LEPTIN ACTION ON OVULATION AND LEPTIN RECEPTORS ACROSS THE RAT OESTROUS CYCLE.

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A thesis submitted to the University of Adelaide in total fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 2001

DEDICATION

This thesis is dedicated to my Parents, **Madhu** and **Ramesh Duggal**, who have worked so hard to get me where I am. You have more courage than anyone I have ever known and I am everything I am, because you love me. I would also like to acknowledge my Sister and best friend, **Preety**, for always being there for me. Thanks Babe.

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Abstract

Leptin, a hormonal product of the *Lep* or *OB* gene, is expressed by adipocytes and circulates in relation to adiposity. Leptin is thought to play a role in regulating food intake and maintaining body weight. Although six isoforms of the leptin receptor exist, leptin exerts its effects by interacting with the long form of the leptin receptor, *OB-RB*. The leptin protein and leptin receptors have been localised in many reproductive tissues, including the ovary. Several publications indicate that the ovary is directly affected by leptin, in particular leptin appears to be able to inhibit ovarian cell steroid production. The high levels of leptin in obese women, and in animal models of obesity, implicate a link between elevated leptin and infertility.

The purpose of this project was to examine the effects of acute leptin treatment on ovulation and other important ovulatory mediators, *in vivo* and *in vitro*. Establishing the relationship between acute leptin concentrations and ovulation may provide insight into the link between the high leptin levels in obese women and infertility. It was also desired that the pattern of leptin receptor expression across the rat oestrous cycle be investigated. The pattern of leptin release in women throughout the menstrual cycle indicates that modulating leptin levels may be a mechanism of altering the sensitivity of leptin across the cycle. Understanding the relationship between leptin and ovarian leptin receptor expression will assist in comprehending the importance of leptin sensitivity in the oestrous cycle of the rat.

The effect of systemic leptin administration on ovulation in the rat ovary, both *in vivo* and *in vitro*, was investigated. Immature gonadotropin-primed rats, injected with leptin

experienced a loss in body weight, food intake and a decline in ovulation *in vivo* and ovaries perfused with leptin also ovulated significantly less. Plasma progesterone and oestradiol levels were unaffected in either model. However, feed restriction alone did not inhibit ovulation.

To investigate the mechanism of leptin-induced inhibition in ovulation, the numbers of follicles entering the pre-ovulatory pool following leptin treatment were established. Leptin treatment did not affect the recruitment of pre-ovulatory follicles from the antral follicle pool. The importance of ovarian leukocytes in ovulation prompted an investigation into the effect of leptin on two leukocytes important in ovulation. A decrease in food intake, either as a result of leptin-treatment or feed restriction, specifically reduced the numbers of neutrophils and monocytes/macrophages infiltrating the theca interna of pre-ovulatory follicles without affecting the numbers found in the stroma. However, this reduction was not solely responsible for the leptin-induced inhibition in ovulation.

In vitro ovarian follicular culture (4 h and 12 h) was used as a tool to investigate if high leptin concentrations could inhibit other factors important to ovulation, such as meiotic competence of oocytes, granulosa cell proliferation, steroid or prostaglandin E_2 synthesis and interleukin-Iβ production. High concentrations of leptin in follicle culture do not inhibit meiotic maturation or steroid synthesis, while an effect on prostaglandin E_2 synthesis may exist. Granulosa cell proliferation was not inhibited by leptin in FSH and IGF-I supplemented culture media, while leptin was able to inhibit the stimulatory effects of IGF-I on FSH-stimulated rat granulosa cell oestradiol production without affecting progesterone production, as previously reported. Leptin did not appear to have

an adverse effect on the components of ovulation tested and therefore impacts the ovulatory cascade in a way that remains to be defined.

Finally, the expression of two isoforms of the leptin receptor (*OB-RB* and *OB-RA*) were investigated throughout the oestrous cycle in order to assess whether ovarian sensitivity to leptin varied throughout the cycle. The isoforms of the leptin receptor were lower in the pro-oestrus and di-oestrus stages than the met-oestrus stages of the rat oestrous cycle in order to modulate leptin sensitivity across the cycle. The fluctuations in the leptin receptors may be a response to the levels of circulating steroid hormones and leptin.

DECLARATION

I declare that the material reported in this thesis has not been applied or accepted for the award of any other degree or diploma in any University and, to the best of my knowledge, contains no material previously published or written by another person, except where due reference is made in the text. The experiments reported in this thesis were performed by myself and any assistance received from others is specifically pointed out and acknowledged.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Priya S. Duggal

(17 / 07 / 01)

ACKNOWLEDGEMENTS

I would like to thank my Supervisor, **Prof. Rob Norman**, for his continued support and encouragement throughout my project. Thank you for being such a fantastic supervisor. It has made all the difference between a chore and a good Ph.D. project.

Thank you especially for the meticulous proofreaders of my work, in particular **Prof. Rob Norman, Dr. Denis Magoffin, Dr. Phil Owens, Dr. Graham Webb** and **Susan Kapsabelis**. Thank you for your continued friendship and advice in the three years of my study.

I appreciate all the help and friendship of the people of the **Department of Obstetrics & Gynaecology**, in particular **Prof. Dave Armstrong, Dr. Lou Warnes, Dr. Rob Gilchrist, Dr. Nicole Hussey, Michael Guerin** and **Jim Wang**. Thank you to my peers and friends in the Department: **Lesley, Theresa, Kylie, Natalie, Helen, Fred and Clyde**, thank you for your friendship, assistance in aspects of my project and words of encouragement.

I would like to acknowledge **Kylie Van der Hoek** and **Carole Woodhouse** for surgical procedures in the *in vitro* perfusion study and **Natalie Ryan** for the maintenance of the perfusions. I would also like to acknowledge **Jenny Hayes** and **Natalie Ryan** for being a part of my blind protocols and the members of the **Endocrine Laboratory (QEH)** for the steroid analysis in the first part of the ovulation study. Thanks to **Ken**, **Bronnie** and **Adrian** from the 'QEH Animal House' for their friendship and advice in relation to my animals.

Special thanks to my friends in Los Angeles, **Dr. Denis Magoffin** and **Stacy Weitsman**, particularly for their help in the real-time experiments. Your friendship and input into my project has been invaluable.

Thank you to **Reproductive Medicine Laboratories** and **The University of Adelaide** for the financial support for my research and Scholarship during my candidature.

Finally, thank you to all of my **friends and family** outside the workplace. I appreciate your friendship more than I can ever describe.

Publications and conference proceedings

Publications

- **Priya S. Duggal**, Natalie K. Ryan, Kylie H. Van der Hoek, Lesley J. Ritter, David T. Armstrong, Denis A. Magoffin and Robert J. Norman. 'The Effects of Leptin Administration and Feed Restriction on Thecal Leukocytes in the Pre-Ovulatory Rat Ovary and the Effects of Leptin on Meiotic Maturation, Granulosa Cell Proliferation, Steroid Hormone and Prostaglandin E_2 Synthesis using Rat Ovarian Follicle Culture.' (submitted [BOR] 2001).
- **Priya S. Duggal**, Stacy R. Weitsman, Denis A. Magoffin and Robert J. Norman. 'The Expression of the Long (OB-RB) and Short (OB-RA) Leptin Receptors throughout the Estrus Cycle in the Mature Rat Ovary.' (submitted [BOR] 2001).
- **Priya S. Duggal**, Kylie H. Van der Hoek, Clyde R. Milner, Natalie K. Ryan, David T. Armstrong, Denis A. Magoffin and Robert J. Norman (2000). 'The *In vivo* and *In vitro* Effects of Exogenous Leptin on Ovulation in the Rat.' Endocrinology 141 (6): 1971-6.

