthermore, NPY mRNA was increased in the pair-fed group compared to both the 2-week vehicle-treated *ob/ob* mice and the 2-week leptin-treated *ob/ob* mice, which is consistent with the effect of leptin on NPY gene expression in food-deprived mice. These observations are consistent with increased leptin resulting in reduced hypothalamic NPY expression and the leptin deficiency in *ob/ob* mice resulting in increased hypothalamic NPY mRNA expression compared to leans.

In summary, there are some significant changes in components of the leptin receptor-mediated signalling pathway after leptin treatment; however some of the changes have not been consistent in magnitude or direction to previous studies. It should be noted that there are other signals capable of

activating the JAK-STAT pathway that may affect the expression of STATs and SOCS genes in the hypothalamus. Moreover, components involved in leptin receptor-mediated signalling are highly expressed in particular areas of the hypothalamus; therefore by examining the gene expression in the hypothalamic block the changes may not be a true reflection of the changes that occur. Of the genes examined, the expression of SOCS-3 mRNA in *ob/ob* mice was the only one that changed after 24-hours or 2-weeks of leptin treatment, but there were significant changes in OB-Rb, STAT3, STAT5 and SOCS-3 in vehicle-treated *ob/ob* mice compared to leans. This suggests regulation of the components of the leptin receptor-mediated signalling pathway may be involved in leptin sensitivity but their role remains unclear. Finally, in discussing the relevance of changes in mRNA, it must be remembered that this may not equate to changes in protein expression whilst the biological effect, if any, of small changes in mRNA expression remains to be determined.

Chapter 6

Quantitative Leptin Receptor Expression in the Hypothalamus of ZDF rats

The animal study described in this chapter was performed by Mohammad Tadayyon and Carolyn Lister at GlaxoSmithKline. mRNA was made available from the hypothalami of ZDF +/? and *fa/fa* rats to investigate leptin receptor expression by TaqMan analysis.

6.1 Introduction

In humans, the consumption of excess food can result in obesity, which over a long period of time can lead to insulin resistance, β-cell failure and to type II diabetes. The diabetes is preceded by an extended period of insulin resistance, due to an increased proportion of adiposity, during which compensatory increased insulin production maintains normal glucose levels (Unger1995). Hyperglycaemia manifests when insulin production fails to keep pace with progressively increasing insulin resistance and type II diabetes ensues. The Zucker Diabetic Fatty (ZDF) rats provide a useful model of adipogenic type II diabetes because they exhibit a similar pattern of increased obesity, insulin resistance followed by β-cell failure (Peterson *et al.*, 1990) to that observed in obese humans.

In ZDF rats there is a marked increase in the triglyceride content in pancreatic islets during the development of the β-cell defects of adipogenic diabetes which leads to increased β-cell death, so-called "lipotoxicity". (Unger1995). Lipotoxicity is observed in several peripheral tissues and is attributed to products of excessive non-β-oxidative metabolism of fatty acid excess in skeletal muscle, pancreatic islets, and myocardium (Lee *et al.*, 1997;

Shimabukuro *et al.*, 1998b; Shimabukuro *et al.*, 1998c). Furthermore, lipoapoptosis of β-cells in obese ZDF *fa/fa* rats is thought to be mediated by overproduction of ceramide, and is induced by high levels of long chain fatty acids (Shimabukuro *et al.*, 1998b). Ceramide increases the expression of inducible nitric oxide synthase (iNOS) through activation of nuclear factor κB (Katsuyama *et al.*, 1998), and thereby augments the production of nitric oxide (NO) (Shimabukuro *et al.*, 1997a). NO forms potent oxidants, such as peroxynitrite (Ghafourifar *et al.*, 1999; Lin *et al.*, 1995), that cause apoptosis. Therefore it is possible that the pathogenesis of β-cell failure is entirely secondary to the overabundance of triglycerides in the pancreatic islets. Experiments to reduce the islet triglyceride content by caloric restriction and administration of inhibitors of inducible iNOS in prediabetic ZDF rats reduce β-cell abnormalities and apoptosis (Ohneda *et al.*, 1995; Unger1995), raising the possibility of a causal relationship between the fat overload in islets and the β-cell dysfunction. It is believed that high levels of these metabolic products may be responsible for the complications of obesity, insulin resistance, cardiovascular disease and diabetes by disrupting cell function and ultimately by promoting programmed cell death (also known as "lipoapoptosis") (Shimabukuro *et al.*, 1998c; Unger1995).

In the Zucker *fa/fa* and ZDF rat, there is a single A → C amino acid substitution at nucleotide 880 in the extracellular domain of the leptin receptor (OB-R(*fa*)), resulting in a glutamine^{269} (Gln^{269}) to proline^{269} (Pro^{269}) amino acid substitution (Iida *et al.*, 1996; Phillips *et al.*, 1996). The resulting phenotype of

the Zucker *fa/fa* rat is obesity, hyperphagia, sterility, hypercholesterolemia, and hyperlipidemia (Zucker & Zucker, 1961; Zucker & Zucker, 1962; Zucker & Zucker, 1963). The Zucker *fa/fa* rat, like the ZDF *fa/fa* rat, is also hyperinsulinaemic and insulin resistant, but does not display fasting hyperglycaemia and is only mildly glucose intolerant. In human terms therefore, the Zucker *fa/fa* rat is probably representative of the obese, pre-diabetic subject. The ZDF rat is similar to, and was derived from, the Zucker *fa/fa* rat, with the exception that the males are hyperglycaemic by the age of about 10 weeks (Clark & Palmer1982; Janssen *et al.*, 1999). Whilst plasma insulin is elevated during the development of diabetes, it drops to below the level of lean controls as the β-cell start to fail and the diabetic state develops (Janssen *et al.*, 1999). The body weight of ZDF rats increases during the development of diabetes, but is reduced as the diabetic state develops (Janssen *et al.*, 1999). The ZDF model is therefore useful for the study of adult onset diabetes.

In vitro experiments have shown reduced OB-R (*fa*) expression compared to wild type OB-R in transfected COS-7 cells (Chua *et al.*, 1996b; Rosenblum *et al.*, 1996; White *et al.*, 1997b). Furthermore, the fatty mutation in short OB-R isoforms results in a 6 to 8-fold reduction in cell surface expression relative to its wild-type (wt) counterpart, whereas OB-Rb (*fa*) exhibits only a 2 to 3-fold reduction in cell surface expression compared with OB-Rb (wt) (White *et al.*, 1997b). In COS-1 cells, overexpression of OB-Rb (wt) activates endogenous STAT1 and –3, or coexpressed STAT1, -3 and -5B upon leptin

binding. In contrast, OB-Rb (*fa*) constitutively activates both endogenous STAT1 and –3, and coexpressed STAT1 and –3, and activation of these STATs was further increased by the addition of leptin (White *et al.*, 1997b). The OB-Rb (*fa*) has a reduced level of STAT1 and –3 activation due to the reduced cell surface expression of this receptor compared with OB-Rb (wt) and a reduced intrinsic ability of OB-Rb (*fa*) to activate STAT1 and –3 compared with OB-Rb (wt) (Rosenblum *et al.*, 1996; White *et al.*, 1997b). Furthermore, OB-Rb (*fa*) is unable to constitutively activate cotransfected STAT5B and exhibits attenuated leptin activation of STAT5B compared with OB-Rb (wt) (White *et al.*, 1997b). The excessive fat accumulation in islets of ZDF rats is the consequence of this amino acid substitution in all isoforms of the leptin receptor, which abolishes the direct lipopenic action (i.e. increase fatty acid oxidation and the expression of genes for fatty acid oxidation) of leptin on the β-cell because of impaired leptin signalling (Shimabukuro *et al.*, 1997b). However, the overexpression of wild-type OB-R in ZDF rats using adenovirus can restore the lipopenic action of leptin, reduce exaggerated iNOS expression by islets and enable FFAs to up-regulate proinsulin gene expression (Wang *et al.*, 1998b).

The aim of this chapter was to investigate whether the mutation in the leptin receptor of ZDF rats changes the expression of OB-Ra and OB-Rb mRNA in the hypothalamus when compared to that of controls (+/?).

6.2 Results

The body weight, blood glucose and plasma insulin levels of ZDF +/? and ZDF *fa/fa* rats were measured between the ages of 7 and 24 weeks, and the plasma leptin levels and pancreatic insulin content was determined at the end of the study. The body weight of ZDF +/? rats and ZDF *fa/fa* rats are shown in Figure 6.1. At the age of 7 weeks, body weights of ZDF *fa/fa* rats were significantly higher than that of ZDF +/? rats (193 ± 5 g versus 147 ± 7.5 g; $P<0.01$). Body weights of the ZDF *fa/fa* rats increased significantly from 193 ± 5 g at 7 weeks of age to 398 ± 13.4 g at 16 weeks of age ($P<0.05$). Thereafter, the body weights of ZDF *fa/fa* rats did not significantly alter whereas ZDF +/? rats showed a small but steady increase in body weight. The body weight of ZDF *fa/fa* rats was the same as ZDF +/? rats at 23 weeks of age, but by the following week, the body weight of the ZDF *fa/fa* rats was lower but not significantly at 409 ± 20.5 g compared to 423 ± 12.3 g for ZDF +/? rats.

The blood glucose concentration of ZDF +/? rats did not increase with age and remained steady during the study period, on average at about 5 mmol/l (Figure 6.2). At the age of 8 weeks, there was no significant difference in blood glucose concentrations of ZDF *fa/fa* rats compared to ZDF +/? rats (7.5 ± 0.6 mmol/l versus 6.2 ± 0.3 mmol/l). However, there was a significant increase in blood glucose levels in ZDF *fa/fa* rats compared to lean controls at the age of 10 weeks (12.9 ± 1.6 mmol/l versus 4.65 ± 0.1 mmol/l; $P<0.01$). Thereafter, blood glucose levels further increased significantly in ZDF *fa/fa* rats compared to ZDF +/? rats to 27 mmol/l ($P<0.01$) at 25 weeks.

Figure 6.1 Changes in body weight of ZDF +/? and *fa/fa* rats

Changes in body weight of ZDF +/? and *fa/fa* rats fed a chow diet between the ages of 7 and 24 weeks. Values are represented as grams ± SEM and * $P<0.05$.

□ ZDF +/? rats

■ ZDF *fa/fa* rats

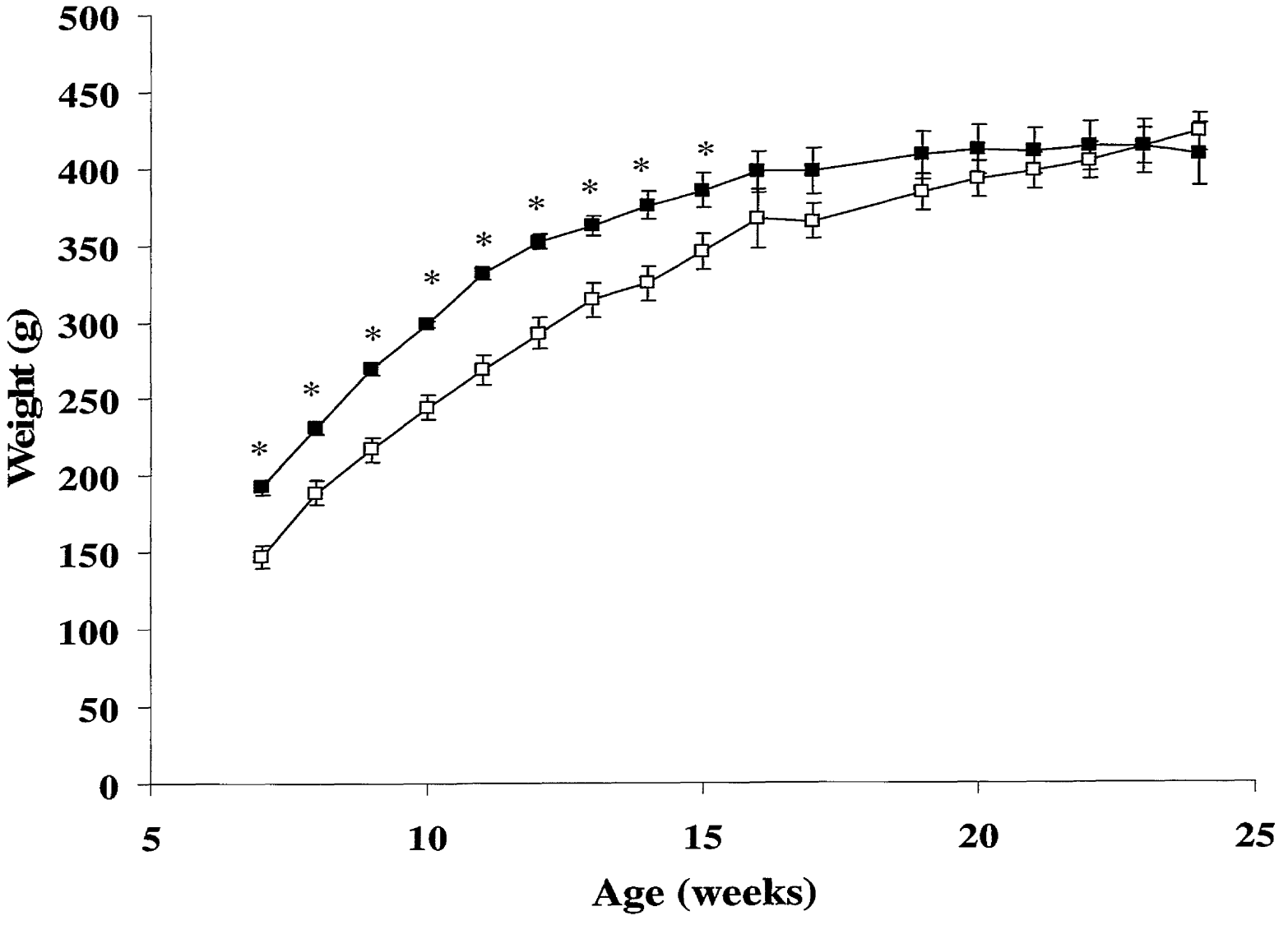
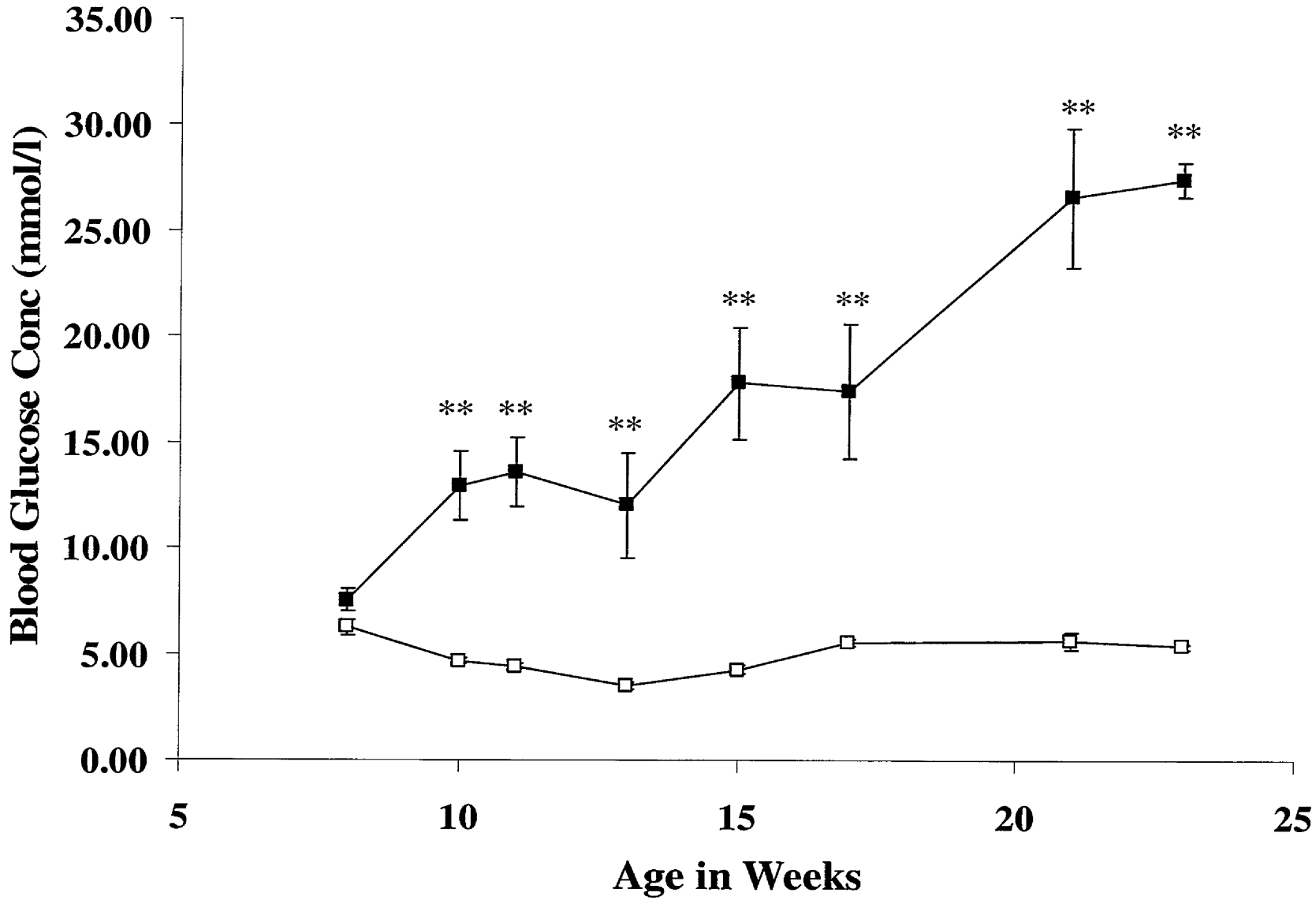

500
450
400
350
300
250
200
150
100
50
0
Weight (g)
5
10
15
20
25
Age (weeks)
*
*
*
*
*
*
*
*
*

Figure 6.2 Changes in blood glucose concentrations of ZDF +/? and *fa/fa* rats

Blood samples were taken from ZDF rats during the course of the study to measure the blood glucose concentration. The blood glucose concentrations (mmol/l) of ZDF +/? and *fa/fa* rats were measured between the ages of 8 and 23 weeks. Values are represented as mmol/l ± SEM and ** $P<0.01$.