Conference Presentations

- **Priya S. Duggal**, Stacy R. Weitsman, Kylie H. Van der Hoek, Denis A. Magoffin and Robert J. Norman. 'The Expression of the Long (OB-RB) and Short (OB-RA) Leptin Receptors throughout the Estrus Cycle in the Mature Rat Ovary.' *Endocrine Society of Australia* (Queensland [submitted] 2001).
- **Priya S. Duggal**, Kylie H. Van der Hoek, David T. Armstrong, Denis A. Magoffin and Robert J. Norman 'Leptin administration and feed restriction causes a reduction in thecal neutrophils and monocytes/macrophages in the pre-ovulatory rat ovary'. *Research Day at the North West Adelaide Heath Service* (Adelaide 2000) & *International Conference of Endocrinology* (Sydney 2000)
- **Priya S. Duggal**, Kylie H. Van der Hoek, David T. Armstrong, Denis A. Magoffin and Robert J. Norman 'Leptin administration and feed restriction causes a reduction in thecal neutrophils and monocytes/macrophages in the pre-ovulatory rat ovary'. *Australian Society for Reproductive Biology* (Canberra 2000)
- **Priya S. Duggal**, Kylie H. Van der Hoek, Clyde R. Milner, Natalie K. Ryan, David T. Armstrong, Denis A. Magoffin and Robert J. Norman. 'The *In vivo* and *In vitro* Effects of Exogenous Leptin on Ovulation in the Rat.' *Research Day at the North West Adelaide Heath Service* (Adelaide 1999)

- **Priya S. Duggal**, Denis A. Magoffin and Robert J. Norman. 'Detection of the long leptin receptor in the rat ovary'. *Australian Society for Reproductive Biology* (Melbourne 1999)
- **Priya S. Duggal**, Kylie H. Van der Hoek, Clyde R. Milner, Natalie K. Ryan, David T. Armstrong, Denis A. Magoffin and Robert J. Norman. 'The *In vivo* and *In vitro* Effects of Exogenous Leptin on Ovulation in the Rat.' *Endocrine Society of Australia* (Melbourne 1999)

Awards

1998 - 2001

Repromed Postgraduate Scholarship

2000

The University of Adelaide Research Abroad Scholarship

Endocrine Society of Australia Travel Award (not accepted)

Australian Society for Reproductive Biology Travel Award

1999

Endocrine Society of Australia Travel Award

NWAHS Research Day Prize Finalist

Glossary/abbreviations

α-MSH - α-melanocyte-stimulating hormone
AGRP - Agouti-related protein
ATP - Adenosine tri phosphate
BAT - Brown adipose tissue
BMI - Body mass index (weight/(height)^2, kg.m^{-2})
bp - Base pairs
BSA - Bovine serum albumin
CART - Cocaine and amphetamine-regulated transcript
cDNA - Complementary DNA
CRH - Corticotropin-releasing hormone
cM - Centi morgan
CNS - Central nervous system
CSF - Cerebrospinal fluid
DHEA-S - Dehydroepiandrosterone sulphate
DHT - Dihydrotestosterone
DNA - Deoxyribonucleic acid
dNTP - DeoxyNucleoside TriPhosphate
ELISA - Enzyme linked immunosorbent assay
FSH - Follicle stimulating hormone
GAL - Galanin
GH - Growth hormone
GnRH - Gonadotrophin release hormone
HCG - Human chorionic gonadotrophin
IGF - Insulin like growth factor
IL - Interleukin
Jak - Janus kinase
KB - Kilo bases
kDa - Kilo daltons
Lep - Leptin
LepR - Leptin receptors
$LepR^{db}/LepR^{db}$ - The genetically diabetic mouse, also known as *db/db* mouse
$LepR^{fa}/LepR^{fa}$ - The Zucker fatty rat, also known as *fa/fa* rat
Lep^{ob}/Lep^{ob} - The genetically obese mouse, also known as *ob/ob* mouse
LH - Luteinising hormone
mRNA - Messenger ribonucleic acid
MC4- Melanocortim receptor
MCH - Melanin-concentrating hormone
mIU - Milli international unit
NPY - Neuropeptide Y
NRS - Normal rat serum
OB - Leptin
OB-R (OB-RA, -RB, -RC, -RD, -RE, -RF) - Leptin receptor isoforms
$P450_{arom}$ - Aromatase gene
$P450_{17\alpha}$ - 17α-hydroxylase
$P450_{scc}$ - Side chain cleavage enzyme
PBS - Phosphate buffered saline
POMC - Pro-opiomelanocortin
PPARγ - Peroxisome proliferator activated receptor γ

RIA - Radio immuno assay
RT-PCR - Reverse transcriptase-polymerase chain reaction
STAT - Signal transducers and activators of transcription
TGF-β - Transforming growth factor-β
UCP - Uncoupling proteins
UV - Ultra-violet
WAT - White adipose tissue

Chapter One

Review of the Literature

1. INTRODUCTION

The importance of the hypothalamus in maintaining energy expenditure, body weight and control of appetite was first hypothesised by Kennedy (1953). Several researchers (Coleman and Hummel, 1969; Coleman, 1973; Harris *et al.*, 1987) further expanded on Kennedy's theory and suggested that a satiety signal or lipostat was responsible for the control of adiposity. In contrast to the well known short-term regulators of food intake, such as glucose, temperature and exercise, the lipostat signal was postulated to monitor a response to deficits in whole animal energy reserves. Therefore, when adipose tissue energy stores were high, the hypothalmic satiety centres responded by reducing food intake (Figure 1.1). In contrast, in times of low food availability, energy reserves were activated and appetite increased.

To further investigate the lipostat theory, Coleman and colleagues performed a series of parabiosis experiments (Coleman and Hummel, 1969; Coleman, 1973), which surgically joined two animals, one lean and one genetically obese, to produce interconnected microvasculature (Figure 1.2). The results indicated that a circulating satiety factor was produced in excess by the obese animal, which resulted in death by starvation of the lean animal. This extensive research into the lipostat theory laid the foundations for the current understanding of leptin biology. The discovery of the hormone leptin, secreted by adipocytes, stimulated research to a level perhaps unrivalled by any other field of research in the present decade. The potential for leptin to be the regulator of weight maintenance or lipostat, being sought after since the late nineteenth century as a control for adiposity, provided a hope for the increasingly prevalent multi-factored condition of obesity involving a significant proportion of the human population in the twenty-first century.