□ ZDF +/?

■ ZDF *fa/fa*


35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Blood Glucose Conc (mmol/l)
**
**
**
**
**
**
**
5
10
15
20
25
Age in Weeks

There was little change in plasma insulin levels of ZDF +/? rats between the ages of 10 and 24 weeks, with levels ranging between 2.2 ± 0.3 ng/ml and 1.3 ± 0.1 ng/ml (Figure 6.3). However, insulin levels of ZDF *fa/fa* rats at the age of 10 weeks were significantly higher than ZDF +/? rats (20.3 ± 2.2 ng/ml versus 1.9 ± 0.2 ng/ml; $P<0.01$). Although plasma insulin levels of ZDF *fa/fa* rats decreased with age, the insulin levels remained significantly higher than ZDF +/? rats until the age of 21 weeks (7.8 ± 2.5 ng/ml versus 1.8 ± 0.2 ng/ml; $P<0.05$).

In this study, plasma leptin levels were measured only from terminal blood samples of ZDF +/? and ZDF *fa/fa* rats (Table 6.1). At the age of 24 weeks, leptin levels in ZDF *fa/fa* rats were significantly higher than ZDF +/? rats by 3.5-fold. Pancreatic insulin levels showed almost a 6.75-fold reduction in ZDF *fa/fa* rats compared to ZDF +/? rats.

Table 6.1 Leptin and pancreatic insulin levels in terminal blood samples of ZDF +/? and *fa/fa* rats

Group	Leptin (ng/ml)	Pancreatic Insulin (ng insulin /mg tissue)
ZDF +/?	**3.6 ± 0.4**	**1836 ± 329**
ZDF *fa/fa*	**12.6 ± 2.1****	**272 ± 94****

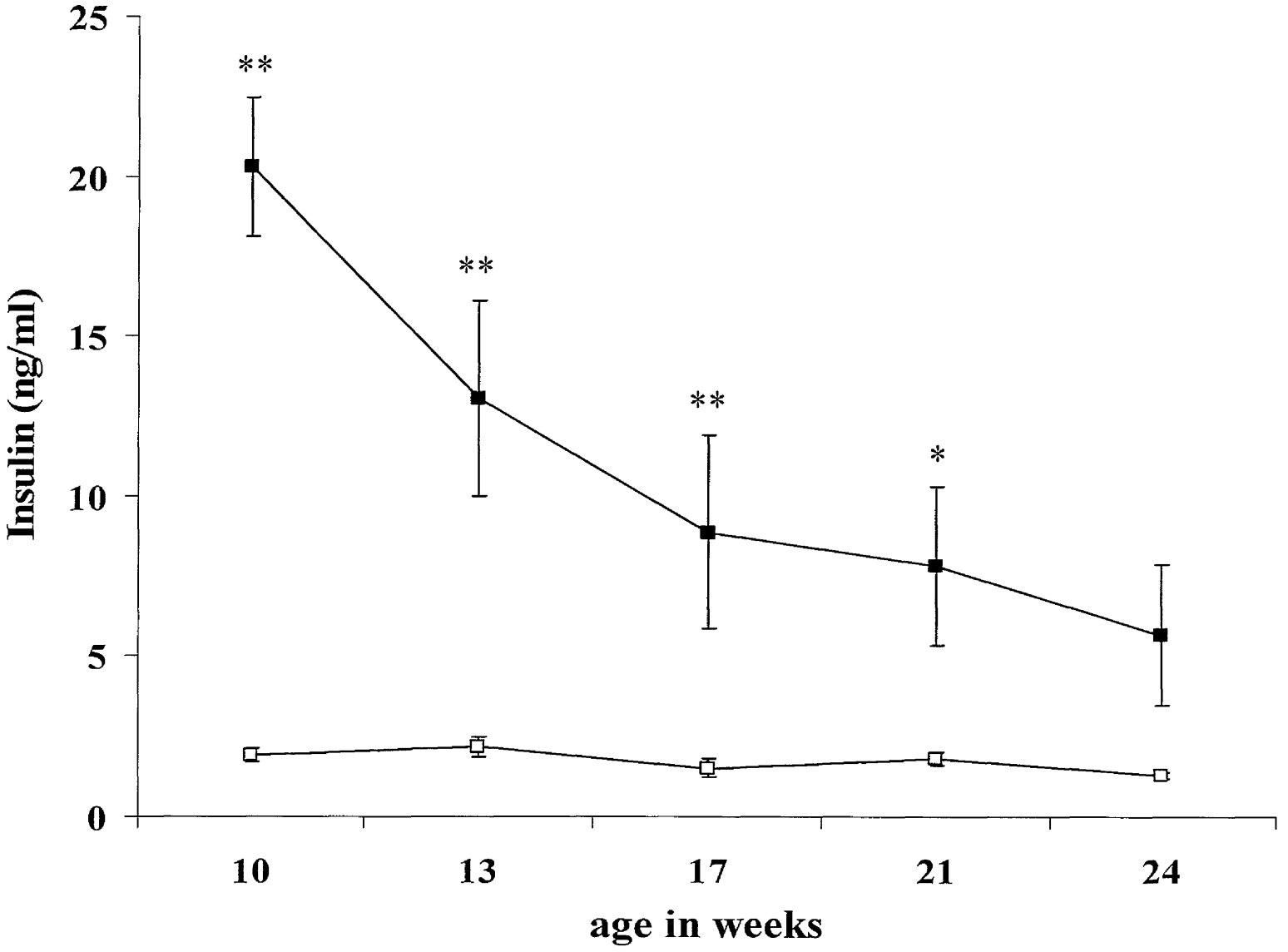
** $P<0.01$

Figure 6.3 Changes in plasma insulin concentration of ZDF +/? and *fa/fa* rats

Plasma insulin concentrations (ng/ml) were measured from blood samples of ZDF +/? and *fa/fa* rats between the ages of 10 to 24 weeks. Values are represented as mmol/l ± SEM, * $P<0.05$ and ** $P<0.01$.

□ ZDF +/? rats

■ ZDF *fa/fa* rats


25
20
15
10
5
0
Insulin (ng/ml)
**
**
**
*
10
13
17
21
24
age in weeks

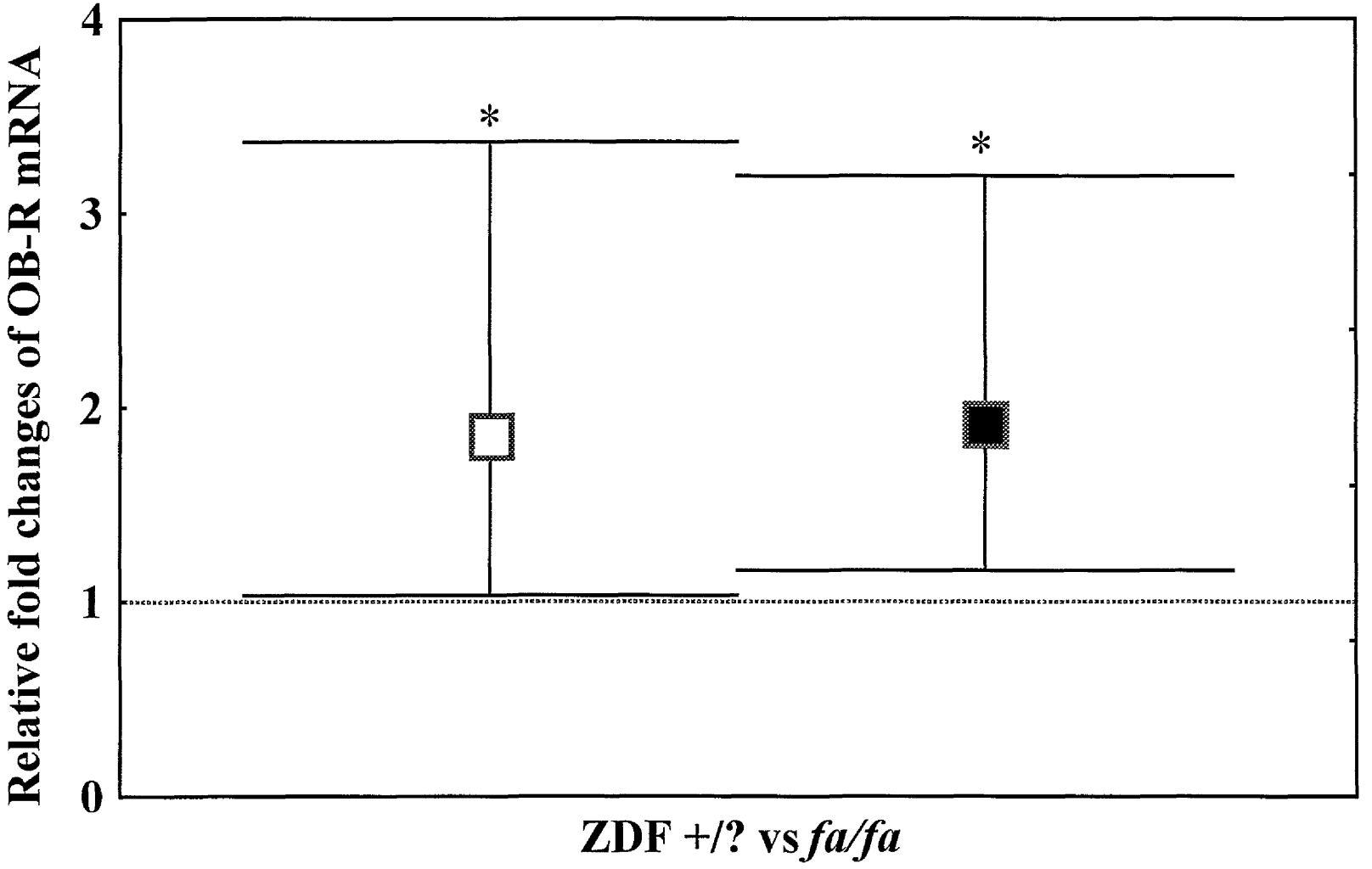
OB-Ra and OB-Rb mRNA expression was investigated in the hypothalamus of ZDF *fa/fa* rats and ZDF +/? rats using β-actin, GAPDH, cylophilin and HPRT as housekeeping genes. The results shown in Figure 6.6 are a pool of the TaqMan runs described above. None of the housekeeping genes were used as a co-variate for ANCOVA analysis because either they were not suitable due to variation in the data or they did not improve the analysis. In the hypothalamus, the mRNA expression of OB-Ra and OB-Rb in ZDF *fa/fa* rats compared to ZDF +/? rats was increased by 86% and 92% ($P<0.05$), respectively (Figure 6.4).

Figure 6.4 OB-Ra and OB-Rb expression in the hypothalamus of ZDF +/? and *fa/fa* rats

The mRNA expression of OB-Ra and OB-Rb in ZDF +/? rats have been ascribed a value of 1 and the symbols indicate fold changes from this baseline (as described in Appendix 1). Comparisons are shown of OB-Ra and OB-Rb mRNA in the hypothalamus of ZDF +/? and ZDF *fa/fa* rats at the age of 24 weeks. Error bars indicate 95% confidence intervals and changes are shown as significant where * $P<0.05$.

☐ OB-Ra

■ OB-Rb


Relative fold changes of OB-R mRNA
4
3
2
1
0
*
*
ZDF +/? vs *fa/fa*

6.3 Discussion

The aim of this study was to investigate if there are any changes in hypothalamic leptin receptor expression in ZDF *fa/fa* rats compared to ZDF +/? rats. This study shows that leptin levels were clearly elevated by 3.5-fold in ZDF *fa/fa* rats compared to ZDF +/? rats at the age of 24 weeks. This can be explained by the fact that ZDF *fa/fa* rats have more fat resulting from their reduced leptin sensitivity. In young ZDF *fa/fa* rats, plasma insulin levels were highly elevated compared to young ZDF +/? rats due to their high glucose levels. However, as β-cells of ZDF *fa/fa* rats start to deteriorate plasma insulin levels in ZDF *fa/fa* rats were reduced with age whereas plasma insulin remained similar in ZDF +/? rats during the 24 week period. At the age of 24 weeks, there was no significant difference in plasma insulin levels between ZDF *fa/fa* rats and ZDF +/? rats. The reduction in ZDF *fa/fa* insulin levels is caused by the failure of β-cells to compensate for the insulin resistant state of the rats due to lipotoxicity.

The changes in body weight, blood glucose, plasma insulin and plasma leptin levels measured in ZDF *fa/fa* rats and ZDF +/? rats are similar to changes observed in a recent study by Janssen *et al.* of animals between 10 and 25 weeks of age (Janssen *et al.*, 1999). In the study by Janssen *et al.*, plasma leptin levels were measured for the duration of the study and correlated with plasma insulin rather than with body weight in ZDF *fa/fa* rats (Janssen *et al.*, 1999). This suggests an important role for insulin in the modulation of leptin secretion which is in accordance with previous studies (Patel *et al.*, 1998; Saladin *et al.*,

1995). Saladin *et al.* showed that insulin administration of fasted rats increased leptin mRNA to the level of fed controls, and Patel *et al.* showed that increased plasma leptin and leptin mRNA following food intake is dependent on plasma insulin levels in rats (Patel *et al.*, 1998; Saladin *et al.*, 1995). In human adipose tissue, insulin appears to induce an early inhibition of leptin secretion by the adipose cell whereas prolonged exposure to insulin increases plasma leptin concentrations (Casabiell *et al.*, 2000). As a feedback mechanism leptin has been shown to modulate insulin secretion. Leptin inhibits the secretion of insulin in isolated islets of *ob/ob* mice (Kieffer *et al.*, 1997) and in the perfused pancreas of *ob/ob* mice (Emilsson *et al.*, 1997).

The differential expression of the OB-Rb transcript in human hypothalamus may be caused by endogenous factors such as insulin and leptin (Hikita *et al.*, 2000). There is growing evidence that insulin functions as an afferent metabolic signal in the CNS (Inui, 1999; Porte *et al.*, 1998; Woods *et al.*, 1998). For example, insulin reduces food intake and body weight in a dose-dependent manner when administered directly into the CNS (Woods *et al.*, 1998). Furthermore, in growth hormone releasing hormone (GHRH) transgenic mice, growth hormone (GH) and/or GHRH as well as leptin and insulin may contribute to changes in OB-Rb mRNA expression in the anterior pituitary and hypothalamus (Cai & Hyde, 1999). It is possible that insulin-mediated intracellular signalling may regulate OB-Rb expression levels in hypothalamic neurons. Porte *et al.* have proposed the hypothesis that intact leptin signalling is required for CNS responses to insulin, and that leptin resistance, therefore,

would lead to a condition in which insulin is less effective as a satiety signal (Porte *et al.*, 1998). Therefore, it is likely that insulin might perform its satiety function in part by regulating leptin signalling in neurons. In the process of such a regulatory mechanism, the differential regulation of OB-Rb by insulin and leptin might play a role in maintaining body weight mass. A previous observation (Iida *et al.*, 1996) that OB-R mRNA expression is increased in the hypothalamus of Zucker *fa/fa* rats by 3.5-fold, is consistent with increased OB-Ra and OB-Rb mRNA expression in the hypothalamus of ZDF *fa/fa* rats. The elevated levels of leptin present in this animal model might be expected to result in reduced hypothalamic leptin receptor expression, however compromised leptin receptor signalling and high insulin levels may be involved in the upregulation of receptor expression. The feedback mechanism could involve another hormone or a downstream signalling factor, such as SOCS-3.

In situ binding experiments using ^{35}S-leptin on brain slices from *fa/fa* rats indicate that the *fa/fa* OB-R is capable of binding leptin (Phillips *et al.*, 1996). Therefore, the Gln to Pro mutation does not render a null state for leptin binding, suggesting that the mutation may have its predominant effect on OB-R expression or dimer formation, a requisite for signalling with this class of receptors (Kishimoto *et al.*, 1994). Rosenblum *et al.* have observed that the mutated *fa* OB-R is functional but there is reduced activation of STATs 1 and 3 (Rosenblum *et al.*, 1996). In addition the efficiency of *fa* OB-R expression is greatly reduced when transfected into either GT1-7 and COS-7 cells and compared to the wild-type OB-R (Rosenblum *et al.*, 1996). The decreased

response to leptin in cells expressing *fa* rat OB-R suggests that the $Gln^{269} \rightarrow Pro^{269}$ mutation plays a role in the cell-surface expression of OB-R and which may be one cause of the leptin resistant phenotype. Therefore, the amount of OB-R present on a cell surface in addition to attenuated signalling capacity may be critical for regulation of body weight. Interestingly, there are examples of missense mutations in the human insulin receptor which cause impaired receptor transport to the cell surface and are partially, if not completely, responsible for insulin resistance (Kadowaki *et al.*, 1990; Kadowaki *et al.*, 1991; Maassen *et al.*, 1991). In addition to the elevated insulin levels and impaired leptin receptor signalling, the increased OB-Ra and OB-Rb mRNA expression may be a compensatory mechanism for reduced cell surface expression.