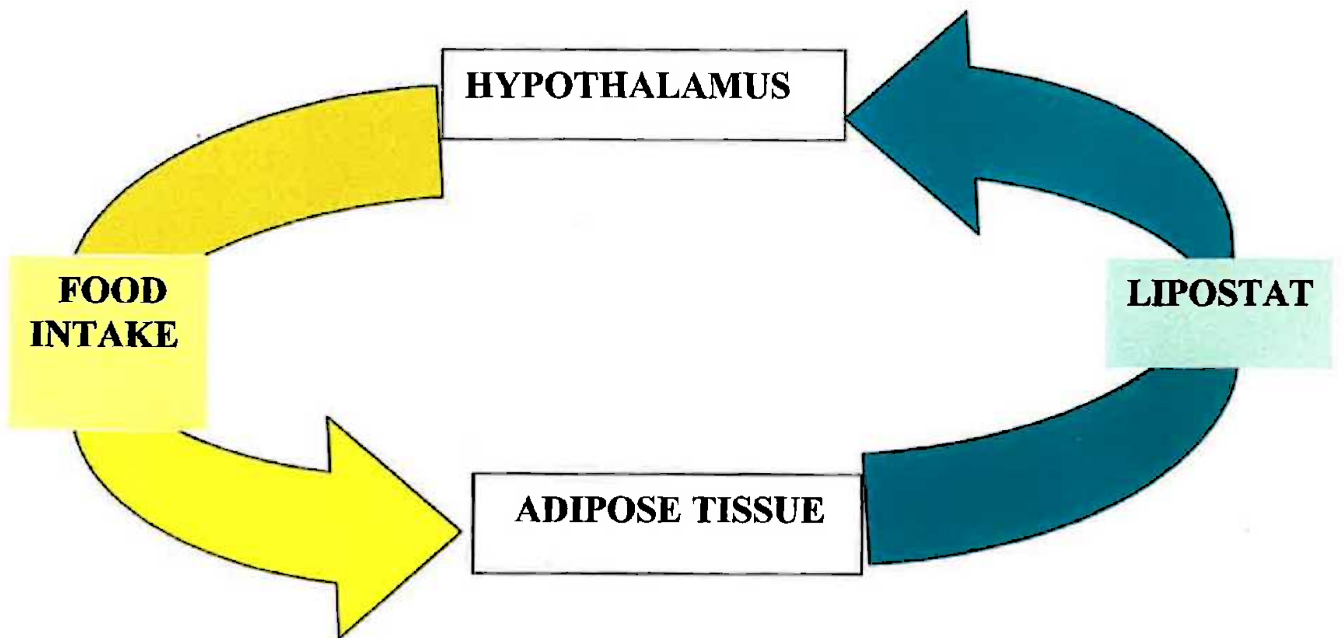


Figure 1.1 A schematic diagram representing the relationship between the hypothalamus and adipose tissue in the regulation of body adiposity. It was postulated that a lipostat signal regulated body adiposity by signalling between food control centres in the brain and adipose tissue.

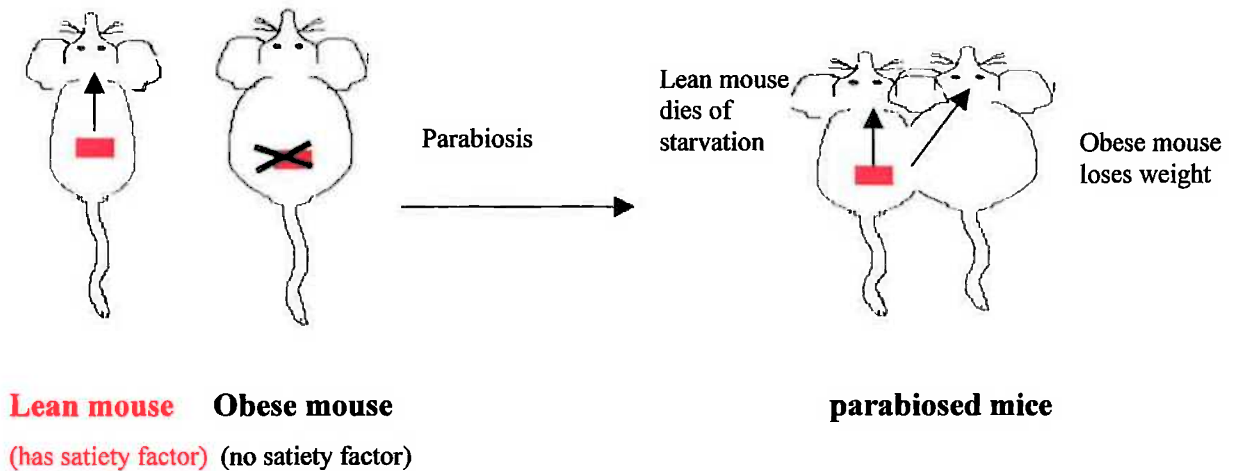


Figure 1.2 A schematic representation of the parabiosis experiments performed by Coleman and colleagues (1969, 1973). When a lean and genetically obese mouse are surgically joined, the lean mouse decreases its food intake while the obese mouse does not. These experiments led to the hypothesis that obese mice failed to produce a satiety factor (indicated by a red square) but their brain remained sensitive to this factor. The production of the satiety factor in the lean mouse acts on both mice and signals the brain to decrease food intake, thereby controlling the amount eaten by the obese animal and leading to death by starvation in the lean animal.

The association of extremes of body mass and reproductive dysfunction has been linked in many species. Both humans and animals experiencing severe dietary restriction or obesity have impaired reproductive systems or dysfunctional reproductive hormone secretion (Cagampang *et al.*, 1990; Cameron, 1991; Henry *et al.*, 2001). The finding that extremely obese individuals with high leptin levels and the genetically obese, leptin deficient Lep^{ob}/Lep^{ob} mouse both experience infertility, initiated research into the effects of leptin on the reproductive system.

The leptin protein has been identified in ovarian tissue, however, its role in this tissue is not well understood. Leptin appears to have both a developmental role in the maturation of the female reproductive system and also a direct effect on ovarian function. Developmentally, the onset of puberty is largely determined by body weight in both humans and other mammals. The link between puberty and body weight, and leptin and body weight has also proven to be important in puberty onset. A sexual dimorphism favours females, resulting in females having higher leptin levels than males. The direct effects of leptin on ovarian function have been inferred from the finding of functional leptin receptors and their mRNA in ovarian tissue. Further investigations have assessed the effects of leptin treatment on isolated components of the ovary, and have shown that leptin has significant effects on compartments of the ovary.

The aim of the following review is to discuss the literature published on leptin (Chapter 1.1, Chapter 1.2), the reproductive system (Chapter 1.3) and the findings on the effects of leptin on the ovary (Chapter 1.3) and the reproductive system (Chapter 1.4). Where possible, the findings discussed are related to rodents; however some sections of this literature review will discuss leptin as it relates to humans because leptin research has clinical relevance in humans.

1.1 Leptin

This section discusses general information on leptin, including the structure of the leptin (*OB* or *Lep*) gene and protein (Chapter 1.1.2), the synthesis of the leptin protein in adipose tissue (Chapter 1.1.3), the leptin receptor isoforms (Chapter 1.1.4) and the importance of the brain in leptin physiology (Chapter 1.1.5).

1.1.1 General information

Leptin is a secretory product of adipocytes (Chapter 1.1.3) and is the protein product of the *OB* or *Lep* gene, first identified in mice by a genetic technique known as positional cloning (Zhang *et al.*, 1994). Leptin has been linked with satiety, energy expenditure and fat store regulation (Halaas *et al.*, 1995). It circulates in the blood and crosses the blood brain barrier to reach its main target, the brain (Hakansson *et al.*, 1998).

1.1.2 Location and structure of the *OB* gene and the leptin protein

The *OB* gene is located on Chromosome 6 in the mouse and chromosome 7 in the human and is organised in a similar manner in rodents and humans, with 3 exons separated by 2 introns (Figure 1.3A) (Chagnon *et al.*, 1998). The *OB* gene is well-conserved among vertebrates: rat and mouse leptin share a 96 per cent amino acid identity, while human leptin shares 85 per cent amino acid identity with mouse and 84 per cent identity with rat (Zhang *et al.*, 1994; Chagnon *et al.*, 1998). The high conservation between human and rodent leptin means that recombinant human leptin is biologically active in rodents (Chehab *et al.*, 1996). Transcription binding motifs on the