There are a number of studies that illustrate reduced OB-R cell surface expression and signalling in the Zucker *fa* rat (Crouse *et al.*, 1998; Rosenblum *et al.*, 1996; White *et al.*, 1997b; Yamashita *et al.*, 1997). White *et al.* have suggested the mutation in OB-Rb (*fa*) induces an incomplete conformational change that partially mimics the ligand bound state, resulting in partial constitutive receptor activation. The OB-Rb (*fa*) may adopt a conformation that prevents efficient interaction with downstream signalling components resulting in constitutively activate STAT5B and impaired for leptin-induced STAT5B activation.

In summary, the mRNA expression of OB-Ra and OB-Rb are increased in the hypothalamus of ZDF *fa/fa* rats, perhaps in an attempt to compensate for reduced OB-R surface expression and leptin signalling. Although it has been

suggested that the reduced OB-R surface expression may result in reduced leptin transport to the CNS, the concentration of cerebral spinal fluid leptin in ZDF *fa/fa* rats is equal to that in non-obese controls (Wu-Peng *et al.*, 1997). Consistent with reduced leptin signalling, this suggests the fatty rat is obese due to diminished leptin responsiveness in hypothalamic neurons. Furthermore, fatty rats respond to i.c.v. leptin injections only at greatly increased doses relative to lean controls (Al Barazanji *et al.*, 1997; Cusin *et al.*, 1996). Moreover, the phenotype of ZDF *fa/fa* may be a result of constitutive OB-Rb (*fa*) signalling, which is not inconsistent with the recessive nature of the fatty mutation since the coexpression with OB-Rb (wt) results in suppression of constitutive OB-Rb (*fa*) signalling (White *et al.*, 1997b). Although the increase in hypothalamic OB-Ra and OB-Rb mRNA expression is small, it is not known what the effects at the protein level will be. Therefore, the resulting phenotype of ZDF *fa/fa* rats may be due to both reduced receptor levels on the cell surface and reduced signalling of the leptin receptor.

Chapter 7

General Discussion

The aim of this research was to investigate whether changes in mRNA expression of genes involved in leptin receptor-mediated signalling plays a role in leptin sensitivity. These studies primarily used leptin-insensitive dietary obese and leptin-sensitive genetically obese mice as model systems. Leptin insensitivity may be a result of one or more defects: the transport of leptin to the hypothalamic nuclei, the expression of leptin receptor isoforms in central and peripheral tissues and/or the expression of signalling components downstream of the leptin receptor. This series of experiments was designed to focus on the latter two possibilities. The expression of six genes that have reportedly been involved in leptin receptor-mediated signalling: OB-Ra, OB-Rb, STAT3, STAT5 SOCS-3 and CIS was examined. Initially, a study was set up to compare the gene expression of components of leptin receptor-mediated signalling in the hypothalamus of leptin-insensitive dietary obese and leptin-sensitive genetically obese (*ob/ob*) mice (Chapter 3). This also raised the opportunity to investigate gene expression in other tissues in which there were increasing number of reports of leptin receptor expression and function. The other tissues examined were the pituitary, pancreas, WAT, BAT and small intestine (Chapter 4).

7.1 AKR/J mice fed a palatable diet compared to chow

7.1.1 Hypothalamus and pituitary

In AKR/J mice fed a palatable diet compared to those fed on chow, hypothalamus and pituitary showed changes in components of leptin receptor-mediated signalling. The expression of OB-Ra and STAT5 mRNA was reduced in the hypothalamus; OB-Ra mRNA was reduced in the pituitary. The reduced expression of OB-Ra mRNA in the hypothalamus and pituitary may implicate reduced leptin access in these tissues and/or reduced OB-Ra-mediated signalling i.e. JAK2 and MAPK activation. Leptin, through OB-Rb, can activate STAT3, but not STAT5, in the hypothalamus of lean and *ob/ob* mice (Vaisse *et al.*, 1996), suggesting the reduced STAT5 mRNA in dietary obese AKR/J mice may be due to obesity-related factors other than leptin. No significant change was observed in SOCS-3 mRNA in the hypothalamus of AKR/J mice fed a palatable diet compared to those fed on a chow diet, although expression tended to increase and is consistent with leptin insensitivity in dietary obese mice. However, a recent study on the development of dietary obesity in C57BL/6J mice showed reduced central leptin sensitivity after 19 weeks on a high-fat diet (Lin *et al.*, 2000). Perhaps in this 14-week study, the mice are still sensitive to the effects of leptin and the effect is more pronounced on the expression of SOCS-3 mRNA than on OB-Rb or STAT3 mRNA. A further explanation may be that there are a number of signals involved in obesity that can activate SOCS-3 mRNA in the hypothalamus, such as NPY and POMC (Elias *et al.*, 1999).

7.1.2 Peripheral tissues

The reduced CIS mRNA in the pancreas of AKR/J mice fed a palatable diet compared to those on chow, albeit not significant, may indicate the pancreas has increased leptin sensitivity. In WAT of AKR/J mice fed a palatable diet compared to chow, the expression of OB-Ra, OB-Rb, STAT5 and CIS mRNA was increased, but SOCS-3 mRNA was reduced. The increased expression of OB-Ra and OB-Rb mRNA was not significant; however, the general increase in components of leptin receptor-mediated signalling suggests this tissue is sensitive to leptin but the increased CIS mRNA may compensate. In BAT of AKR/J mice fed a palatable diet compared to chow, the expression of OB-Rb mRNA was reduced but STAT3 and STAT5 mRNA was increased. Whilst these changes were not significant, they show that although OB-Rb mRNA is reduced consistent with low leptin sensitivity, the expression of STAT3 and STAT5 mRNA is increased. Perhaps in compensation these changes suggest the interplay of other obesity-related factors as well as tissue-specific changes, which are most likely related to the biological function of leptin in these tissues. In contrast to WAT, all of the components of the leptin receptor-mediated signalling pathway examined, except CIS, were significantly reduced in the small intestine of AKR/J mice fed a palatable diet compared to chow. This suggests that the small intestine has low leptin sensitivity and may have reduced leptin signalling.

7.2 *ob/ob* compared to lean mice

7.2.1 Hypothalamus and pituitary

In *ob/ob* compared to lean mice in this study, the expression of components of the leptin receptor-mediated signalling pathway may be expected to increase due to the lack of circulating leptin; however they were generally increased in some tissues and reduced in others. In the hypothalamus of *ob/ob* mice, only STAT3 and SOCS-3 mRNA was reduced. These changes contrast to those observed in the study of leptin-treated *ob/ob* mice where OB-Rb, STAT3, STAT5 and NPY mRNA were increased in *ob/ob* mice compared to leans. An increase in expression of components of leptin receptor-mediated signalling is expected since the *ob/ob* mice are leptin-hypersensitive. It is possible that the reduced SOCS-3 mRNA in *ob/ob* compared to lean mice may compensate for the increased STAT3 mRNA, where the change in STAT3 mRNA may involve other obesity-related factors. There was no significant increase in the expression of hypothalamic NPY mRNA, which is expected due to the lack of circulating leptin, although there was tendency towards an increase. In the pituitary of *ob/ob* mice compared to leans, the expression of OB-Rb, STAT3, STAT5 and SOCS-3 mRNA was reduced, which may be due to leptin deficiency. Furthermore, CIS mRNA was reduced in the pituitary of *ob/ob* mice, but not significantly, and may also be due to leptin deficiency.

7.2.2 Peripheral tissues

In the pancreas, the expression of STAT3, STAT5 and SOCS-3 mRNA was increased, perhaps due to high leptin sensitivity and/or increased insulin signalling in the pancreas, which can activate these components. As expected, insulin and PDX-1 mRNA expression was increased in the pancreas of *ob/ob* mice compared to leans, which is consistent with the elevated pancreatic insulin levels. Furthermore, the reduced GLUT2 mRNA in the pancreas may be associated with the reduced glucose tolerance of *ob/ob* mice. In BAT of *ob/ob* mice compared to leans, the increased OB-Ra, OB-Rb and STAT3 mRNA expression and reduced CIS mRNA expression is consistent with elevated leptin sensitivity in this tissue. However, the increased expression of SOCS-3 mRNA may be due to other obesity-related factors. In the small intestine of *ob/ob* mice compared to leans, the expression of OB-Rb and CIS mRNA was reduced and STAT3 mRNA was increased. The changes of these genes in different tissues do not follow any particular pattern and may be due to the effect of other obesity-related factors. It is difficult to distinguish effects of no leptin, tissue-specific effects and other obesity-related factors.

7.3 AKR/J comapared to C57BL/6 lean mice

In comparing obesity-prone AKR/J lean mice to C57BL/6 lean mice, the expression of leptin receptor-mediated signalling components was generally increased in all tissues of AKR/J mice examined (except WAT where there was no comparison made). In the hypothalamus, only OB-Ra mRNA was increased

significantly but the expression of CIS mRNA also showed a trend towards an increase. In the pituitary, the expression of OB-Ra, OB-Rb, STAT5 and SOCS-3 mRNA was increased significantly while STAT3 mRNA expression tended to increase. In the pancreas, STAT3, STAT5 and SOCS-3 mRNA was increased while in BAT, the expression of OB-Ra, OB-Rb and CIS mRNA was increased. In the small intestine, there was a significant increase in OB-Rb and STAT3 mRNA, however the increases in OB-Ra, STAT5, and SOCS-3 mRNA were close to significance. Interestingly, in all of these tissues, the expression of OB-Ra mRNA was increased, whilst OB-Rb mRNA was increased in all of the tissues except the hypothalamus. Therefore, the increased expression in components of leptin receptor-mediated signalling is tissue-specific, which may be related to the function of the leptin in these tissues and contribute to their phenotype.

7.4 Leptin treatment of *ob/ob* mice

A recent paper by Emilsson *et al.* (1999) had looked at the gene expression of components of leptin receptor-mediated signalling in central and peripheral tissues after 48-hr intraperitoneal leptin treatment of lean and *ob/ob* mice and observed increased expression of SOCS-3 and CIS mRNA in leptin-treated lean mice in the hypothalamus, liver and small intestine. Furthermore, a study by Bjorbaek *et al.* showed increased SOCS-3 mRNA expression in the hypothalamus of *ob/ob* mice after peripheral leptin administration but no change in CIS mRNA (Bjorbaek *et al.*, 1998). In order to expand our understanding of

the regulation of leptin sensitivity, the study presented in chapter 5 was designed to examine the expression of components of leptin receptor-mediated signalling after short and long-term leptin treatment in *ob/ob* mice and attempt to reduce the body weight of *ob/ob* mice considerably to see if any changes in the genes of interest were reversed.

In the study of *ob/ob* mice treated with leptin for 24 hours and 2 weeks, an increase of OB-Rb, STAT3, STAT5 and NPY mRNA was observed in the hypothalamus of vehicle-treated *ob/ob* mice compared to leans. However, the hypothalamic expression of SOCS-3 mRNA was reduced in *ob/ob* mice compared to leans. The treatment of *ob/ob* mice with leptin for 24 hours showed a significant reduction of SOCS-3 mRNA, whilst the expression of both OB-Ra and NPY mRNA showed a non-significant trend towards a reduction. However, after *ob/*ob mice were treated with leptin for 2 weeks, the expression of SOCS-3 mRNA was increased and NPY mRNA was decreased, and STAT3 and STAT5 mRNA expression was unchanged. Furthermore, food restriction of *ob/ob* mice for 2 weeks showed an increased expression of STAT3 and STAT5 mRNA, whilst SOCS-3 and NPY mRNA was increased. This implies that increased SOCS-3 mRNA after 2 weeks of leptin treatment is due to reduced food intake rather than leptin effects. Previously, it was shown that SOCS-3 mRNA was increased between 1-3 hours after leptin treatment of *ob/ob* mice (Bjorbaek *et al.*, 1998), but a subsequent study in CHO cells showed a continued increase up to 20 hours after treatment (Bjorbaek *et al.*, 1999). It is

not clear why expression of SOCS-3 mRNA was reduced after 24 hours of leptin treatment.

7.5 *fa/fa* compared to +/? rats

An opportunity arose to examine the expression of leptin receptor isoforms OB-Ra and OB-Rb in the hypothalamus of ZDF *fa/fa* rats, a rat model of diabetes that has a mutation in the leptin receptor. Results from this study are presented in Chapter 6. In the hypothalamus of ZDF +/? rats compared to ZDF *fa/fa* rats, the expression of OB-Ra and OB-Rb mRNA was increased by almost 2-fold. This may be due to reduced leptin receptor-mediated signalling and cell surface receptor expression, as observed in the *fatty* rat, and as a result there is a compensatory increase in the expression of leptin receptor isoforms.

7.6 Future Work

In the dietary and genetically obese mice, there were a number of tissues yet to be examined, such as the liver, kidney, soleus muscle and adrenals. In addition to examining other tissues, there is much scope to determine changes in expression of other relevant genes. For example, in the hypothalamus there are a number of genes involved in energy balance that would be interesting to examine, such as melanin-concentrating hormone (MCH), orexins, CART, CRH, the melanocortin system i.e. POMC, ACTH, α-melanocyte stimulating hormone (α-MSH), melanocortin-4 (MC-4) receptor and AGRP. The analysis of these genes and more may provide further insight in the genes that are

involved in leptin sensitivity. In the pituitary, it would be interesting to analyse the genes expressed by gonadotropes, somatotropes and corticotropes that may be involved in leptin effects, such as adrenocorticotropin hormone (ACTH), follicle stimulating hormone (FSH), luteinizing hormone (LH), thyroid stimulating hormone (TSH) and GH. Furthermore, it would have been useful to analyse the expression of components of the leptin receptor-mediated signalling pathway in the islets of the pancreas, where most of the leptin receptor isoform expression is concentrated and there is cross-talk of leptin and insulin signalling pathways. In adipose tissue, leptin is believed to increase the expression of UCP mRNA and shift fuel consumption to fat oxidation. Therefore, it would be interesting to analyse the expression of UCP mRNA as well as genes that may be involved in fatty acid metabolism.

In the study described in Chapter 5 of *ob/ob* mice treated with leptin, the expression of components of leptin receptor-mediated signalling pathway in the hypothalamus could have been followed up by the analysis of the genes involved in energy balance described above. Furthermore, the pancreas and adipose tissue were also extracted from the mice but the tissues remain to be analysed. The expression of OB-Ra and OB-Rb mRNA was analysed in the hypothalamus of ZDF +/? and *fa/fa* rats, however there were no other genes analysed. It would be interesting to see the effects on expression of components of the leptin receptor mediated signalling pathway in ZDF *fa/fa* rats given that the *fatty* rat performs reduced signal transduction and compare results to those in other animal models.

Most of the studies to date that examined leptin sensitivity in obesity have investigated the effects of leptin on feeding, however few studies have examined leptin sensitivity at a functional or physiological level with respect to factors other than feeding. The changes in leptin sensitivity may not be restricted to the hypothalamus alone, but perhaps some of the peripheral tissues also exhibit changes in leptin sensitivity. Therefore, leptin access to the brain may not be the only factor involved in leptin sensitivity, but the reduced effects of leptin in peripheral tissues may affect metabolic functions as observed in obesity. For example, a reduced effect of leptin in adipose tissue may have an effect on UCP genes and reduce energy expenditure, and in pancreatic islets, a reduced effect of leptin may result in increased insulin secretion and an increase in fatty acids due to reduced fatty acid oxidation. The importance of the changes in gene expression involved in leptin receptor-mediated signalling in some tissues may be better understood knowing the effects of leptin insensitivity on other metabolic effects in obesity.

Whilst I have only looked at the mRNA expression of leptin receptor-mediated signalling components in the tissues of these obese models, it would be interesting and very informative to investigate the changes in protein expression. It goes without saying that changes of mRNA do not necessarily reflect changes in protein expression nor activity of the gene expression.

7.7 Concluding Remarks

I have presented data suggesting the changes in expression of components of the leptin receptor-mediated signalling pathway may contribute to how cells respond to leptin in a tissue- and strain-specific manner. Furthermore, analysis of tissues taken from dietary obese and genetically obese mice suggests some tissues may be more sensitive to the effects of leptin compared to others. Some strains of mice are more prone to diet-induced obesity compared to others, which may be reflected by the increased expression of components of the leptin receptor-mediated signalling pathway in certain tissues. The specific pattern of gene expression changes were different dependent on the strain and conditions of the experiment. In many cases, changes due to leptin sensitivity could not be definitively distinguished from those due to other obesity-related factors.

References

Ahima, R. S., Prabakaran, D., Mantzoros, C., Qu, D. Q., Lowell, B., Maratosflier, E., & Flier, J. S. (1996) Role of leptin in the neuroendocrine response to fasting. *Nature*, **382** (6588), 250-252.

Ahren, B. & Havel, P. J. (1999) Leptin inhibits insulin secretion induced by cellular cAMP in a pancreatic B cell line (INS-1 cells). *American Journal of Physiology - Regulatory Integrative & Comparative Physiology*, **277** (4), R959-R966.

Al Barazanji, K. A., Buckingham, R. E., Arch, J. R. S., Haynes, A., Mossakowska, D. E., Mcbay, D. L., Holmes, S. D., Mchale, M. T., Wang, X. M., & Gloger, I. S. (1997) Effects of intracerebroventricular infusion of leptin in obese Zucker rats. *Obesity Research*, **5** (5), 387-394.

Aliaga, J. C., Deschenes, C., Beaulieu, J. F., Calvo, E. L., & Rivard, N. (1999) Requirement of the MAP kinase cascade for cell cycle progression and differentiation of human intestinal cells. *American Journal of Physiology*, **277** (3 Pt 1), G631-G641.

Allison, D. B., Fontaine, K. R., Manson, J. E., Stevens, J., & VanItallie, T. B. (1999) Annual deaths attributable to obesity in the United States. *Jama: Journal of the American Medical Association*, **282** (16), 1530-1538.

Ashcroft, F. M. & Rorsman, P. (1989) Electrophysiology of the pancreatic beta-cell. *Progress in Biophysics & Molecular Biology*, **54** (2), 87-143.

Auernhammer, C. J., Bousquet, C., & Melmed, S. (1999) Autoregulation of pituitary corticotroph SOCS-3 expression: Characterization of the murine SOCS-3 promoter. *Proceedings of the National Academy of Sciences of the United States of America*, **96** (12), 6964-6969.

Bado, A., Levasseur, S., Attoub, S., Kermorgant, S., Laigneau, J. P., Bortoluzzi, M. N., Moizo, L., Lehy, T., Guerremillo, M., Lemarchandbrustel, Y., & Lewin, M. J. M. (1998) The stomach is a source of leptin. *Nature*, **394** (6695), 790-793.

Banks, A. S., Davis, S. M., Bates, S. H., & Myers, M. G. (2000) Activation of downstream signals by the long form of the leptin receptor. *Journal of Biological Chemistry*, **275** (19), 14563-14572.

Banks, W. A., DiPalma, C. R., & Farrell, C. L. (1999) Impaired transport of leptin across the blood-brain barrier in obesity. *Peptides*, **20** (11), 1341-1345.

Barash, I. A., Cheung, C. C., Weigle, D. S., Ren, H. P., Kabigting, E. B., Kuijper, J. L., Clifton, D. K., & Steiner, R. A. (1996) Leptin is a metabolic signal to the reproductive system. *Endocrinology*, **137** (7), 3144-3147.

Barr, V. A., Malide, D., Zarnowski, M. J., Taylor, S. I., & Cushman, S. W. (1997) Insulin stimulates both leptin secretion and production by rat white adipose tissue. *Endocrinology*, **138** (10), 4463-4472.

Baskin, D. G., Schwartz, M. W., Seeley, R. J., Woods, S. C., Porte, D., Breininger, J. F., Jonak, Z., Schaefer, J., Krouse, M., Burghardt, C., Campfield, L. A., Burn, P., & Kochan, J. P. (1999) Leptin receptor long-form splice-variant protein expression in neuron cell bodies of the brain and co-localization with neuropeptide Y mRNA in the arcuate nucleus. *Journal of Histochemistry & Cytochemistry*, **47** (3), 353-362.

Baskin, D. G., Seeley, R. J., Kuijper, J. L., Lok, S., Weigle, D. S., Erickson, J. C., Palmiter, R. D., & Schwartz, M. W. (1998) Increased expression of mRNA for the long form of the leptin receptor in the hypothalamus is associated with leptin hypersensitivity and fasting. *Diabetes*, **47** (4), 538-543.

Baumann, H., Morella, K. K., White, D. W., Dembski, M., Bailon, P. S., Kim, H. K., Lai, C. F., & Tartaglia, L. A. (1996) The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. *Proceedings of the National Academy of Sciences of the United States of America*, **93** (16), 8374-8378.

Bazan, J. F. (1989) A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor beta-chain. *Biochemical & Biophysical Research Communications*, **164** (2), 788-795.

Begum, N., Song, Y., Rienzie, J., & Ragolia, L. (1998) Vascular smooth muscle cell growth and insulin regulation of mitogen-activated protein kinase in hypertension. *American Journal of Physiology*, **275** (1 Pt 1), C42-C49.

Bendinelli, P., Maroni, P., Giraldi, F. P., & Piccoletti, R. (2000) Leptin activates Stat3, Stat1 and AP-1 in mouse adipose tissue. *Molecular & Cellular Endocrinology*, **168** (1-2), 11-20.

Berti, L., Kellerer, M., Capp, E., & Haring, H. U. (1997) Leptin stimulates glucose transport and glycogen synthesis in C_2 C_{12} myotubes - evidence for a PI3-kinase mediated effect. *Diabetologia*, **40** (5), 606-609.

Bjorbaek, C., Buchholz, R. M., Davis, S. M., Bates, S. H., Pierroz, D. D., Gu, H., Neel, B. G., Myers, M. G., & Flier, J. S. (2001) Divergent roles of SHP-2 in ERK activation by leptin receptors. *Journal of Biological Chemistry*, **276** (7), 4747-4755.

Bjorbaek, C., El Haschimi, K., Frantz, J. D., & Flier, J. S. (1999) The role of SOCS-3 in leptin signaling and leptin resistance. *Journal of Biological Chemistry*, **274** (42), 30059-30065.

Bjorbaek, C., Elmquist, J. K., Frantz, J. D., Shoelson, S. E., & Flier, J. S. (1998b) Identification of SOCS-3 as a potential mediator of central leptin resistance. *Molecular Cell*, **1** (4), 619-625.

Bjorbaek, C., Elmquist, J. K., Michl, P., Ahima, R. S., Vanbueren, A., Mccall, A. L., & Flier, J. S. (1998a) Expression of leptin receptor isoforms in rat brain microvessels. *Endocrinology*, **139** (8), 3485-3491.

Bjorbaek, C., Lavery, H. J., Bates, S. H., Olson, R. K., Davis, S. M., Flier, J. S., & Myers, M. G. (2000) SOCS3 mediates feedback inhibition of the leptin receptor via Tyr(985). *Journal of Biological Chemistry*, **275** (51), 40649-40657.

Bjorbaek, C., Uotani, S., Dasilva, B., & Flier, J. S. (1997) Divergent signaling capacities of the long and short isoforms of the leptin receptor. *Journal of Biological Chemistry*, **272** (51), 32686-32695.

Boado, R. J., Golden, P. L., Levin, N., & Pardridge, W. M. (1998) Up-regulation of blood-brain barrier short-form leptin receptor gene products in rats fed a high fat diet. *Journal of Neurochemistry*, **71** (4), 1761-1764.

Boden, G., Chen, X., Mozzoli, M., & Ryan, I. (1996) Effect of fasting on serum leptin in normal human subjects. *Journal of Clinical Endocrinology & Metabolism*, **81** (9), 3419-3423.

Boden, G., Chen, X. H., Kolaczynski, J. W., & Polansky, M. (1997) Effects of prolonged hyperinsulinemia on serum leptin in normal human subjects. *Journal of Clinical Investigation*, **100** (5), 1107-1113.

Bouloumie, A., Drexler, H. C. A., Lafontan, M., & Busse, R. (1998) Leptin, the product of ob gene, promotes angiogenesis. *Circulation Research*, **83** (10), 1059-1066.

Briscoe, C. P., Hanif, S., Arch, J. R. S., & Tadayyon, M. (2001a) Fatty acids inhibit leptin signalling in BRIN-BD11 insulinoma cells. *Journal of Molecular Endocrinology*, **26** (2), 145-154.

Briscoe, C. P., Hanif, S., Arch, J. R. S., & Tadayyon, M. (2001b) Leptin receptor long-form signalling in a human liver cell line. *Cytokine*, **14** (4), 225-229.

Burguera, B., Couce, M. E., Long, J., Lamsam, J., Laakso, K., Jensen, M. D., Parisi, J. E., & Lloyd, R. V. (2000) The long form of the leptin receptor (OB-Rb) is widely expressed in the human brain. *Neuroendocrinology*, **71** (3), 187-195.

Cai, A. H. & Hyde, J. F. (1998) Upregulation of leptin receptor gene expression in the anterior pituitary of human growth hormone-releasing hormone transgenic mice. *Endocrinology*, **139** (1), 420-423.

Cai, A. H. & Hyde, J. F. (1999) The human growth hormone-releasing hormone transgenic mouse as a model of modest obesity: Differential changes in leptin receptor (OBR) gene expression in the anterior pituitary and hypothalamus after fasting and OBR localization in somatotrophs. *Endocrinology*, **140** (8), 3609-3614.

Campfield, L. A., Smith, F. J., Guisez, Y., Devos, R., & Burn, P. (1995) Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science*, **269** (5223), 546-549.

Caro, J. F., Kolaczynski, J. W., Nyce, M. R., Ohannesian, J. P., Opentanova, I., Goldman, W. H., Lynn, R. B., Zhang, P. L., Sinha, M. K., & Considine, R. V. (1996) Decreased cerebrospinal-fluid/serum leptin ratio in obesity - a possible mechanism for leptin resistance. *Lancet*, **348** (9021), 159-161.

Carpenter, L. R., Farruggella, T. J., Symes, A., Karow, M. L., Yancopoulos, G. D., & Stahl, N. (1998) Enhancing leptin response by preventing SH2-containing phosphatase 2 interaction with Ob receptor. *Proceedings of the National Academy of Sciences of the United States of America*, **95** (11), 6061-6066.

Casabiell, X., Pineiro, V., De la Cruz, L. F., Gualillo, O., Folgar, L., Dieguez, C., & Casanueva, F. F. (2000) Dual effect of insulin on in vitro leptin secretion by adipose tissue. *Biochemical & Biophysical Research Communications*, **276** (2), 477-482.

Casabiell, X., Pineiro, V., Tome, M. A., Peino, R., Dieguez, C., & Casanueva, F. F. (1997) Presence of leptin in colostrum and/or breast milk from lactating mothers - a potential role in the regulation of neonatal food intake. *Journal of Clinical Endocrinology & Metabolism*, **82** (12), 4270-4273.

Ceddia, R. B., William, W. N., Lima, F. B., & Curi, R. (1998) Leptin inhibits insulin-stimulated incorporation of glucose into lipids and stimulates glucose decarboxylation in isolated rat adipocytes. *Journal of Endocrinology*, **158** (3), R7-R9.

Ceresa, B. P., Horvath, C. M., & Pessin, J. E. (1997) Signal transducer and activator of transcription-3 serine phosphorylation by insulin is mediated by a RAS/RAF/MEK-dependent pathway. *Endocrinology*, **138** (10), 4131-4137.

Ceresa, B. P. & Pessin, J. E. (1996) Insulin stimulates the serine phosphorylation of the signal transducer and activator of transcription (STAT3) isoform. *Journal of Biological Chemistry*, **271** (21), 12121-12124.

Chehab, F. E., Lim, M. E., & Lu, R. H. (1996) Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nature Genetics*, **12** (3), 318-320.

Chen, H., Charlat, O., Tartaglia, L. A., Woolf, E. A., Weng, X., Ellis, S. J., Lakey, N. D., Culpepper, J., Moore, K. J., Breitbart, R. E., Duyk, G. M., Tepper, R. I., & Morgenstern, J. P. (1996) Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in *db/db* mice. *Cell*, **84** (3), 491-495.

Chen, N. G., Swick, A. G., & Romsos, D. R. (1997) Leptin constrains acetylcholine-induced insulin secretion from pancreatic islets of *ob/ob* mice. *Journal of Clinical Investigation*, **100** (5), 1174-1179.

Chua, S. C., Chung, W. K., Wupeng, X. S., Zhang, Y. Y., Liu, S. M., Tartaglia, L., & Leibel, R. L. (1996a) Phenotypes of mouse diabetes and rat fatty due to mutations in the ob (leptin) receptor. *Science*, **271** (5251), 994-996.

Chua, S. C., White, D. W., Wupeng, X. S., Liu, S. M., Okada, N., Kershaw, E. E., Chung, W. K., Powerkehoe, L., Chua, M., Tartaglia, L. A., & Leibel, R. L. (1996b) Phenotype of fatty due to Gln269Pro mutation in the leptin receptor (Lepr). *Diabetes*, **45** (8), 1141-1143.

Cizza, G., Lotsikas, A. J., Licinio, J., Gold, P. W., & Chrousos, G. (1997) Plasma leptin levels do not change in patients with cushings-disease shortly after correction of hypercortisolism - comment. *Journal of Clinical Endocrinology & Metabolism*, **82** (8), 2747-2750.

Clark, J. B. & Palmer, C. J. (1982) The diabetic Zucker rat - a new model for non-insulin dependent diabetes. *Diabetes*, **30** 126A.

Clement, K., Vaisse, C., Lahlou, N., Cabrol, S., Pelloux, V., Cassuto, D., Gourmelen, M., Dina, C., Chambaz, J., Lacorte, J. M., Basdevant, A., Bougneres, P., Lebouc, Y., Froguel, P., & Guygrand, B. (1998) A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature*, **392** (6674), 398-401.

Coffer, P. J., Vanpuijenbroek, A., Burgering, B. M. T., Klopdejonge, M., Koenderman, L., Bos, J. L., & Kruijer, W. (1997) Insulin activates STAT3 independently of P21RAS-ERK and PI-3K signal transduction. *Oncogene*, **15** (21), 2529-2539.

Cohen, B., Novick, D., & Rubinstein, M. (1996b) Modulation of insulin activities by leptin. *Science*, **274** (5290), 1185-1188.

Cohen, S. L., Halaas, J. L., Friedman, J. M., Chait, B. T., Bennett, L., Chang, D., Hecht, R., & Collins, F. (1996a) Human leptin characterization. *Nature*, **382** (6592), 589.

Coleman, D. (1973) Effects of parabiosis of obese with diabetes and normal mice. *Diabetologia*, **9** 294-298.

Coleman, D. & Hummel, K. (1969) Effects of parabiosis of normal with genetically diabetic mice. *American Journal of Physiology*, **217** 1298-1304.

Commins, S. P., Watson, P. M., Frampton, I. C., & Gettys, T. W. (2001) Leptin selectively reduces white adipose tissue in mice via a UCP1-dependent mechanism in brown adipose tissue. *American Journal of Physiology - Endocrinology & Metabolism*, **280** (2), E372-E377.

Commins, S. P., Watson, P. M., Levin, N., Beiler, R. J., & Gettys, T. W. (2000) Central leptin regulates the UCP1 and *ob* genes in brown and white adipose tissue via different beta-adrenoceptor subtypes. *Journal of Biological Chemistry*, **275** (42), 33059-33067.

Considine, R. V., Cooksey, R. C., Williams, L. B., Fawcett, R. L., Zhang, P. L., Ambrosius, W. T., Whitfield, R. M., Jones, R., Inman, M., Huse, J., & McClain, D. A. (2000) Hexosamines regulate leptin production in human subcutaneous adipocytes. *Journal of Clinical Endocrinology & Metabolism*, **85** (10), 3551-3556.

Considine, R. V., Sinha, M. K., Heiman, M. L., Kriauciunas, A., Stephens, T. W., Nyce, M. R., Ohannesian, J. P., Marco, C. C., Mckee, L. J., Bauer, T. L., & Caro, J. F. (1996) Serum immunoreactive leptin concentrations in normal-weight and obese humans. *New England Journal of Medicine*, **334** (5), 292-295.

Couce, M. E., Burguera, B., Parisi, J. E., Jensen, M. D., & Lloyd, R. V. (1997) Localization of leptin receptor in the human brain. *Neuroendocrinology*, **66** (3), 145-150.

Crawley, J. B., Rawlinson, L., Lali, F. V., Page, T. H., Saklatvala, J., & Foxwell, B. M. (1997) T cell proliferation in response to interleukins 2 and 7 requires p38MAP kinase activation. *Journal of Biological Chemistry*, **272** (23), 15023-15027.

Crouse, J. A., Elliott, G. E., Burgess, T. L., Chiu, L., Bennett, L., Moore, J., Nicolson, M., & Pacifici, R. E. (1998) Altered cell surface expression and signaling of leptin receptors containing the fatty mutation. *Journal of Biological Chemistry*, **273** (29), 18365-18373.

Cusin, I., Rohnerjeanrenaud, F., Strickerkrongrad, A., & Jeanrenaud, B. (1996) The weight-reducing effect of an intracerebroventricular bolus injection of leptin in genetically obese *fa/fa* rats - reduced sensitivity compared with lean animals. *Diabetes*, **45** (10), 1446-1451.

Da Silva, B. A., Bjorbaek, C., Uotani, S., & Flier, J. S. (1998) Functional properties of leptin receptor isoforms containing the Gln->Pro extracellular domain mutation of the fatty rat. *Endocrinology*, **139** (9), 3681-3690.

Davies, L. & Marks, J. L. (1994) Role of hypothalamic neuropeptide Y gene expression in body weight regulation. *American Journal of Physiology*, **266** (5 Part 2), R1687-R1691.

Devos, R., Guisez, Y., Vanderheyden, J., White, D. W., Kalai, M., Fountoulakis, M., & Plaetinck, G. (1997) Ligand-independent dimerization of the extracellular domain of the leptin receptor and determination of the stoichiometry of leptin binding. *Journal of Biological Chemistry*, **272** (29), 18304-18310.

Dieterich, K. D. & Lehnert, H. (1998) Expression of leptin receptor mRNA and the long form splice variant in human anterior pituitary and pituitary adenoma. *Experimental & Clinical Endocrinology & Diabetes*, **106** (6), 522-525.

El Haschimi, K., Pierroz, D. D., Hileman, S. M., Bjorbaek, C., & Flier, J. S. (2000) Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. *Journal of Clinical Investigation*, **105** (12), 1827-1832.

Elias, C. F., Aschkenasi, C., Lee, C., Kelly, J., Ahima, R. S., Bjorbaek, C., Flier, J. S., Saper, C. B., & Elmquist, J. K. (1999) Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. *Neuron*, **23** (4), 775-786.

Elmquist, J. K., Ahima, R. S., Maratosflier, E., Flier, J. S., & Safer, C. B. (1997) Leptin activates neurons in ventrobasal hypothalamus and brainstem. *Endocrinology*, **138** (2), 839-842.

Elmquist, J. K., Bjorbaek, C., Ahima, R. S., Flier, J. S., & Saper, C. B. (1998) Distributions of leptin receptor mRNA isoforms in the rat brain. *Journal of Comparative Neurology*, **395** (4), 535-547.

Elmquist, J. K., Elias, C. F., & Saper, C. B. (1999) From lesions to leptin: Hypothalamic control of food intake and body weight. *Neuron*, **22** (2), 221-232.