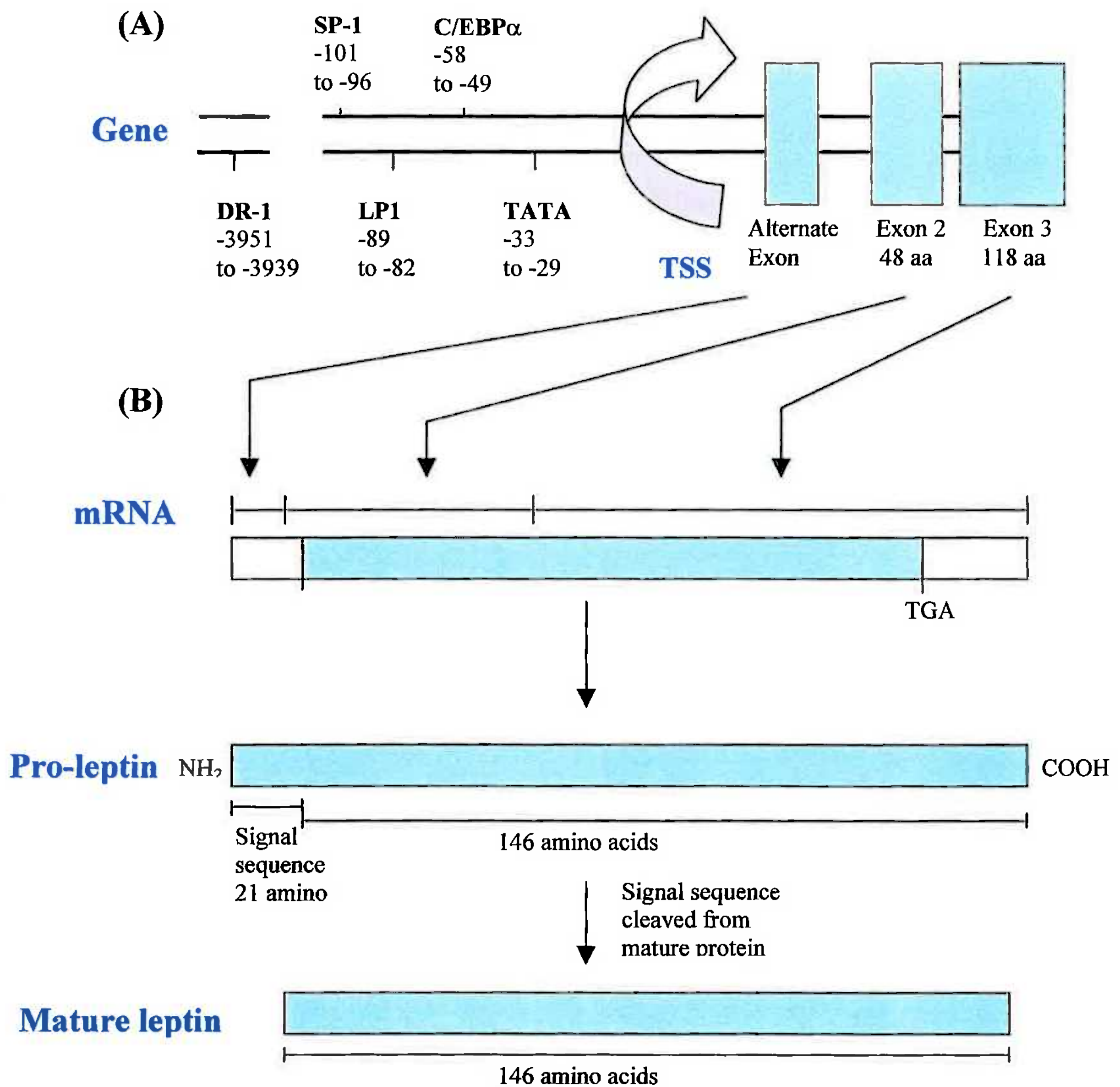


Figure 1.3 The mouse leptin gene, encoded mRNA and protein. (A) The mouse leptin gene consists of three exons (shaded boxes) separated by two introns. The promoter contains transcription response elements including a TATA box, a CAAT/enhancer binding protein (C/EBPα) element, a leptin promoter specific factor (LP1), a SP1 site and a DR-1 site. All positions indicated are relative to the transcription start site (TSS) with exon size indicated in amino acids (aa). (B) The two coding exons (2 and 3) transcribe a leptin message that codes for a pro-leptin of 167 amino acids. The 21 amino acid signal sequence is removed prior to release of leptin (146 aa) from adipocytes. The leptin protein in circulation is 16 kDa. Adapted from Houseknecht and Portocarrero, 1998.

leptin promoter indicate that there are several regulatory domains contributing to leptin gene transcription (Figure 1.3A).

Rodents produce a 4.5 Kb mRNA while humans produce a 3.5 Kb mRNA (Zhang *et al.*, 1994; Chagnon *et al.*, 1998). The *OB* mRNA is translated into a 167 amino acid protein, with the first 21 amino acids acting as a signal peptide at the N-terminus, resulting in a mature un-glycosylated protein of 146 amino acids with a molecular weight of 16 kDa (Figure 1.3B) (Zhang *et al.*, 1994). The tertiary structure of leptin resembles a class I cytokine (such as interleukins and granulocyte-macrophage colony stimulating factor) without consensus sites for N-linked glycosylation (Di Francesco *et al.*, 1997; Kline *et al.*, 1997). However, the primary structure of leptin is unique (Di Francesco *et al.*, 1997; Kline *et al.*, 1997) and the secondary structure is comprised of four α-helices and two short β-sheets held together by an intramolecular disulphide link of 2 cysteines (at position 96 and 146) in the carboxyterminal region (Figure 1.4) (Rock *et al.*, 1996; Di Francesco *et al.*, 1997; Kline *et al.*, 1997). The disulphide link is essential for the structural integrity of the molecule as it minimises exposure of the hydrophobic core and creates a compact molecule with thermal stability (Rock *et al.*, 1996; Kline *et al.*, 1997; Zhang *et al.*, 1997). The disulphide bridge and the removal of the signal peptide are the only two post-translational modifications required for a functional leptin protein (Zhang *et al.*, 1994; Cohen and Chait, 1997).

The major biological effect of an increase in the exposure to leptin protein in rodents is to decrease food intake, a response which stimulates utilisation of fat stores (Mounzih *et al.*, 1997). Genetic deletion experiments show that the most potent part of the leptin protein in inhibiting feeding comprises the N-terminal amino acids 22-56. The 116-167 amino acid region causes low levels of inhibition of feeding while amino acids 57-92 do not affect feeding at all (Samson *et al.*, 1996; Grasso *et al.*, 1999). Therefore, amino