Emanuelli, B., Peraldi, P., Filloux, C., Sawka-Verhelle, D., Hilton, D., & Van Obberghen, E. (2000) SOCS-3 is an insulin-induced negative regulator of insulin signaling. *Journal of Biological Chemistry*, **275** (21), 15985-15991.

Emilsson, V., Arch, J. R. S., de Groot, R. P., Lister, C. A., & Cawthorne, M. A. (1999) Leptin treatment increases suppressors of cytokine signaling in central and peripheral tissues. *FEBS Letters*, **455** (1-2), 170-174.

Emilsson, V., Liu, Y. L., Cawthorne, M. A., Morton, N. M., & Davenport, M. (1997) Expression of the functional leptin receptor mRNA in pancreatic islets and direct inhibitory action of leptin on insulin secretion. *Diabetes*, **46** (2), 313-316.

Emilsson, V., O'Dowd, J., Nolan, A. L., & Cawthorne, M. A. (2001) Hexosamines and nutrient excess induce leptin production and leptin receptor activation in pancreatic islets and clonal beta-cells. *Endocrinology*, **142** (10), 4414-4419.

Farooqi, I. S., Jebb, S. A., Langmack, G., Lawrence, E., Cheetham, C. H., Prentice, A. M., Hughes, I. A., McCamish, M. A., & O'Rahilly, S. (1999) Effects of recombinant leptin therapy in a child with congenital leptin deficiency. *New England Journal of Medicine*, **341** (12), 879-884.

Faust, I. M., Johnson, P. R., & Hirsch, J. (1977) Adipose tissue regeneration following lipectomy. *Science*, **197** 391-393.

Fehmann, H. C., Berghofer, P., Brandhorst, D., Brandhorst, H., Hering, B., Bretzel, R. G., & Goke, B. (1997a) Leptin inhibition of insulin secretion from isolated human islets. *Acta Diabetologica*, **34** (4), 249-252.

Fehmann, H. C., Peiser, C., Bode, H. P., Stamm, M., Staats, P., Hedetoft, C., Lang, R. E., & Goke, B. (1997b) Leptin - a potent inhibitor of insulin secretion. *Peptides*, **18** (8), 1267-1273.

Fei, H., Okano, H. J., Li, C., Lee, G. H., Zhao, C., Darnell, R., & Friedman, J. M. (1997) Anatomic localization of alternatively spliced leptin receptors (OB-R) in mouse brain and other tissues. *Proceedings of the National Academy of Sciences of the United States of America*, **94** (13), 7001-7005.

Firmbach-Kraft, I., Byers, M., Shows, T., Dalla-Favera, R., & Krolewski, J. J. (1990) Tyk2, prototype of a novel class of non-receptor tyrosine kinase genes. *Oncogene*, **5** (9), 1329-1336.

Flegal, K. M., Carroll, M. D., Kuczmarski, R. J., & Johnson, C. L. (1998) Overweight and obesity in the United States - prevalence and trends, 1960-1994. *International Journal of Obesity*, **22** (1), 39-47.

Frederich, R. C., Hamann, A., Anderson, S., Lollmann, B., Lowell, B. B., & Flier, J. S. (1995) Leptin levels reflect body lipid content in mice - evidence for diet-induced resistance to leptin action. *Nature Medicine*, **1** (12), 1311-1314.

Friedman, J. M. (2000) Obesity in the new millennium. *Nature*, **404** (6778), 632-634.

Friedman, J. M. & Halaas, J. L. (1998) Leptin and the regulation of body weight in mammals. *Nature*, **395** (6704), 763-770.

Fukuda, H. & Iritani, N. (1999) Transcriptional regulation of leptin gene promoter in rat. *FEBS Letters*, **455** (1-2), 165-169.

Fukuda, H., Iritani, N., Sugimoto, T., & Ikeda, H. (1999) Transcriptional regulation of fatty acid synthase gene by insulin/glucose, polyunsaturated fatty acid and leptin in hepatocytes and adipocytes in normal and genetically obese rats. *European Journal of Biochemistry*, **260** (2), 505-511.

Gainsford, T., Willson, T. A., Metcalf, D., Handman, E., Mcfarlane, C., Ng, A., Nicola, N. A., Alexander, W. S., & Hilton, D. J. (1996) Leptin can induce proliferation, differentiation, and functional activation of hemopoietic cells. *Proceedings of the National Academy of Sciences of the United States of America*, **93** (25), 14564-14568.

Gavrilova, O., Barr, V., Marcussamuels, B., & Reitman, M. (1997) Hyperleptinemia of pregnancy associated with the appearance of a circulating form of the leptin receptor. *Journal of Biological Chemistry*, **272** (48), 30546-30551.

Gearing, D. P., King, J. A., Gough, N. M., & Nicola, N. A. (1989) Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor. *EMBO Journal*, **8** (12), 3667-3676.

Ghafourifar, P., Schenk, U., Klein, S. D., & Richter, C. (1999) Mitochondrial nitric-oxide synthase stimulation causes cytochrome c release from isolated mitochondria. Evidence for intramitochondrial peroxynitrite formation. *Journal of Biological Chemistry*, **274** (44), 31185-31188.

Ghilardi, N. & Skoda, R. C. (1997) The leptin receptor activates janus kinase 2 and signals for proliferation in a factor-dependent cell line. *Molecular Endocrinology*, **11** (4), 393-399.

Ghilardi, N., Ziegler, S., Wiestner, A., Stoffel, R., Heim, M. H., & Skoda, R. C. (1996) Defective STAT signaling by the leptin receptor in diabetic mice. *Proceedings of the National Academy of Sciences of the United States of America*, **93** (13), 6231-6235.

Gobert, S., Chretien, S., Gouilleux, F., Muller, O., Pallard, C., Dusanter-Fourt, I., Groner, B., Lacombe, C., Gisselbrecht, S., & Mayeux, P. (1996) Identification of tyrosine residues within the intracellular domain of the erythropoietin receptor crucial for STAT5 activation. *EMBO Journal*, **15** (10), 2434-2441.

Golden, P. L., Maccagnan, T. J., & Pardridge, W. M. (1997) Human blood-brain barrier leptin receptor. Binding and endocytosis in isolated human brain microvessels. *Journal of Clinical Investigation*, **99** (1), 14-18.

Gong, D. W., Bi, S., Pratley, R. E., & Weintraub, B. D. (1996) Genomic structure and promoter analysis of the human obese gene. *Journal of Biological Chemistry*, **271** (8), 3971-3974.

Gotoda, T., Manning, B. S., Goldstone, A. P., Imrie, H., Evans, A. L., Strosberg, A. D., Mckeigue, P. M., Scott, J., & Aitman, T. J. (1997) Leptin receptor gene variation and obesity: lack of association in a white british male population. *Human Molecular Genetics*, **6** (6), 869-876.

Gouilleux, F., Pallard, C., Dusanterfourt, I., Wakao, H., Haldosen, L. A., Norstedt, G., Levy, D., & Groner, B. (1995) Prolactin, growth hormone, erythropoietin and granulocyte-macrophage colony stimulating factor induce MGF-Stat5 DNA binding activity. *EMBO Journal*, **14** (9), 2005-2013.

Grasso, P., Leinung, M. C., Ingher, S. P., & Lee, D. W. (1997) *In vivo* effects of leptin-related synthetic peptides on body weight and food intake in female *ob/ob* mice: localization of leptin activity to domains between amino acid residues 106-140. *Endocrinology*, **138** (4), 1413-1418.

Gremlich, S., Bonny, C., Waeber, G., & Thorens, B. (1997) Fatty acids decrease IDX-1 expression in rat pancreatic islets and reduce GLUT2, glucokinase, insulin, and somatostatin levels. *Journal of Biological Chemistry*, **272** (48), 30261-30269.

Gu, H., Pratt, J. C., Burakoff, S. J., & Neel, B. G. (1998) Cloning of p97/Gab2, the major SHP2-binding protein in hematopoietic cells, reveals a novel pathway for cytokine-induced gene activation. *Molecular Cell*, **2** (6), 729-740.

Guan, X. M., Hess, J. F., Yu, H., Hey, P. J., & Vanderploeg, L. H. T. (1997) Differential expression of mRNA for leptin receptor isoforms in the rat brain. *Molecular & Cellular Endocrinology*, **133** (1), 1-7.

Hahn, T. M., Breininger, J. F., Baskin, D. G., & Schwartz, M. W. (1998) Coexpression of AGRP and NPY in fasting-activated hypothalamic neurons. *Nature Neuroscience*, **1** (4), 271-272.

Hakansson-Ovesjo, M. L., Collin, M., & Meister, B. (2000) Down-regulated STAT3 messenger ribonucleic acid and STAT3 protein in the hypothalamic arcuate nucleus of the obese leptin-deficient (*ob/ob*) mouse. *Endocrinology*, **141** (11), 3946-3955.

Hakansson, M. L., Brown, H., Ghilardi, N., Skoda, R. C., & Meister, B. (1998) Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. *Journal of Neuroscience*, **18** (1), 559-572.

Hakansson, M. L. & Meister, B. (1998) Transcription factor STAT3 in leptin target neurons of the rat hypothalamus. *Neuroendocrinology*, **68** (6), 420-427.

Halaas, J. L., Boozer, C., Blairwest, J., Fidahusein, N., Denton, D. A., & Friedman, J. M. (1997) Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. *Proceedings of the National Academy of Sciences of the United States of America*, **94** (16), 8878-8883.

Halaas, J. L., Gajiwala, K. S., Maffei, M., Cohen, S. L., Chait, B. T., Rabinowitz, D., Lallone, R. L., Burley, S. K., & Friedman, J. M. (1995) Weight-reducing effects of the plasma protein encoded by the obese gene. *Science*, **269** (5223), 543-546.

Haniu, M., Arakawa, T., Bures, E. J., Young, Y., Hui, J. O., Rohde, M. F., Welcher, A. A., & Horan, T. (1998) Human leptin receptor: determination of disulfide structure and N-glycosylation sites of the extracellular domain. *Journal of Biological Chemistry*, **273** (44), 28691-28699.

Hansen, J. A., Lindberg, K., Hilton, D. J., Nielsen, J. H., & Billestrup, N. (1999) Mechanism of inhibition of growth hormone receptor signaling by suppressor of cytokine signaling proteins. *Molecular Endocrinology*, **13** (11), 1832-1843.

Hardwick, J. C. H., Van den Brink, G. R., Offerhaus, G. J., Van Deventer, S. J. H., & Peppelenbosch, M. P. (2001) Leptin is a growth factor for colonic epithelial cells. *Gastroenterology*, **121** (1), 79-90.

Harris, R. B. S. (1990) Role of set point theory in regulation of body weight. *FASEB Journal*, **4** 3310-3318.

Harris, R. B. S., Kasser, T. R., & Martin, R. J. (1986) Dynamics of recovery of body composition after overfeeding, food restriction or starvation of mature female rats. *Journal of Nutrition*, **116** 2536-2546.

Harris, R. B. S., Ramsay, T. G., Smith, S. R., & Bruch, R. C. (1996) Early and late stimulation of *ob* mRNA expression in meal-fed and overfed rats. *Journal of Clinical Investigation*, **97** (9), 2020-2026.

Harvey, J. & Ashford, M. L. J. (1998) Insulin occludes leptin activation of ATP-sensitive K^+ channels in rat CRI-G1, insulin secreting cells. *Journal of Physiology-London*, **511** (3), 695-706.

Harvey, J., Hardy, S. C., Irving, A. J., & Ashford, M. L. J. (2000b) Leptin activation of ATP-sensitive K+ (K_{ATP}) channels in rat CRI-G1 insulinoma cells involves disruption of the actin cytoskeleton. *Journal of Physiology-London*, **527** (1), 95-107.

Harvey, J., McKay, N. G., Walker, K. S., Van der, K. J., Downes, C. P., & Ashford, M. L. J. (2000a) Essential role of phosphoinositide 3-kinase in leptin-induced K_{ATP} channel activation in the rat CRI-G1 insulinoma cell line. *Journal of Biological Chemistry*, **275** (7), 4660-4669.

Harvey, J., Mckenna, F., Herson, P. S., Spanswick, D., & Ashford, M. L. J. (1997) Leptin activates ATP-sensitive potassium channels in the rat insulin-secreting cell line, CRI-G1. *Journal of Physiology-London*, **504** (3), 527-535.

Hausberger, F. X. (1959) Parabiosis and transplantation experiments in hereditary obese mice. *The Anatomical Record*, **130** 313.

Heid, C. A., Stevens, J., Livak, K. J., & Williams, M. P. Real time quantitative PCR. Genome Methods 6, 986-994. 1996.

Hervey, G. R. (1958) The effects of lesions in the hypothalamus in parabiotic rats. *Journal of Physiology*, **145** 336-352.

Hikita, M., Bujo, H., Hirayama, S., Takahashi, K., Morisaki, N., & Saito, Y. (2000) Differential regulation of leptin receptor expression by insulin and leptin in neuroblastoma cells. *Biochemical & Biophysical Research Communications*, **271** (3), 703-709.

Hilton, D. J., Richardson, R. T., Alexander, W. S., Viney, E. M., Willson, T. A., Sprigg, N. S., Starr, R., Nicholson, S. E., Metcalf, D., & Nicola, N. A. (1998) Twenty proteins containing a C-terminal SOCS box form five structural classes. *Proceedings of the National Academy of Sciences of the United States of America*, **95** (1), 114-119.

Hoggard, N., Hunter, L., Duncan, J. S., Williams, L. M., Trayhurn, P., & Mercer, J. G. (1997a) Leptin and leptin receptor mRNA and protein expression in the murine fetus and placenta. *Proceedings of the National Academy of Sciences of the United States of America*, **94** (20), 11073-11078.

Hoggard, N., Mercer, J. G., Rayner, D. V., Moar, K., Trayhurn, P., & Williams, L. M. (1997b) Localization of leptin receptor mRNA splice variants in murine peripheral tissues by RT-PCR and *in situ* hybridization. *Biochemical & Biophysical Research Communications*, **232** (2), 383-387.

Holz, G. G. & Habener, J. F. (1992) Signal transduction crosstalk in the endocrine system: pancreatic beta-cells and the glucose competence concept. *Trends in Biochemical Sciences*, **17** (10), 388-393.

Huang, X. F., Lin, S., & Zhang, R. (1997) Upregulation of leptin receptor mRNA expression in obese mouse brain. *Neuroreport*, **8** (4), 1035-1038.

Hwa, J. J., Fawzi, A. B., Graziano, M. P., Ghibaudi, L., Williams, P., Van Heek, M., Davis, H., Rudinski, M., Sybertz, E., & Strader, C. D. (1997) Leptin increases energy expenditure and selectively promotes fat metabolism in *ob/ob* mice. *American Journal of Physiology - Regulatory Integrative & Comparative Physiology*, **41** (4), R1204-R1209.

Hwa, J. J., Ghibaudi, L., Compton, D., Fawzi, A. B., & Strader, C. D. (1996) Intracerebroventricular injection of leptin increases thermogenesis and mobilizes fat metabolism in *ob/ob* mice. *Hormone & Metabolic Research*, **28** (12), 659-663.

Hwang, C. S., Mandrup, S., Macdougald, O. A., Geiman, D. E., & Lane, M. D. (1996) Transcriptional activation of the mouse obese (*Ob*) gene by CCAAT enhancer binding protein alpha. *Proceedings of the National Academy of Sciences of the United States of America*, **93** (2), 873-877.

Idzerda, R. L., March, C. J., Mosley, B., Lyman, S. D., Vanden Bos, T., Gimpel, S. D., Din, W. S., Grabstein, K. H., Widmer, M. B., & Park, L. S. (1990) Human interleukin 4 receptor confers biological responsiveness and defines a novel receptor superfamily. *Journal of Experimental Medicine*, **171** (3), 861-873.

Iida, M., Murakami, T., Ishida, K., Mizuno, A., Kuwajima, M., & Shima, K. (1996) Substitution at codon 269 (Glutamine-Proline) of the leptin receptor (OB-R) cDNA is the only mutation found in the Zucker fatty (*fa/fa*) rat. *Biochemical & Biophysical Research Communications*, **224** (2), 597-604.

Ingalls, A. M., Dickie, M. M., & Snell, G. D. (1950) Obesity, a new mutation in the house mouse. *Journal of Heredity*, **41** 317-318.

Inui, A. (1999) Feeding and body-weight regulation by hypothalamic neuropeptides: mediation of the actions of leptin. *Trends in Neurosciences*, **22** (2), 62-67.

Ishida, K., Murakami, T., Mizuno, A., Iida, M., Kuwajima, M., & Shima, K. (1997) Leptin suppresses basal insulin secretion from rat pancreatic islets. *Regulatory Peptides*, **70** (2-3), 179-182.

Ishihara, H., Tashiro, F., Ikuta, K., Asano, T., Katagiri, H., Inukai, K., Kikuchi, M., Yazaki, Y., Oka, Y., & Miyazaki, J. (1995) Inhibition of pancreatic beta-cell glucokinase by antisense RNA expression in transgenic mice: mouse strain-dependent alteration of glucose tolerance. *FEBS Letters*, **371** (3), 329-332.

Islam, M. S., Morton, N. M., Hansson, A., & Emilsson, V. (1997) Rat insulinoma-derived pancreatic beta-cells express a functional leptin receptor that mediates a proliferative response. *Biochemical & Biophysical Research Communications*, **238** (3), 851-855.

Isse, N., Ogawa, Y., Tamura, N., Masuzaki, H., Mori, K., Okazaki, T., Satoh, N., Shigemoto, M., Yoshimasa, Y., Nishi, S., Hosoda, K., Inazawa, J., & Nakao, K. (1995) Structural organization and chromosomal assignment of the human obese gene. *Journal of Biological Chemistry*, **270** (46), 27728-27733.

Itoh, N., Yonehara, S., Schreurs, J., Gorman, D. M., Maruyama, K., Ishii, A., Yahara, I., Arai, K., & Miyajima, A. (1990) Cloning of an interleukin-3 receptor gene: a member of a distinct receptor gene family. *Science*, **247** (4940), 324-327.

Jackson, E. K. & Li, P. (1997) Human leptin has natriuretic activity in the rat. *American Journal of Physiology - Renal Fluid & Electrolyte Physiology*, **41** (3), F-F.

Janssen, S. W. J., Martens, G. J. M., Sweep, C. G. J., Ross, H. A., & Hermus, A. R. M. M. (1999) In Zucker diabetic fatty rats plasma leptin levels are correlated with plasma insulin levels rather than with body weight. *Hormone & Metabolic Research*, **31** (11), 610-615.

Jin, L., Burguera, B. G., Couce, M. E., Scheithauer, B. W., Lamsan, J., Eberhardt, N. L., Kulig, E., & Lloyd, R. V. (1999) Leptin and leptin receptor expression in normal and neoplastic human pituitary: evidence of a regulatory role for leptin on pituitary cell proliferation. *Journal of Clinical Endocrinology & Metabolism*, **84** (8), 2903-2911.

Jin, L., Zhang, S., Burguera, B. G., Couce, M. E., Osamura, R. Y., Kulig, E., & Lloyd, R. V. (2000) Leptin and leptin receptor expression in rat and mouse pituitary cells. *Endocrinology*, **141** (1), 333-339.

Kadowaki, T., Kadowaki, H., Accili, D., & Taylor, S. I. (1990) Substitution of lysine for asparagine at position 15 in the alpha-subunit of the human insulin receptor. A mutation that impairs transport of receptors to the cell surface and decreases the affinity of insulin binding. *Journal of Biological Chemistry*, **265** (31), 19143-19150.

Kadowaki, T., Kadowaki, H., Accili, D., Yazaki, Y., & Taylor, S. I. (1991) Substitution of arginine for histidine at position 209 in the alpha-subunit of the human insulin receptor. A mutation that impairs receptor dimerization and transport of receptors to the cell surface. *Journal of Biological Chemistry*, **266** (31), 21224-21231.

Kamohara, S., Burcelin, R., Halaas, J. L., Friedman, J. M., & Charron, M. J. (1997) Acute stimulation of glucose metabolism in mice by leptin treatment. *Nature*, **389** (6649), 374-377.

Karlsson, C., Lindell, K., Svensson, E., Bergh, C., Lind, P., Billig, H., Carlsson, L. M. S., & Carlsson, B. (1997) Expression of functional leptin receptors in the human ovary. *Journal of Clinical Endocrinology & Metabolism*, **82** (12), 4144-4148.

Karlsson, E., Stridsberg, M., & Sandler, S. (1998) Leptin regulation of islet amyloid polypeptide secretion from mouse pancreatic islets. *Biochemical Pharmacology*, **56** (10), 1339-1346.

Kastin, A. J., Pan, W. H., Maness, L. M., Koletsky, R. J., & Ernsberger, P. (1999) Decreased transport of leptin across the blood-brain barrier in rats lacking the short form of the leptin receptor. *Peptides*, **20** (12), 1449-1453.

Katsuyama, K., Shichiri, M., Marumo, F., & Hirata, Y. (1998) Role of nuclear factor-kappa-b activation in cytokine- and sphingomyelinase-stimulated inducible nitric oxide synthase gene expression in vascular smooth muscle cells. *Endocrinology*, **139** (11), 4506-4512.

Kellerer, M., Koch, M., Metzinger, E., Mushack, J., Capp, E., & Haring, H. U. (1997) Leptin activates PI-3 kinase in C_2C_{12} myotubes via janus kinase-2 (JAK-2) and insulin receptor substrate-2 (IRS-2) Dependent pathways. *Diabetologia*, **40** (11), 1358-1362.

Kennedy, G. C. (1953) The role of depot fat in the hypothalamic control of food intake in the rat. *Proc.R.Soc.London Ser.B*, **140** 578-596.

Kieffer, T. J., Heller, R. S., & Habener, J. F. (1996) Leptin receptors expressed on pancreatic beta-cells. *Biochemical & Biophysical Research Communications*, **224** (2), 522-527.

Kieffer, T. J., Keller, R. S., Leech, C. A., Holz, G. G., & Habener, J. F. (1997) Leptin suppression of insulin secretion by the activation of ATP-sensitive K^+ channels in pancreatic beta-cells. *Diabetes*, **46** (6), 1087-1093.

Kim, H. & Baumann, H. (1999) Dual signaling role of the protein tyrosine phosphatase SHP-2 in regulating expression of acute-phase plasma proteins by interleukin-6 cytokine receptors in hepatic cells. *Molecular & Cellular Biology*, **19** (8), 5326-5338.

Kishimoto, T., Taga, T., & Akira, S. (1994) Cytokine signal transduction. *Cell*, **76** (2), 253-262.

Klingmuller, U., Bergelson, S., Hsiao, J. G., & Lodish, H. F. (1996) Multiple tyrosine residues in the cytosolic domain of the erythropoietin receptor promote activation of STAT5. *Proceedings of the National Academy of Sciences of the United States of America*, **93** (16), 8324-8328.

Kolaczynski, J. W., Nyce, M. R., Considine, R. V., Boden, G., Nolan, J. J., Henry, R., Mudaliar, S. R., Olefsky, J., & Caro, J. F. (1996b) Acute and chronic effect of insulin on leptin production in humans - studies *in vivo* and *in vitro*. *Diabetes*, **45** (5), 699-701.

Kolaczynski, J. W., Ohannesian, J. P., Considine, R. V., Marco, C. C., & Caro, J. F. (1996a) Response of leptin to short-term and prolonged overfeeding in humans. *Journal of Clinical Endocrinology & Metabolism*, **81** (11), 4162-4165.

Kopelman, P. G. (2000) Obesity as a medical problem. *Nature*, **404** (6778), 635-643.

Krebs, D. L. & Hilton, D. J. (2000) SOCS: physiological suppressors of cytokine signaling. *Journal of Cell Science*, **113** (Pt 16), 2813-2819.

Kristensen, P., Judge, M. E., Thim, L., Ribel, U., Christjansen, K. N., Wulff, B. S., Clausen, J. T., Jensen, P. B., Madsen, O. D., Vrang, N., Larsen, P. J., & Hastrup, S. (1998) Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature*, **393** (6680), 72-76.

Kulkarni, R. N., Wang, Z. L., Wang, R. M., Hurley, J. D., Smith, D. M., Ghatei, M. A., Withers, D. J., Gardiner, J. V., Bailey, C. J., & Bloom, S. R. (1997) Leptin rapidly suppresses insulin release from insulinoma cells, rat and human islets and, *in vivo*, in mice. *Journal of Clinical Investigation*, **100** (11), 2729-2736.

Kutoh, E., Boss, O., Levasseur, F., & Giacobino, J. P. (1997) Quantification of the full length leptin receptor (OB-Rb) in human brown and white adipose tissue. *Life Sciences*, **62** (5), 445-451.

Leclercq-Meyer, V., Considine, R. V., Sener, A., & Malaisse, W. J. (1996) Do leptin receptors play a functional role in the endocrine pancreas. *Biochemical & Biophysical Research Communications*, **229** (3), 794-798.

Leclercq-Meyer, V. & Malaisse, W. J. (1997) Failure of leptin to counteract the effects of glucose on insulin and glucagon release by the perfused rat pancreas. *Medical Science Research*, **25** (4), 257-259.

Leclercq-Meyer, V. & Malaisse, W. J. (1998) Failure of human and mouse leptin to affect insulin, glucagon and somatostatin secretion by the perfused rat pancreas at physiological glucose concentration. *Molecular & Cellular Endocrinology*, **141** (1-2), 111-118.

Lee, G. H., Proenca, R., Montez, J. M., Carroll, K. M., Darvishzadeh, J. G., Lee, J. I., & Friedman, J. M. (1996) Abnormal splicing of the leptin receptor in diabetic mice. *Nature*, **379** (6566), 632-635.

Lee, Y., Hirose, H., Zhou, Y. T., Esser, V., Mcgarry, J. D., & Unger, R. H. (1997) Increased lipogenic capacity of the islets of obese rats: a role in the pathogenesis of NIDDM. *Diabetes*, **46** (3), 408-413.

Leibowitz, G., Melloul, D., Yuli, M., Gross, D. J., Apelqvist, A., Edlund, H., Cerasi, E., & Kaiser, N. (2001) Defective glucose-regulated insulin gene expression associated with PDX-1 deficiency in the Psammomys obesus model of type 2 diabetes. *Diabetes*, **50** (Suppl 1), S138-S139.

Leonard, W. J. & O'Shea, J. J. (1998) JAKs and STATs: biological implications. *Annual Review of Immunology*, **16** 293-322.

Li, C. & Friedman, J. M. (1999) Leptin receptor activation of SH2 domain containing protein tyrosine phosphatase 2 modulates Ob receptor signal transduction. *Proceedings of the National Academy of Sciences of the United States of America*, **96** (17), 9677-9682.

Lin, K. T., Xue, J. Y., Nomen, M., Spur, B., & Wong, P. Y. K. (1995) Peroxynitrite-induced apoptosis in HL-60 cells. *Journal of Biological Chemistry*, **270** (28), 16487-16490.

Lin, S., Thomas, T. C., Storlien, L. H., & Huang, X. F. (2000) Development of high fat diet-induced obesity and leptin resistance in C57BI/6J mice. *International Journal of Obesity*, **24** (5), 639-646.

Liu, L. S., Karkanias, G. B., Morales, J. C., Hawkins, M., Barzilai, N., Wang, J. L., & Rossetti, L. (1998) Intracerebroventricular leptin regulates hepatic but not peripheral glucose fluxes. *Journal of Biological Chemistry*, **273** (47), 31160-31167.

Liu, M. T., Seino, S., & Kirchgessner, A. L. (1999) Identification and characterization of glucoresponsive neurons in the enteric nervous system. *Journal of Neuroscience*, **19** 10305-10317.

Liu, Y. L., Emilsson, V., & Cawthorne, M. A. (1997) Leptin inhibits glycogen synthesis in the isolated soleus muscle of obese (*ob/ob*) Mice. *FEBS Letters*, **411** (2-3), 351-355.

Lollmann, B., Gruninger, S., Stricker-Krongrad, A., & Chiesi, M. (1997) Detection and quantification of the leptin receptor splice variants Ob-Ra, b, and, e in different mouse tissues. *Biochemical & Biophysical Research Communications*, **238** (2), 648-652.

Lord, G. M., Matarese, G., Howard, L. K., Baker, R. J., Bloom, S. R., & Lechler, R. I. (1998) Leptin modulates the t-cell immune response and reverses starvation-induced immunosuppression. *Nature*, **394** (6696), 897-901.

Lostao, M. P., Urdaneta, E., Martinezanso, E., Barber, A., & Martinez, J. A. (1998) Presence of leptin receptors in rat small intestine and leptin effect on sugar absorption. *FEBS Letters*, **423** (3), 302-306.

Luo, G. & Yu-Lee, L. (1997) Transcriptional activation by STAT5. Differential activities at growth-related versus differentiation-specic promoters. *Journal of Biological Chemistry*, **272** 26841-26849.

Maassen, J. A., Van der Vorm, E. R., Van der Zon, G. C., Klinkhamer, M. P., Krans, H. M., & Moller, W. (1991) A leucine to proline mutation at position 233 in the insulin receptor inhibits cleavage of the proreceptor and transport to the cell surface. *Biochemistry*, **30** (44), 10778-10783.

Macdougald, O. A., Hwang, C. S., Fan, H. Y., & Lane, M. D. (1995) Regulated expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes. *Proceedings of the National Academy of Sciences of the United States of America*, **92** (20), 9034-9037.

Madej, T., Boguski, M. S., & Bryant, S. H. (1995) Threading analysis suggests that the obese gene product may be a helical cytokine. *FEBS Letters*, **373** (1), 13-18.

Maffei, M., Halaas, J., Ravussin, E., Pratley, R. E., Lee, G. H., Zhang, Y., Fei, H., Kim, S., Lallone, R., Ranganathan, S., Kern, P. A., & Friedman, J. M. (1995) Leptin levels in human and rodent: measurement of plasma leptin and *ob* RNA in obese and weight-reduced subjects. *Nature Medicine*, **1** (11), 1155-1161.

Malmstrom, R., Taskinen, M. R., Karonen, S. L., & Ykijarvinen, H. (1996) Insulin increases plasma leptin concentrations in normal subjects and patients with NIDDM. *Diabetologia*, **39** (8), 993-996.

Maness, L. M., Banks, W. A., & Kastin, A. J. (2000) Persistence of blood-to-brain transport of leptin in obese leptin-deficient and leptin receptor-deficient mice. *Brain Research*, **873** (1), 165-167.

Maness, L. M., Kastin, A. J., Farrell, C. L., & Banks, W. A. (1998) Fate of leptin after intracerebroventricular injection into the mouse brain. *Endocrinology*, **139** (11), 4556-4562.

Martin, R. L., Perez, E., He, Y. J., Dawson, R., & Millard, W. J. (2000) Leptin resistance is associated with hypothalamic leptin receptor mRNA and protein downregulation. *Metabolism: Clinical & Experimental*, **49** (11), 1479-1484.

Masuzaki, H., Ogawa, Y., Sagawa, N., Hosoda, K., Matsumoto, T., Mise, H., Nishimura, H., Yoshimasa, Y., Tanaka, I., Mori, T., & Nakao, K. (1997) Nonadipose tissue production of leptin: leptin as a novel placenta-derived hormone in humans. *Nature Medicine*, **3** (9), 1029-1033.

Matsumoto, A., Masuhara, M., Mitsui, K., Yokouchi, M., Ohtsubo, M., Misawa, H., Miyajima, A., & Yoshimura, A. (1997) CIS, a cytokine inducible SH2 protein, is a target of the JAK-STAT5 pathway and modulates STAT5 activation. *Blood*, **89** (9), 3148-3154.

McClain, D. A., Alexander, T., Cooksey, R. C., & Considine, R. V. (2000) Hexosamines stimulate leptin production in transgenic mice. *Endocrinology*, **141** (6), 1999-2002.

Mccowen, K. C., Chow, J. C., & Smith, R. J. (1998) Leptin signaling in the hypothalamus of normal rats *in vivo*. *Endocrinology*, **139** (11), 4442-4447.

Mercer, J. G., Hoggard, N., Williams, L. M., Lawrence, C. B., Hannah, L. T., & Trayhurn, P. (1996) Localization of leptin receptor mRNA and the long form splice variant (OB-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. *FEBS Letters*, **387** (2-3), 113-116.

Mercer, J. G., Moar, K. M., & Hoggard, N. (1998) Localization of leptin receptor (OB-R) messenger ribonucleic acid in the rodent hindbrain. *Endocrinology*, **139** (1), 29-34.

Miller, S. G., Devos, P., Guerremillo, M., Wong, K., Hermann, T., Staels, B., Briggs, M. R., & Auwerx, J. (1996) The adipocyte specific transcription factor C/EBP-alpha modulates human *ob* gene expression. *Proceedings of the National Academy of Sciences of the United States of America*, **93** (11), 5507-5511.

Mise, H., Sagawa, N., Matsumoto, T., Yura, S., Nanno, H., Itoh, H., Mori, T., Masuzaki, H., Hosoda, K., Ogawa, Y., & Nakao, K. (1998) Augmented placental production of leptin in preeclampsia: possible involvement of placental hypoxia. *Journal of Clinical Endocrinology & Metabolism*, **83** (9), 3225-3229.

Mix, H., Widjaja, A., Jandl, O., Cornberg, M., Kaul, A., Goke, M., Beil, W., Kuske, M., Brabant, G., Manns, M. P., & Wagner, S. (2000) Expression of leptin and leptin receptor isoforms in the human stomach. *Gut*, **47** (4), 481-486.

Mizuno, T. M. & Mobbs, C. V. (1999) Hypothalamic Agouti-related protein messenger ribonucleic acid is inhibited by leptin and stimulated by fasting. *Endocrinology*, **140** (2), 814-817.

Montague, C. T., Farooqi, I. S., Whitehead, J. P., Soos, M. A., Rau, H., Wareham, N. J., Sewter, C. P., Digby, J. E., Mohammed, S. N., Hurst, J. A., Cheetham, C. H., Earley, A. R., Barnett, A. H., Prins, J. B., & Orahilly, S. (1997) Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature*, **387** (6636), 903-908.

Morton, N. M., Emilsson, V., de Groot, R. P., Pallett, A. L., & Cawthorne, M. A. (1999) Leptin signalling in pancreatic islets and clonal insulin-secreting cells. *Journal of Molecular Endocrinology*, **22** (2), 173-184.

Morton, N. M., Emilsson, V., Liu, Y. L., & Cawthorne, M. A. (1998) Leptin action in intestinal cells. *Journal of Biological Chemistry*, **273** (40), 26194-26201.

Murakami, T., Iida, M., & Shima, K. (1995) Dexamethasone regulates obese expression in isolated rat adipocytes. *Biochemical & Biophysical Research Communications*, **214** (3), 1260-1267.

Murakami, T., Yamashita, T., Iida, M., Kuwajima, M., & Shima, K. (1997) A short form of leptin receptor performs signal transduction. *Biochemical & Biophysical Research Communications*, **231** (1), 26-29.

Must, A., Spadano, J., Coakley, E. H., Field, A. E., Colditz, G., & Dietz, W. H. (1999) The disease burden associated with overweight and obesity. *Jama: Journal of the American Medical Association*, **282** (16), 1523-1529.