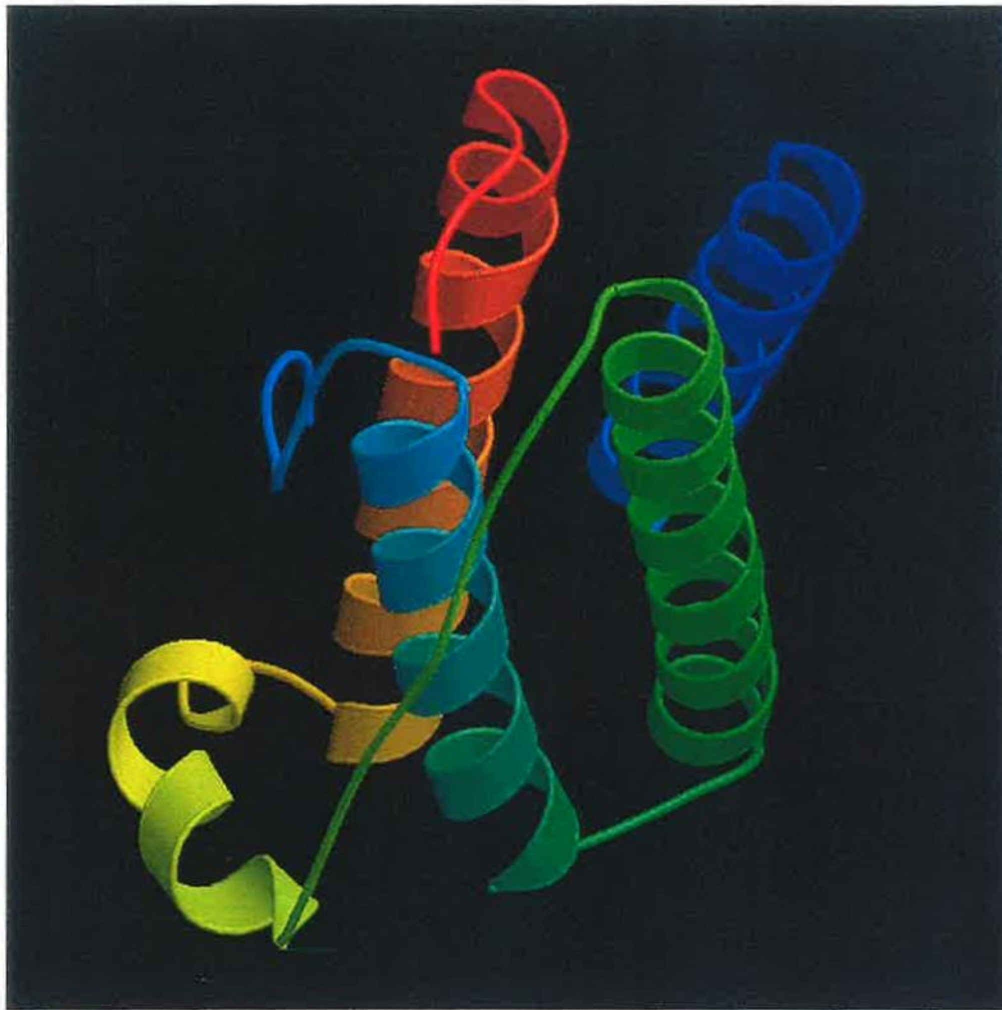


Figure 1.4 X-ray diffraction ribbon diagram of the human leptin protein with α-helices and β-sheets. Source: Protein Data Bank (Id: 1AX8), Zhang *et al.*, 1997.

acids 22-56 of leptin are important in interactions of leptin and its receptor for the effects of leptin on food intake.

1.1.3 Leptin synthesis in adipose tissue

Leptin gene expression and the leptin protein are synthesised and secreted from white adipose tissue (WAT), with lower expression in brown adipose tissue (BAT) (Cinti *et al.*, 1997). Leptin detected in BAT, the main fat source for thermogenesis, is possibly attributed to the small numbers of white adipocytes in BAT, as classical brown adipocytes differ from white adipocytes due to their morphology and their apparent lack of detectable leptin expression (Cinti *et al.*, 1997). Overall, humans have very little BAT, with larger amounts found in newborns (Himms-Hagen, 1995; Gura, 1998). Leptin mRNA levels in both BAT and WAT increase slightly in the suckling phase of humans (35-60 days old, when a high fat milk diet may result in building fat stores) and at the time of puberty (Chapter 1.4.2) and sexual maturation (Devaskar *et al.*, 1997). It was initially hypothesised that, for Kennedy's (1953) 'lipostat' theory to work, leptin would be produced exclusively in adipose tissue. It is now known that there is rapid activation of *OB* gene expression and leptin release from skeletal muscle in response to glucosamine and leptin secretion from the human placenta during pregnancy, indicating that leptin production is not exclusive to fat cells (Senaris *et al.*, 1997; Wang *et al.*, 1998).

Leptin treatment results in autocrine and paracrine effects on adipocytes and therefore leptin in circulation can regulate leptin protein production (Siegrist Kaiser *et al.*, 1997). *In vivo* leptin treatment of leptin deficient, Lep^{ob}/Lep^{ob} mice indicates that leptin can down-regulate endogenous *OB* mRNA in adipocytes (Slieker *et al.*, 1996). However, *in vitro* treatment of rat adipocytes with leptin shows that there is no direct effect on

endogenous leptin mRNA expression, indicating that leptin controls *OB* mRNA expression by an indirect non-autocrine mechanism (Slieker *et al.*, 1996). The finding that human adipose tissue expresses the leptin receptor for leptin signal transduction also indicates that leptin production may be auto-regulated (Kielar *et al.*, 1998; Kutoh *et al.*, 1998). Adipocytes with a high density of fat cells have higher expression of leptin mRNA than smaller fat cells (Hamilton *et al.*, 1995). Since large adipocytes express more *OB* mRNA, cell wall stretching may initiate a regulatory signal: exogenously applied tension to the plasma membrane leads to the induction of gene expression in other cell systems (Caro *et al.*, 1996).

1.1.4 Leptin receptors

The leptin receptors (*OB-R* or *LepR*) are class I cytokine receptors with extracellular, transmembrane and intracellular domains. The gene encoding rat leptin receptor was cloned from choroid plexus cDNA and it was found that leptin receptors share amino acid sequence similarity with class I cytokine receptor proteins like glycoprotein gp130 (Tartaglia *et al.*, 1995). Multiple splice variants of the leptin receptor have been identified with the leptin receptor gene encoding at least 6 alternatively spliced isoforms (Figure 1.5). Isoforms have a strong homology in the long cytoplasmic domain, a region important in intracellular signalling (Figure 1.5, Figure 1.6) (Tartaglia *et al.*, 1995). The *OB-R* gene has Janus kinase 1 and 2 interaction motifs (Figure 1.5, Figure 1.6), which are required for signal transduction (Chapter 1.1.4.1). One leptin receptor isoform, known as the functional leptin receptor or *OB-RB,* has a long cytoplasmic domain which has the ability to transduce signals using homo-oligomerisation. Other isoforms of the transmembrane receptor have short cytoplasmic domains with no known signal transduction ability (*OB-RA*, *-RC*, *-RD*, *-RF*), and there is a soluble receptor isoform (*OB-RE*) which acts as a carrier molecule and circulates bound to leptin (Figure 1.6)

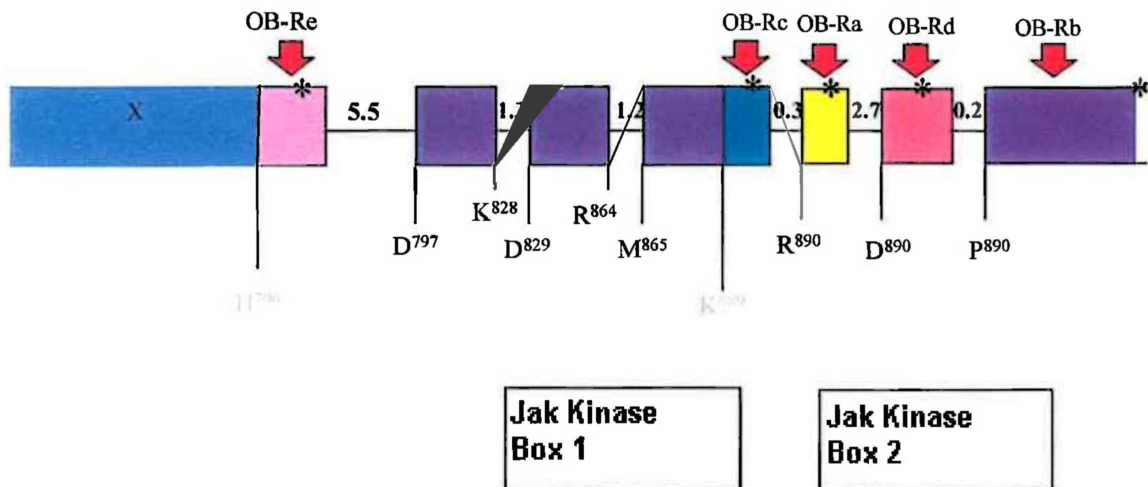
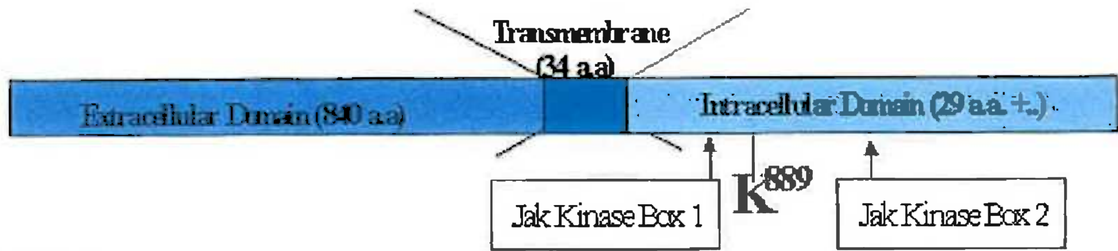