Naka, T., Narazaki, M., Hirata, M., Matsumoto, T., Minamoto, S., Aono, A., Nishimoto, N., Kajita, T., Taga, T., Yoshizaki, K., Akira, S., & Kishimoto, T. (1997) Structure and function of a new STAT-induced STAT inhibitor. *Nature*, **387** (6636), 924-929.

Nakashima, K., Narazaki, M., & Taga, T. (1997) Leptin receptor (OB-R) Oligomerizes with itself but not with its closely related cytokine signal transducer gp130. *FEBS Letters*, **403** (1), 79-82.

Nicholson, S. E., De Souza, D., Fabri, L. J., Corbin, J., Willson, T. A., Zhang, J. G., Silva, A., Asimakis, M., Farley, A., Nash, A. D., Metcalf, D., Hilton, D. J., Nicola, N. A., & Baca, M. (2000) Suppressor of cytokine signaling-3 preferentially binds to the SHP-2-binding site on the shared cytokine receptor subunit gp130. *Proceedings of the National Academy of Sciences of the United States of America*, **97** (12), 6493-6498.

Nicholson, S. E., Willson, T. A., Farley, A., Starr, R., Zhang, J. G., Baca, M., Alexander, W. S., Metcalf, D., Hilton, D. J., & Nicola, N. A. (1999) Mutational analyses of the SOCS proteins suggest a dual domain requirement but distinct mechanisms for inhibition of LIF and IL-6 signal transduction. *EMBO Journal*, **18** (2), 375-385.

Ohlsson, H., Karlsson, K., & Edlund, T. (1993) IPF1, a homeodomain-containing transactivator of the insulin gene. *EMBO Journal*, **12** (11), 4251-4259.

Ohneda, M., Inman, L. R., & Unger, R. H. (1995) Caloric restriction in obese pre-diabetic rats prevents beta-cell depletion, loss of beta-cell GLUT2 and glucose incompetence. *Diabetologia*, **38** (2), 173-179.

Ookuma, M., Ookuma, K., & York, D. A. (1998) Effects of leptin on insulin secretion from isolated rat pancreatic islets. *Diabetes*, **47** (2), 219-223.

Orci, L., Thorens, B., Ravazzola, M., & Lodish, H. F. (1989) Localization of the pancreatic beta cell glucose transporter to specific plasma membrane domains. *Science*, **245** (4915), 295-297.

Pallett, A. L., Morton, N. M., Cawthorne, M. A., & Emilsson, V. (1997) Leptin inhibits insulin secretion and reduces insulin mRNA levels in rat isolated pancreatic islets. *Biochemical & Biophysical Research Communications*, **238** (1), 267-270.

Pardridge, W. M. (1986) Receptor-mediated peptide transport through the blood-brain barrier. *Endocrine Reviews*, 7 (3), 314-330.

Partanen, J., Makela, T. P., Alitalo, R., Lehvaslaiho, H., & Alitalo, K. (1990) Putative tyrosine kinases expressed in K-562 human leukemia cells. *Proceedings of the National Academy of Sciences of the United States of America*, **87** (22), 8913-8917.

Patel, B. K., Koenig, J. I., Kaplan, L. M., & Hooi, S. C. (1998) Increase in plasma leptin and *lep* mRNA concentrations by food intake is dependent on insulin. *Metabolism: Clinical & Experimental*, **47** (5), 603-607.

Peiser, C., McGregor, G. P., & Lang, R. E. (2000) Leptin receptor expression and suppressor of cytokine signaling transcript levels in high-fat-fed rats. *Life Sciences*, **67** (24), 2971-2981.

Pellegrini, S. & Dusanter-Fourt, I. (1997) The structure, regulation and function of the Janus kinases (JAKs) and the signal transducers and activators of transcription (STATs). *European Journal of Biochemistry*, **248** (3), 615-633.

Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T., & Collins, F. (1995) Effects of the obese gene product on body weight regulation in *ob/ob* mice. *Science*, **269** (5223), 540-543.

Peraldi, P., Filloux, C., Emanuelli, B., Hilton, D. J., & Van Obberghen, E. (2001) Insulin induces suppressor of cytokine signaling-3 tyrosine phosphorylation through janus-activated kinase. *Journal of Biological Chemistry*, **276** (27), 24614-24620.

Peterson, R. G., Shaw, W. N., Neel, M., Little, L. A., & Eichberg, J. (1990) Zucker diabetic fatty rat as a model for non-insulin-dependent diabetes mellitus. *ILAR News*, **32** 16-19.

Phillips, M. S., Liu, Q. Y., Hammond, H. A., Dugan, V., Hey, P. J., Caskey, C. T., & Hess, J. F. (1996) Leptin receptor missense mutation in the fatty Zucker rat. *Nature Genetics*, **13** (1), 18-19.

Poitout, V., Rouault, C., Guerremillo, M., Briaud, I., & Reach, G. (1998) Inhibition of insulin secretion by leptin in normal rodent islets of langerhans. *Endocrinology*, **139** (3), 822-826.

Porte, D., Seeley, R. J., Woods, S. C., Baskin, D. G., Figlewicz, D. P., & Schwartz, M. W. (1998) Obesity, diabetes and the central nervous system [Review]. *Diabetologia*, **41** (8), 863-881.

Powis, J. E., Bains, J. S., & Ferguson, A. V. (1998) Leptin depolarizes rat hypothalamic paraventricular nucleus neurons. *American Journal of Physiology - Regulatory Integrative & Comparative Physiology*, **43** (5), R1468-R1472.

Prentki, M., Tornheim, K., & Corkey, B. E. (1997) Signal transduction mechanisms in nutrient-induced insulin secretion. *Diabetologia*, **40** (Suppl 2), S32-S41.

Ram, P. A. & Waxman, D. J. (1999) SOCS/CIS protein inhibition of growth hormone-stimulated STAT5 signaling by multiple mechanisms. *Journal of Biological Chemistry*, **274** (50), 35553-35561.

Rau, H., Reaves, B. J., O'Rahilly, S., & Whitehead, J. P. (1999) Truncated human leptin (Delta 133) associated with extreme obesity undergoes proteasomal degradation after defective intracellular transport. *Endocrinology*, **140** (4), 1718-1723.

Rentsch, J. & Chiesi, M. (1996) Regulation of ob gene mRNA levels in cultured adipocytes. *FEBS Letters*, **379** (1), 55-59.

Roduit, R. & Thorens, B. (1997) Inhibition of glucose-induced insulin secretion by long-term pre-exposure of pancreatic islets to leptin. *FEBS Letters*, **415** (2), 179-182.

Rosenblum, C. I., Tota, M., Cully, D., Smith, T., Collum, R., Qureshi, S., Hess, J. F., Phillips, M. S., Hey, P. J., Vongs, A., Fong, T. M., Xu, L., Chen, H. Y., Smith, R. G., Schindler, C., & Vanderploeg, L. H. T. (1996) Functional STAT 1 and 3 signaling by the leptin receptor (OB-R); reduced expression of the rat fatty leptin receptor in transfected cells. *Endocrinology*, **137** (11), 5178-5181.

Rossetti, L., Massillon, D., Barzilai, N., Vuguin, P., Chen, W., Hawkins, M., Wu, J., & Wang, J. L. (1997) Short term effects of leptin on hepatic gluconeogenesis and *in vivo* insulin action. *Journal of Biological Chemistry*, **272** (44), 27758-27763.

Saladin, R., Devos, P., Guerremillo, M., Leturque, A., Girard, J., Staels, B., & Auwerx, J. (1995) Transient increase in obese gene expression after food intake or insulin administration. *Nature*, **377** (6549), 527-529.

Savioz, A., Charnay, Y., Huguenin, C., Graviou, C., Greggio, B., & Bouras, C. (1997) Expression of leptin receptor mRNA (long form splice variant) in the human cerebellum. *Neuroreport*, **8** (14), 3123-3126.

Scarpace, P. J., Nicolson, M., & Matheny, M. (1998) UCP2, UCP3 and leptin gene expression: modulation by food restriction and leptin. *Journal of Endocrinology*, **159** (2), 349-357.

Schiemann, W. P. & Nathanson, N. M. (1998) Raf-1 independent stimulation of mitogen-activated protein kinase by leukemia inhibitory factor in 3T3-L1 cells. *Oncogene*, **16** (20), 2671-2679.

Schwartz, M. W., Peskind, E., Raskind, M., Boyko, E. J., & Porte, D. (1996b) Cerebrospinal fluid leptin levels - relationship to plasma levels and to adiposity in humans. *Nature Medicine*, **2** (5), 589-593.

Schwartz, M. W., Seeley, R. J., Campfield, L. A., Burn, P., & Baskin, D. G. (1996a) Identification of targets of leptin action in rat hypothalamus. *Journal of Clinical Investigation*, **98** (5), 1101-1106.

Schwartz, M. W., Seeley, R. J., Woods, S. C., Weigle, D. S., Campfield, L. A., Burn, P., & Baskin, D. G. (1997) Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. *Diabetes*, **46** (12), 2119-2123.

Segal, K. R., Landt, M., & Klein, S. (1996) Relationship between insulin sensitivity and plasma leptin concentration in lean and obese men. *Diabetes*, **45** (7), 988-991.

Seufert, J., Kieffer, T. J., & Habener, J. F. (1999b) Leptin inhibits insulin gene transcription and reverses hyperinsulinemia in leptin-deficient *ob/ob* mice. *Proceedings of the National Academy of Sciences of the United States of America*, **96** (2), 674-679.

Seufert, J., Kieffer, T. J., Leech, C. A., Holz, G. G., Moritz, W., Ricordi, C., & Habener, J. F. (1999a) Leptin suppression of insulin secretion and gene expression in human pancreatic islets: implications for the development of adipogenic diabetes mellitus. *Journal of Clinical Endocrinology & Metabolism*, **84** (2), 670-676.

Sharma, K. & Considine, R. V. (1998) The ob protein (leptin) and the kidney. *Kidney International*, **53** (6), 1483-1487.

Shekhawat, P. S., Garland, J. S., Shivpuri, C., Mick, G. J., Sasidharan, P., Pelz, C. J., & McCormick, K. L. (1998) Neonatal cord blood leptin - its relationship to birth weight, body mass index, maternal diabetes, and steroids. *Pediatric Research*, **43** (3), 338-343.

Shi, Z. Q., Lu, W., & Feng, G. S. (1998a) The SHP-2 tyrosine phosphatase has opposite effects in mediating the activation of extracellular signal-regulated and c-Jun NH2-terminal mitogen-activated protein kinases. *Journal of Biological Chemistry*, **273** (9), 4904-4908.

Shi, Z. Q., Nelson, A., Whitcomb, L., Wang, J. L., & Cohen, A. M. (1998b) Intracerebroventricular administration of leptin markedly enhances insulin sensitivity and systemic glucose utilization in conscious rats. *Metabolism: Clinical & Experimental*, **47** (10), 1274-1280.

Shimabukuro, M., Higa, M., Zhou, Y. T., Wang, M. Y., Newgard, C. B., & Unger, R. H. (1998b) Lipoapoptosis in beta-cells of obese prediabetic *fa/fa* rats: role of serine palmitoyltransferase overexpression. *Journal of Biological Chemistry*, **273** (49), 32487-32490.

Shimabukuro, M., Koyama, K., Chen, G. X., Wang, M. Y., Trieu, F., Lee, Y., Newgard, C. B., & Unger, R. H. (1997b) Direct antidiabetic effect of leptin through triglyceride depletion of tissues. *Proceedings of the National Academy of Sciences of the United States of America*, **94** (9), 4637-4641.

Shimabukuro, M., Ohneda, M., Lee, Y., & Unger, R. H. (1997a) Role of nitric oxide in obesity-induced beta cell disease. *Journal of Clinical Investigation*, **100** (2), 290-295.

Shimabukuro, M., Zhou, Y. T., Levi, M., & Unger, R. H. (1998c) Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. *Proceedings of the National Academy of Sciences of the United States of America*, **95** (5), 2498-2502.

Shimabukuro, M., Zhou, Y. T., Levi, M., & Unger, R. H. (1998a) Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. *Proceedings of the National Academy of Sciences of the United States of America*, **95** 2498-2502.

Shimizu, H., Ohtani, K., Tsuchiya, T., Takahashi, H., Uehara, Y., Sato, N., & Mori, M. (1997) Leptin stimulates insulin secretion and synthesis in HIT-T15 cells. *Peptides*, **18** (8), 1263-1266.

Shinozuka, Y., Okada, M., Oki, T., Sagane, K., Mizui, Y., Tanaka, I., Katayama, K., & Murakami-Murofushi, K. (2001) Altered expression of HES-1, BETA2/NeuroD, and PDX-1 is involved in impaired insulin synthesis induced by glucocorticoids in HIT-T15 cells. *Biochemical & Biophysical Research Communications*, **287** (1), 229-235.

Siegrist-Kaiser, C. A., Pauli, V., Jugeaubry, C. E., Boss, O., Pernin, A., Chin, W. W., Cusin, I., Rohnerjeanrenaud, F., Burger, A. G., Zapf, J., & Meier, C. A. (1997) Direct effects of leptin on brown and white adipose tissue. *Journal of Clinical Investigation*, **100** (11), 2858-2864.

Sierra-Honigmann, M. R., Nath, A. K., Murakami, C., Garcia-Cardena, G., Papapetropoulos, A., Sessa, W. C., Madge, L. A., Schechner, J. S., Schwabb, M. B., Polverini, P. J., & Flores-Riveros, J. R. (1998) Biological action of leptin as an angiogenic factor. *Science*, **281** (5383), 1683-1686.

Sinha, M. K., Sturis, J., Ohannesian, J., Magosin, S., Stephens, T., Heiman, M. L., Polonsky, K. S., & Caro, J. F. (1996) Ultradian oscillations of leptin secretion in humans. *Biochemical & Biophysical Research Communications*, **228** (3), 733-738.

Slieker, L. J., Sloop, K. W., Surface, P. L., Kriauciunas, A., Laquier, F., Manetta, J., Buevalleskey, J., & Stephens, T. W. (1996) Regulation of expression of ob mRNA and protein by glucocorticoids and cAMP. *Journal of Biological Chemistry*, **271** (10), 5301-5304.

Sone, M., Nagata, H., Takekoshi, S., & Osamura, R. Y. (2001) Expression and localization of leptin receptor in the normal rat pituitary gland. *Cell & Tissue Research*, **305** (3), 351-356.

Spanswick, D., Smith, M. A., Groppi, V. E., Logan, S. D., & Ashford, M. L. J. (1997) Leptin inhibits hypothalamic neurons by activation of ATP-sensitive potassium channels. *Nature*, **390** (6659), 521-525.

Sparmann, G., Jaschke, A., Loehre, M., Liebe, S., & Emmrich, J. Tissue homogenization as a key step in extracting RNA from human and rat pancreatic tissue. Biotechniques 22, 408-412. 1997.

Starr, R. & Hilton, D. J. (1998) SOCS - Suppressors of cytokine signalling. *International Journal of Biochemistry & Cell Biology*, **30** (10), 1081-1085.

Starr, R., Willson, T. A., Viney, E. M., Murray, L. J. L., Rayner, J. R., Jenkins, B. J., Gonda, T. J., Alexander, W. S., Metcalf, D., Nicola, N. A., & Hilton, D. J. (1997) A family of cytokine-inducible inhibitors of signalling. *Nature*, **387** (6636), 917-921.

Steppan, C. M. & Swick, A. G. (1999) A role for leptin in brain development. *Biochemical & Biophysical Research Communications*, **256** (3), 600-602.

Storz, P., Doppler, H., Pfizenmaier, K., & Muller, G. (1999) Insulin selectively activates STAT5b, but not STAT5a, via a JAK2-independent signalling pathway in Kym-1 rhabdomyosarcoma cells. *FEBS Letters*, **464** (3), 159-163.

Strobel, A., Issad, T., Camoin, L., Ozata, M., & Strosberg, A. D. (1998) A leptin missense mutation associated with hypogonadism and morbid obesity. *Nature Genetics*, **18** (3), 213-215.

Sun, Q. Y., Breitbart, H., & Schatten, H. (1999) Role of the MAPK cascade in mammalian germ cells. *Reproduction, Fertility, & Development*, **11** (7-8), 443-450.

Taha, C. & Klip, A. (1999) The insulin signaling pathway. *Journal of Membrane Biology*, **169** (1), 1-12.

Takahashi, Y., Okimura, Y., Mizuno, I., Iida, K., Takahashi, T., Kaji, H., Abe, H., & Chihara, K. (1997) Leptin induces mitogen-activated protein kinase-dependent proliferation of C3H10T1/2 cells. *Journal of Biological Chemistry*, **272** (20), 12897-12900.

Takahashi, Y., Okimura, Y., Mizuno, I., Takahashi, T., Kaji, H., Uchiyama, T., Abe, H., & Chihara, K. (1996) Leptin induces tyrosine phosphorylation of cellular proteins including STAT-1 in human renal adenocarcinoma cells, ACHN. *Biochemical & Biophysical Research Communications*, **228** (3), 859-864.

Takaya, K., Ogawa, Y., Hiraoka, J., Hosoda, K., Yamori, Y., Nakao, K., & Koletsky, R. J. (1996) Nonsense mutation of leptin receptor in the obese spontaneously hypertensive Koletsky rat. *Nature Genetics*, **14** (2), 130-131.

Tanabe, K., Okuya, S., Tanizawa, Y., Matsutani, A., & Oka, Y. (1997) Leptin induces proliferation of pancreatic beta cell line MIN6 through activation of mitogen-activated protein kinase. *Biochemical & Biophysical Research Communications*, **241** (3), 765-768.

Tanizawa, Y., Okuya, S., Ishihara, H., Asano, T., Yada, T., & Oka, Y. (1997) Direct stimulation of basal insulin secretion by physiological concentrations of leptin in pancreatic beta cells. *Endocrinology* , **138** (10), 4513-4516.

Tartaglia, L. A., Dembski, M., Weng, X., Deng, N. H., Culpepper, J., Devos, R., Richards, G. J., Campfield, L. A., Clark, F. T., Deeds, J., Muir, C., Sanker, S., Moriarty, A., Moore, K. J., Smutko, J. S., Mays, G. G., Woolf, E. A., Monroe, C. A., & Tepper, R. I. (1995) Identification and expression cloning of a leptin receptor, OB-R. *Cell*, **83** (7), 1263-1271.

Tena-Sempere, M., Pinilla, L., Gonzalez, L. C., Casanueva, F. F., Dieguez, C., & Aguilar, E. (2000) Homologous and heterologous down-regulation of leptin receptor messenger ribonucleic acid in rat adrenal gland. *Journal of Endocrinology*, **167** (3), 479-486.

Tena-Sempere, M., Pinilla, L., Zhang, F. P., Gonzalez, L. C., Huhtaniemi, I., Casanueva, F. F., Dieguez, C., & Aguilar, E. (2001) Developmental and hormonal regulation of leptin receptor (OB-R) messenger ribonucleic acid expression in rat testis. *Biology of Reproduction*, **64** (2), 634-643.

Thorens, B., Sarkar, H. K., Kaback, H. R., & Lodish, H. F. (1988) Cloning and functional expression in bacteria of a novel glucose transporter present in liver, intestine, kidney, and beta-pancreatic islet cells. *Cell*, **55** (2), 281-290.

Troiano, R. P. & Flegal, K. M. (1998) Overweight children and adolescents - description, epidemiology, and demographics. *Pediatrics*, **101** (3 Suppl S), 497-504.

Uehara, Y., Shimizu, H., Ohtani, K., Sato, N., & Mori, M. (1998) Hypothalamic corticotropin-releasing hormone is a mediator of the anorexigenic effect of leptin. *Diabetes*, **47** (6), 890-893.

Unger, R. H. (1995) Lipotoxicity in the pathogenesis of obesity-dependent NIDDM: genetic and clinical implications. *Diabetes* , **44** (8), 863-870.

Unger, R. H. & Orci, L. (2001) Diseases of liporegulation: new perspective on obesity and related disorders. *FASEB Journal*, **15** (2), 312-321.

Unger, R. H., Zhou, Y. T., & Orci, L. (1999) Regulation of fatty acid homeostasis in cells: novel role of leptin. *Proceedings of the National Academy of Sciences of the United States of America*, **96** (5), 2327-2332.

Vaisse, C., Halaas, J. L., Horvath, C. M., Darnell, J. E., Stoffel, M., & Friedman, J. M. (1996) Leptin activation of STAT3 in the hypothalamus of wildtype and *ob/ob* mice but not *db/db* mice. *Nature Genetics*, **14** (1), 95-97.

Valera, A., Solanes, G., Fernandezalvarez, J., Pujol, A., Ferrer, J., Asins, G., Gomis, R., & Bosch, F. (1994) Expression of GLUT-2 antisense RNA in beta cells of transgenic mice leads to diabetes. *Journal of Biological Chemistry*, **269** (46), 28543-28546.

Van Heek, M., Compton, D. S., France, C. F., Tedesco, R. P., Fawzi, A. B., Graziano, M. P., Sybertz, E. J., Strader, C. D., & Davis, H. R. (1997) Diet-induced obese mice develop peripheral, but not central, resistance to leptin. *Journal of Clinical Investigation*, **99** (3), 385-390.

Verdier, F., Chretien, S., Muller, O., Varlet, P., Yoshimura, A., Gisselbrecht, S., Lacombe, C., & Mayeux, P. (1998b) Proteasomes regulate erythropoietin receptor and signal transducer and activator of transcription 5 (STAT5) activation. Possible involvement of the ubiquitinated CIS protein. *Journal of Biological Chemistry*, **273** (43), 28185-28190.

Verdier, F., Rabionet, R., Gouilleux, F., Beisenherz-Huss, C., Varlet, P., Muller, O., Mayeux, P., Lacombe, C., Gisselbrecht, S., & Chretien, S. (1998a) A sequence of the CIS gene promoter interacts preferentially with two associated STAT5A dimers: a distinct biochemical difference between STAT5A and STAT5B. *Molecular & Cellular Biology*, **18** (10), 5852-5860.

Vinkemeier, U., Cohen, S. L., Moarefi, I., Chait, B. T., Kuriyan, J., & Darnell, J. E., Jr. (1996) DNA binding of in vitro activated Stat1 alpha, Stat1 beta and truncated Stat1: interaction between NH2-terminal domains stabilizes binding of two dimers to tandem DNA sites. *EMBO Journal*, **15** (20), 5616-5626.

Waeber, G., Thompson, N., Nicod, P., & Bonny, C. (1996) Transcriptional activation of the GLUT2 gene by the IPF-1/STF-1/IDX-1 homeobox factor. *Molecular Endocrinology*, **10** (11), 1327-1334.

Wang, J. L., Liu, R., Hawkins, M., Barzilai, N., & Rossetti, L. (1998a) A nutrient-sensing pathway regulates leptin gene expression in muscle and fat. *Nature*, **393** (6686), 684-688.

Wang, J. L., Liu, R., Liu, L. S., Chowdhury, R., Barzilai, N., Tan, J. Z., & Rossetti, L. (1999b) The effect of leptin on Lep expression is tissue-specific and nutritionally regulated. *Nature Medicine*, **5** (8), 895-899.

Wang, M. Y., Koyama, K., Shimabukuro, M., Newgard, C. B., & Unger, R. H. (1998b) Ob-Rb gene transfer to leptin-resistant islets reverses diabetogenic phenotype. *Proceedings of the National Academy of Sciences of the United States of America*, **95** (2), 714-718.

Wang, M. Y., Lee, Y., & Unger, R. H. (1999a) Novel form of lipolysis induced by leptin. *Journal of Biological Chemistry*, **274** (25), 17541-17544.

Wang, M. Y., Zhou, Y. T., Newgard, C. B., & Unger, R. H. (1996) A novel leptin receptor isoform in rat. *FEBS Letters*, **392** (2), 87-90.

Wang, Y. H., Tache, Y., Sheibel, A. B., Go, V. L. W., & Wei, J. Y. (1997b) Two types of leptin-responsive gastric vagal afferent terminals: an *in vitro* single-unit study in rats. *American Journal of Physiology - Regulatory Integrative & Comparative Physiology*, **42** (2), R-R.

Wang, Y. P., Kuropatwinski, K. K., White, D. W., Hawley, T. S., Hawley, R. G., Tartaglia, L. A., & Baumann, H. (1997a) Leptin receptor action in hepatic cells. *Journal of Biological Chemistry*, **272** (26), 16216-16223.

Wang, Z. W., Zhou, Y. T., Kakuma, T., Lee, Y., Kalra, S. P., Kalra, P. S., Pan, W. T., & Unger, R. H. (2000) Leptin resistance of adipocytes in obesity: role of suppressors of cytokine signaling. *Biochemical & Biophysical Research Communications*, **277** (1), 20-26.

Watada, H., Kajimoto, Y., Umayahara, Y., Matsuoka, T., Kaneto, H., Fujitani, Y., Kamada, T., Kawamori, R., & Yamasaki, Y. (1996) The human glucokinase gene beta-cell-type promoter: an essential role of insulin promoter factor 1/PDX-1 in its activation in HIT-T15 cells. *Diabetes*, **45** (11), 1478-1488.

Watowich, S. S., Wu, H., Socolovsky, M., Klingmuller, U., Constantinescu, S. N., & Lodish, H. F. (1996) Cytokine receptor signal transduction and the control of hematopoietic cell development. *Annual Review of Cell & Developmental Biology*, **12** 91-128.

Watson, P. M., Commins, S. P., Beiler, R. J., Hatcher, H. C., & Gettys, T. W. (2000) Differential regulation of leptin expression and function in A/J vs. C57BL/6J mice during diet-induced obesity. *American Journal of Physiology - Endocrinology & Metabolism*, **279** (2), E356-E365.

Weir, G. C., Sharma, A., Zangen, D. H., & Bonnerweir, S. (1997) Transcription factor abnormalities as a cause of beta cell dysfunction in diabetes: a hypothesis. *Acta Diabetologica*, **34** (3), 177-184.

White, D. W., Kuropatwinski, K. K., Devos, R., Baumann, H., & Tartaglia, L. A. (1997a) Leptin receptor (OB-R) signalling: cytoplasmic domain mutational analysis and evidence for receptor homo-oligomerization. *Journal of Biological Chemistry*, **272** (7), 4065-4071.

White, D. W. & Tartaglia, L. A. (1996) Leptin and OB-R: body weight regulation by a cytokine receptor. *Cytokine & Growth Factor Reviews*, 7 (4), 303-309.

White, D. W., Wang, Y. P., Chua, S. C., Morgenstern, J. P., Leibel, R. L., Baumann, H., & Tartaglia, L. A. (1997b) Constitutive and impaired signaling of leptin receptors containing the Gln-Pro extracellular domain fatty mutation. *Proceedings of the National Academy of Sciences of the United States of America*, **94** (20), 10657-10662.

Widdowson, P. S., Upton, R., Buckingham, R., Arch, J., & Williams, G. (1997) Inhibition of food response to intracerebroventricular injection of leptin is attenuated in rats with diet induced obesity. *Diabetes*, **46** (11), 1782-1785.

Wilks, A. F. (1989) Two putative protein-tyrosine kinases identified by application of the polymerase chain reaction. *Proceedings of the National Academy of Sciences of the United States of America*, **86** (5), 1603-1607.

Winston, L. A. & Hunter, T. (1995) JAK2, Ras, and Raf are required for activation of extracellular signal-regulated kinase/mitogen-activated protein kinase by growth hormone. *Journal of Biological Chemistry*, **270** (52), 30837-30840.

Wolf, A. M. & Colditz, G. A. (1998) Current estimates of the economic cost of obesity in the United States. *Obesity Research*, **6** (2), 97-106.

Woods, S. C., Seeley, R. J., Porte, D., & Schwartz, M. W. (1998) Signals that regulate food intake and energy homeostasis. *Science*, **280** (5368), 1378-1383.

Wu-Peng, X. S., Chua, S. C., Okada, N., Liu, S. M., Nicolson, M., & Leibel, R. L. (1997) Phenotype of the obese Koletsky (F) rat due to Tyr763Stop mutation in the extracellular domain of the leptin receptor (LEPR): evidence for deficient plasma-to-CSF transport of leptin in both the Zucker and Koletsky obese rat. *Diabetes*, **46** (3), 513-518.

Xu, X., Sun, Y. L., & Hoey, T. (1996) Cooperative DNA binding and sequence-selective recognition conferred by the STAT amino-terminal domain. *Science*, **273** (5276), 794-797.

Yamashita, T., Murakami, T., Iida, M., Kuwajima, M., & Shima, K. (1997) Leptin receptor of Zucker fatty rat performs reduced signal transduction. *Diabetes*, **46** (6), 1077-1080.

Yamashita, T., Murakami, T., Otani, S., Kuwajima, M., & Shima, K. (1998) Leptin receptor signal transduction: OB-Ra and OB-Rb of *fa* type. *Biochemical & Biophysical Research Communications*, **246** (3), 752-759.

Yasukawa, H., Misawa, H., Sakamoto, H., Masuhara, M., Sasaki, A., Wakioka, T., Ohtsuka, S., Imaizumi, T., Matsuda, T., Ihle, J. N., & Yoshimura, A. (1999) The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop. *EMBO Journal*, **18** (5), 1309-1320.

Yoshimura, A., Ohkubo, T., Kiguchi, T., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., Hara, T., & Miyajima, A. (1995) A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin-3 and erythropoietin receptors. *EMBO Journal*, **14** (12), 2816-2826.

You, M., Yu, D. H., & Feng, G. S. (1999) SHP-2 tyrosine phosphatase functions as a negative regulator of the interferon-stimulated JAK/STAT pathway. *Molecular & Cellular Biology*, **19** (3), 2416-2424.

Yu, W. H., Kimura, M., Walczewska, A., Karanth, S., & McCann, S. M. (1997a) Role of leptin in hypothalamic-pituitary function. *Proceedings of the National Academy of Sciences of the United States of America*, **94** (3), 1023-1028.

Yu, W. H., Walczewska, A., Karanth, S., & McCann, S. M. (1997b) Nitric oxide mediates leptin-induced luteinizing hormone-releasing hormone (LHRH) and LHRH and leptin-induced LH release from the pituitary gland. *Endocrinology*, **138** (11), 5055-5058.

Zamorano, P. L., Mahesh, V. B., Desevilla, L. M., Chorich, L. P., Bhat, G. K., & Brann, D. W. (1997) Expression and localization of the leptin receptor in endocrine and neuroendocrine tissues of the rat. *Neuroendocrinology*, **65** (3), 223-228.

Zawalich, W. S., Bonnet-Eymard, M., & Zawalich, K. C. (1997) Signal transduction in pancreatic ß-cells: regulation of insulin secretion by information flow in the phospholipase C/protein kinase C pathway. *Frontiers in Bioscience*, **2** d160-d172.

Zhang, F. M., Basinski, M. B., Beals, J. M., Briggs, S. L., Churgay, L. M., Clawson, D. K., Dimarchi, R. D., Furman, T. C., Hale, J. E., Hsiung, H. M., Schoner, B. E., Smith, D. P., Zhang, X. Y., Wery, J. P., & Schevitz, R. W. (1997) Crystal structure of the obese protein leptin-e100. *Nature*, **387** (6629), 206-209.

Zhang, Y. Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., & Friedman, J. M. (1994) Positional cloning of the mouse obese gene and its human homologue. *Nature*, **372** (6505), 425-432.

Zhao, A. Z., Bornfeldt, K. E., & Beavo, J. A. (1998) Leptin inhibits insulin secretion by activation of phosphodiesterase-3B. *Journal of Clinical Investigation*, **102** (5), 869-873.

Zlokovic, B. V., Jovanovic, S., Miao, W., Samara, S., Verma, S., & Farrell, C. L. (2000) Differential regulation of leptin transport by the choroid plexus and blood-brain barrier and high affinity transport systems for entry into hypothalamus and across the blood-cerebrospinal fluid barrier. *Endocrinology*, **141** (4), 1434-1441.

Zucker, L. M. & Zucker, T. F. (1961) Fatty, a new mutation in the rat. *Journal of Heredity*, **52** 275-278.

Zucker, T. F. & Zucker, L. M. (1962) Hereditary obesity in the rat associated with high serum fat and cholesterol. *Proceedings of the Society for Experimental Biology and Medecine*, **110** 165-171.

Zucker, T. F. & Zucker, L. M. (1963) Fat accretion and growth in the rat. *Journal of Nutrition*, **80** 6-19.

Appendix 1

The level of mRNA expressed for a particular transcript has a common element or component of variation which may be due to sample specific differences such as sample or tissue quality. Housekeeping or endogenous control genes are measured in parallel to test genes to quantify such a component. They are used in a number of ways, with the intention of removing such differences, leaving a better estimate of the treatment effect. A log-log plot of the gene of interest against housekeeping gene would expect to result in a strong correlation between the two genes. However, if the housekeeping gene is also affected by treatment, subsequent use of the housekeeper could remove "real" treatment effects in the test gene or even introduce "false" effects.

The calculation of ratios (test/housekeeper cDNA quantity) is the most common way of adjusting for sample specific differences. An improvement on the ratio analysis is the Analysis of Covariance (ANOCOVA), as this takes into account different types of relationships between test and reference genes. ANOCOVA still runs the risk of introducing false positive and negative results due to the use of a housekeeping gene that is affected by treatment. However, covariate efficiency factors (c.e.f.) can help to assess housekeeping genes not only by quantifying the degree to which a housekeeping gene is affected by treatment (c.e.f. (Trt)), but also whether they have been successful in adjusting for variation caused by sample specific differences such as sample or tissue quality (c.e.f. (Res)). The c.e.f. (Trt) value ranges between zero and one, where

a value of one indicates the housekeeping gene is independent of treatment and zero indicates the housekeeping gene is affected by the treatment. The c.e.f. (Res) ranges from zero to infinity, where values greater than one improve the precision of the analysis and the larger the value the greater the improvement. The gain in reducing variation by using a housekeeping gene can be offset by the inefficiency of a housekeeper with respect to the treatment. An overall assessment of the usefulness of housekeeping genes can be performed by comparing the product of the two efficiency factors (c.e.f. (Product)) across analyses using different housekeeping genes.

The ANOCOVA analysis was performed for each test gene without using a housekeeping gene as a co-variate as well as using each of the housekeeping genes (GAPDH, cyclophilin, 36B4 and HPRT) as co-variates. The most suitable housekeeping gene was selected not only by comparing the values of c.e.f. (Product), but also by taking into consideration the c.e.f. (Trt) and c.e.f. (Res) values. In the study investigating dietary and genetically obese mice, the analysis showed that HPRT was the most suitable housekeeping gene in the hypothalamus and small intestine, whereas cyclophilin was the most suitable housekeeping gene in the pituitary, pancreas and WAT. There was no suitable housekeeping gene in BAT that improved the analysis, therefore the changes were reported without using a housekeeping gene as a co-variate. In the study of leptin treatment of *ob/ob* mice, the housekeeping gene used for the hypothalamus was 36B4 since it was the most consistent of the housekeeping genes, although cyclophilin and HPRT could also have been used. In the

hypothalamus of +/? and *fa/fa* ZDF rats, the two housekeeping genes analysed to use as a co-variate were β-actin and GAPDH. However, neither of these housekeeping genes were used as a co-variate since they did not improve the analysis.