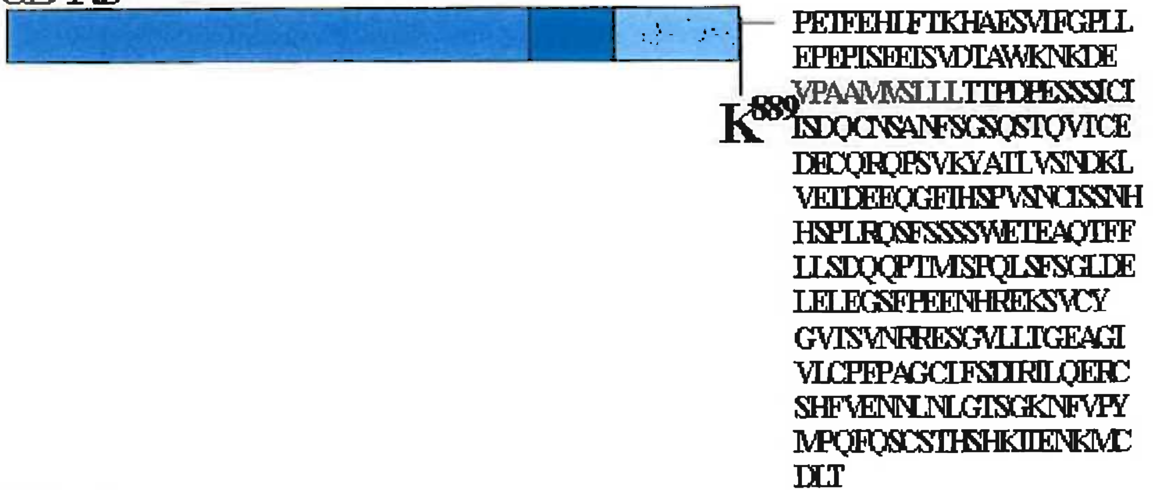
Figure 1.5 Schematic representation of the leptin receptor gene. (A) The leptin receptor has at least 6 splice variants. The most common (*OB-RA, OB-RB, OB-RC, OB-RD, OB-RE*) are shown above. The region labelled X is common to all isoforms. *OB-RE* is the shortest of all the leptin receptors, the purple regions splice to form the longest of the *OB-R*: *OB-RB* mRNA; the other short isoforms (*OB-RC, OB-RA* and *OB-RD*) are indicated in green, yellow and red respectively. The asterisks indicate the termination of the mRNA splice and the black numerals indicate intron size between exons in kb. The genetic information for the Janus kinase interaction motifs (Boxes 1 & 2) surround K^{889} as indicated.

Figure 1.6 Schematic Diagram of the various forms of the leptin receptor. The leptin receptor has three main domains: an extracellular domain, common to all isoforms; a transmembrane domain, common to all but the soluble receptor (*OB-RE*) and an intracellular domain of varied length which diverges at Lysine^{889} in all but *OB-RE*. The 6 isoforms of the leptin receptor with their domains are shown. Arrows indicate positions of Jak Kinase Boxes 1 and 2. Adapted from Lee *et al*., 1996.



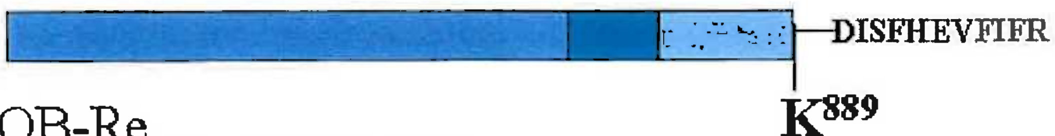
OB-Ra

OB-Rb



OB-Rc

OB-Rd



OB-Re

OB-Rf

(Bjorbaek *et al.*, 1997; Nakashima *et al.*, 1997).

1.1.4.1 Functional leptin receptor (*OB-RB*) signalling

The signal transduction cascade of class 1 cytokine receptors, such as the functional leptin receptor (*OB-RB)* requires the involvement of a class of cytoplasmic tyrosine kinases, known as the Janus kinases (Jaks) (Ghilardi *et al.*, 1996). The cytoplasmic domain of *OB-RB* possesses the Jak and signal transducers and activators of transcription (STAT) interaction motifs (Figure 1.7) (Bjorbaek *et al.*, 1997). All *OB-R* isoforms possess the Jak box 1 interaction motif for Jak interaction and the box 2 motif, which, in association with the box 1 motif, is essential for inducing DNA synthesis (Figure 1.5, Figure 1.6). In addition, there is a box 3 motif containing a tyrosine-X-X-glutamine motif for STAT-3 docking (Stahl *et al.*, 1995). Binding of the leptin ligand to the functional leptin receptor activates Jaks, which are auto-phosphorylated in response to ligand-receptor binding (Ghilardi *et al.*, 1996; Nakashima *et al.*, 1997). The activated Jak proteins then phosphorylate specific tyrosine residues on the cytoplasmic tail of the receptor, providing binding sites for STAT proteins (Figure 1.7) (Ihle, 1995; Ghilardi *et al.*, 1996).

The functional leptin receptor must exist as a homo-dimer, in a receptor-ligand stoichiometry of 2:2, for signal transduction of leptin (De Vos *et al.*, 1997). A hetero-dimer of a long and short leptin receptor isoform appears to be inadequate for signal transduction (De Vos *et al.*, 1997; Nakashima *et al.*, 1997). Leptin has direct effects on the adipose tissues, BAT and WAT, resulting in the activation of the Jak/STAT pathway and the increased expression of specific target genes (Siegrist Kaiser *et al.*, 1997). Leptin induces tyrosine phosphorylation of several cellular proteins, such as STAT-1, -3, -5 and -6 (Takahashi *et al.*, 1997; McCowen *et al.*, 1998).

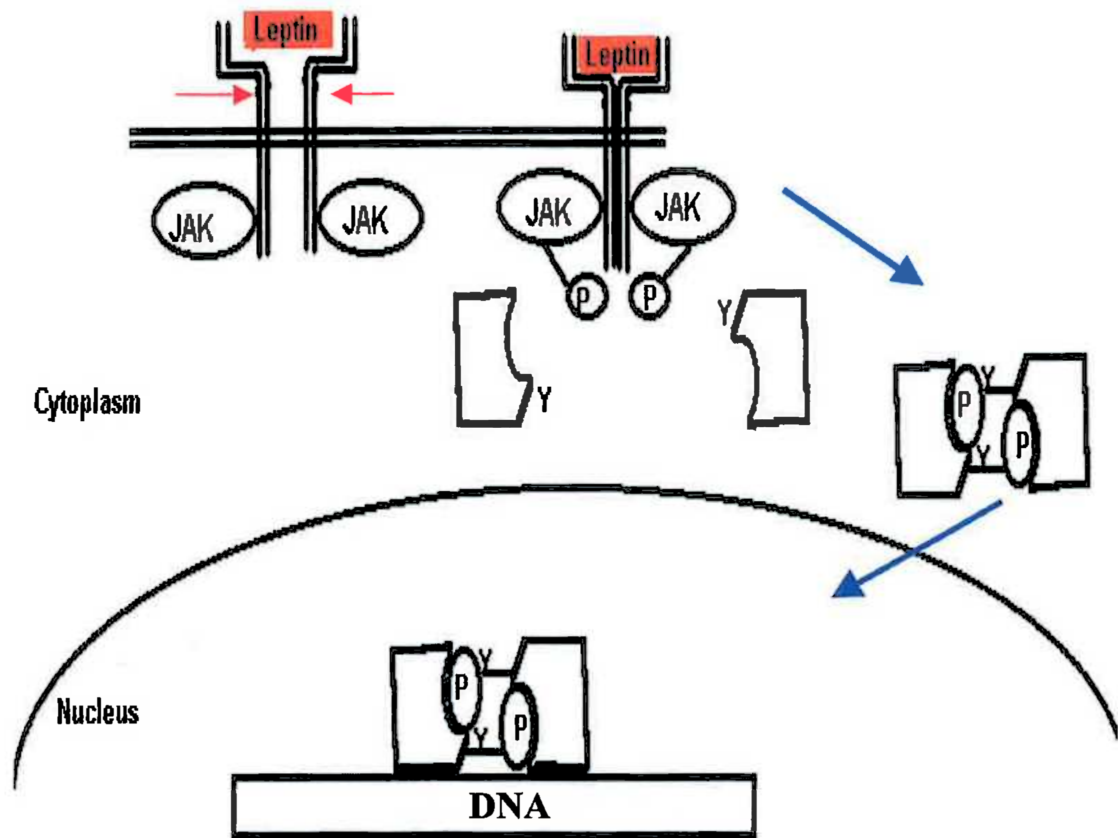


Figure 1.7 The leptin receptor uses Janus kinases (Jak) to phosphorylate tyrosine residues. Binding of leptin to its functional receptor will induce dimerisation of the receptor (represented by two red arrows). The janus kinases associated with the receptor will induce phosphorylation of tyrosine residues (Y), creating phosphotyrosine docking sites for the STAT proteins (represented by clear blocks). After phosphorylation of tyrosine residues, these STAT proteins will dissociate from the receptor and form dimers, resulting in the active transcriptional regulator unit. After transport into the nucleus, the unit binds STAT responsive elements on DNA and stimulates transcription of responsive target genes. Adapted from Auwerx and Staels, 1998.

1.1.4.2 The short isoforms of the leptin receptor

Multiple short isoforms of the leptin receptor exist: *OB-RA*, *OB-RC*, *OB-RD* and *OB-RF* (Figure 1.5). Unlike the long isoform of the leptin receptor, the short isoforms of the leptin receptor lack STAT binding sites, however there is some evidence that these may still transduce STATs (Cohen *et al.*, 1996; Ghilardi *et al.*, 1996; Bjorbaek *et al.*, 1997; White *et al.*, 1997; Yamashita *et al.*, 1998). *OB-RA* is expressed at high levels ubiquitously, while *OB-RC* and *OB-RD* are only detectable using a sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) assay (Wang *et al.*, 1996; Fei *et al.*, 1997; Kielar *et al.*, 1998). High levels of the short *OB-R* mRNA have been localised in the rat uterus, ovary and testis (Zamorano *et al.*, 1997). The short isoform of the leptin receptor is expressed at high levels in the choroid plexus, the site of cerebrospinal fluid (CSF) production, indicating that the receptor may function as a transporter for leptin, (Stephens and Caro, 1998).

1.1.4.3 The soluble isoform of the leptin receptor (*OB-RE*)

The soluble leptin receptor (*OB-RE*) has a coding sequence that predicts a molecular weight of 100 kDa in rodents and 92 kDa in humans, however, the receptor is larger (120 kDa in rodents and 130 kDa in humans) than the predicted size (Liu *et al.*, 1997; Li *et al.*, 1998). This may be a result of glycosylation or the participation of more than one receptor subunit in the receptor-ligand binding complex (Liu *et al.*, 1997; Li *et al.*, 1998). Rodents have three macromolecules which circulate bound to leptin, with molecular masses of 85, 176 and 240 kDa (Houseknecht *et al.*, 1996). The soluble leptin receptor binds the leptin protein with a higher affinity than the functional leptin receptor, supporting the hypothesis that *OB-RE* binds leptin as a dimer, resulting in a

340 kDa product in humans when leptin is added (Liu *et al.*, 1997). In support of this, leptin binding factors of 450 kDa (dimer), 176 kDa and 240kDa (single receptors), which specifically bind leptin at concentrations inversely proportional to serum leptin levels, have been described in human serum (Houseknecht *et al.*, 1996; Diamond *et al.*, 1997).

1.1.5 Leptin and the brain

The brain has an important role in leptin action, with *OB-RB* expression being approximately 50 per cent of total leptin receptors expressed in regions of the brain linked with food intake and energy balance (Lee *et al.*, 1996; Mercer *et al.*, 1996; Corp *et al.*, 1998). Systemic leptin administration activates nuclear groups in many regions of the brain, including the median eminence, anterior pituitary and ventrobasal hypothalamus (Elmquist *et al.*, 1997; Zamorano *et al.*, 1997; Cai and Hyde, 1998). The localisation of the functional leptin receptors in these regions is consistent with their involvement in food intake, nutritional regulation, energy balance and physiological responses during substrate availability (Elmquist *et al.*, 1997). This preferential distribution of the functional leptin receptor demonstrates the importance of the brain in the proposed feedback loop between the adipose tissue and the brain (Figure 1.1) (Ghilardi *et al.*, 1996; Hoggard *et al.*, 1997).

Studies have shown that leptin administered directly into the cerebrospinal fluid or the brain, exerts a more potent effect than systemic administration and results in negative control of feeding and energy balance, causing body weight loss (Campfield *et al.*, 1995; Stephens *et al.*, 1995). In addition, hypothalmic lesions may result in hyperphagia, decreased energy expenditure and increased adipocyte leptin mRNA (Frederich *et al.*, 1995; Funahashi *et al.*, 1995; Maffei *et al.*, 1995). It is therefore

believed that leptin is transported via the cerebrospinal fluid to reach the brain where it exerts effects on food intake and energy metabolism (Figure 1.8). In support of this theory, central infusion of leptin results in an up-regulation of leptin receptor gene expression within the hypothalamus, while down-regulating the gene expression of neuropeptide Y (NPY), a potent stimulator of food intake (Chapter 1.2.2.1) (Dickson and Bennett, 1999). The binding of leptin may release or transport uncharacterised factors into the cerebrospinal fluid, allowing a cascade of events to 'control' feeding and energy expenditure (Figure 1.8) (Malik and Young, 1996).

Although the main target of leptin is thought to be the brain, the functional leptin receptor is highly expressed in non-neural tissues, such as the lung, kidney, epithelium of the jejunum and ovary (Chapter 1.3.2.1) (Tartaglia *et al.*, 1995; Lollmann *et al.*, 1997; Morton *et al.*, 1998; Breidert *et al.*, 1999; Fruhbeck *et al.*, 1999).

1.2 Leptin and obesity

Leptin appears to be involved in many biological processes. In particular, leptin has a clear involvement in obesity regulation. The following section discusses leptin and leptin receptor mutations which result in rodent obesity (Chapter 1.2.1), the involvement of leptin in anabolism and catabolism (Chapter 1.2.2), factors important in the regulation of leptin (Chapter 1.2.3) and the role of leptin in human obesity (Chapter 1.2.4). In addition, this section also addresses the involvement of leptin in other biological processes, including hematopoiesis, angiogenesis, removal of leptin from the body and diurnal variation of leptin (Chapter 1.2.5).

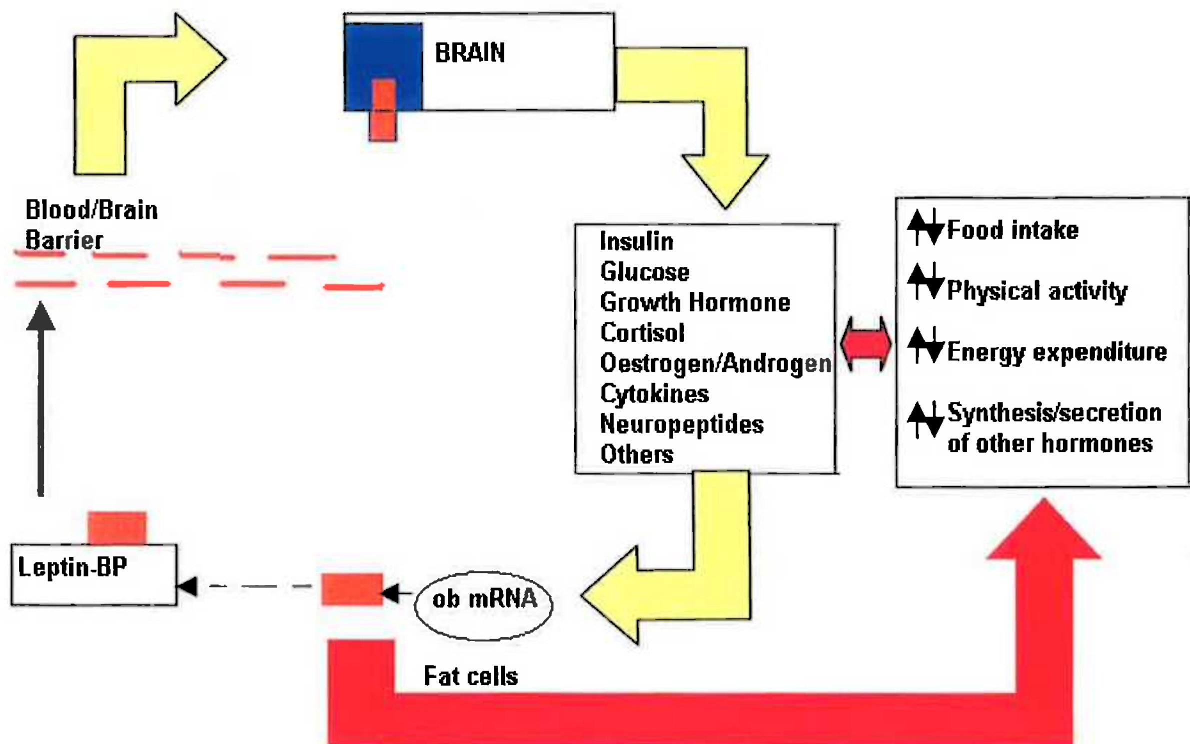


Figure 1.8 A schematic model of some important elements that may be involved in the leptin signalling pathway to regulate energy balance. Leptin (red square) produced from fat cells binds leptin binding proteins (BP) and travels across the blood brain barrier to the brain, where it binds to the functional leptin receptors (blue square) in the hypothalamus. The binding of leptin to the food regulatory regions of the brain may signal various other hormones and neuropeptides, which may then regulate fat cells, thereby forming an auto-regulatory loop. Both leptin and these other regulatory hormones and neuropeptides may therefore be able to control food intake, physical activity, energy expenditure and the secretion of other hormones.

1.2.1 Mutations of the *OB* & *OB-R* genes in rodents

Leptin mRNA and/or protein levels are altered in many models of genetic obesity in rodents. The three most relevant to leptin, the Lep^{ob}/Lep^{ob} mutation, the $LepR^{db}/LepR^{db}$ mutation and the $LepR^{fa}/LepR^{fa}$ mutation, are discussed below. Reviews (Igel *et al.*, 1997; Leibel *et al.*, 1997) detailing other mutations in rodent models have been explored, but they are not specifically relevant to this literature review.

1.2.1.1 The obese Lep^{ob}/Lep^{ob} mouse

The original C57BL/6J Lep^{ob}/Lep^{ob} obese mouse has a mutation in codon 105 of its *OB* gene, resulting in a thymidine replacing a cytosine to create a premature stop in the leptin protein where an arginine is normally present (Zhang *et al.*, 1994). This results in an inactive short leptin protein. In Lep^{ob}/Lep^{ob} mice there is a twenty-fold increase in *OB* mRNA relative to wild type mice, suggesting that the absence of a functional leptin protein up-regulates *OB* gene expression (Zhang *et al.*, 1994). The inactive protein fails to prevent obesity in the mouse and the absence of a functional protein makes the mouse infertile. However, unlike the Lep^{ob}/Lep^{ob} mouse, which has low levels of circulating leptin, obese humans have high levels of leptin in circulation. A second type of *OB* mutant mouse, the SM/Ckc-+ ^{Dac} $Lep^{ob2J}/LepR^{ob2J}$ strain, does not synthesise *OB* mRNA, resulting in an identical phenotype to C57BL/6J Lep^{ob}/Lep^{ob} mice (Zhang *et al.*, 1994).

Leptin treatment (5 μg/g body weight, given daily intraperitoneally for 2 weeks) to Lep^{ob}/Lep^{ob} mice decreases food intake and body weight by 30 per cent (Halaas *et al.*, 1995). However, this treatment does not affect their diabetic ($LepR^{db}/LepR^{db}$) relatives

(Chapter 1.2.1.2) which do not possess a functional leptin receptor (Halaas *et al.*, 1995). Male and female obese (*Lep^ob^/Lep^ob^*) mice treated twice a day with leptin lose body weight, consume less food, have lower plasma glucose levels and possess less body fat (0.67 per cent as compared with 12.22 per cent controls) (Halaas *et al.*, 1995; Mounzih *et al.*, 1997). Food intake in leptin-treated pre-pubertal females decreases to 80 per cent of that consumed by free-fed (ad-libitum fed) animals, resulting in retarded growth in leptin-treated and food restricted animals (pair-fed) (Cheung *et al.*, 1997).

Early sexual development in Lep^{ob}/Lep^{ob} female mice is normal, however the mice never experience oestrous cycles or ovulation (Chehab *et al.*, 1996). Lep^{ob}/Lep^{ob} mice have low levels of growth hormone (GH) (Larson *et al.*, 1976), gonadotrophins (luteinising hormone and follicle stimulating hormone), and prolactin (Swerdloff *et al.*, 1978). Sterility of Lep^{ob}/Lep^{ob} female and male mice is caused by an insufficient level of hormones at the hypothalmic-pituitary level, rather than the inhibition of copulatory activity (Ahima *et al.*, 1996; Chehab *et al.*, 1996; Mounzih *et al.*, 1997). With leptin treatment, copulatory plugs are detected 1-6 days after the introduction of breeder males, demonstrating the occurrence of oestrus and ovulation with leptin administration, while food restricted or saline treated control mice are unaffected (Chehab *et al.*, 1996). Lep^{ob}/Lep^{ob} leptin treated mice give birth to normal pups, however if leptin is stopped, these pups do not survive due to the failure of lactation (Chehab *et al.*, 1996). Leptin treatment to Lep^{ob}/Lep^{ob} mice improves ovarian parameters by increasing primordial, primary, secondary and graffian follicles, improved LH levels, ovarian and uterine histology when compared with controls (Barash *et al.*, 1996). Uterine cross sectional area, epithelial height, endometrial area and glandular area are also increased with leptin administration (Barash *et al.*, 1996). Leptin treatment tends to increase uterine weight by promoting proliferative growth of the uterine glands, epithelium and endometrium, which are typical responses to oestrogen stimulation (Barash *et al.*, 1996). Likewise,

leptin treatment rescues male sterility of Lep^{ob}/Lep^{ob} mice (Mounzih *et al.*, 1997), indicating that leptin acts as a metabolic signal to the reproductive system of both sexes. The withdrawal of leptin administration results in an increase in the amount of food consumed and body weight (Chehab *et al.*, 1996), indicating the requirement of leptin for maintaining homeostasis. Hence, leptin administration restores fertility in both sexes and results in ovulation, pregnancy, parturition and lactation in obese Lep^{ob}/Lep^{ob} female mice (Chehab *et al.*, 1996).

1.2.1.2 The diabetic ($LepR^{db}/LepR^{db}$) mouse

A leptin receptor cloned from mouse choroid plexus maps to 6 cM on the $LepR^{db}$ gene on Chromosome 4 (Tartaglia *et al.*, 1995; Chua *et al.*, 1996). The diabetic $LepR^{db}/LepR^{db}$ mouse has an abnormal splice leading to a 106 bp insertion into the 3' end of its *OB-RB* mRNA. This insertion is identical to the C-terminal exon of *OB-RA* and predicts a premature stop codon, changing the amino acid sequence of *OB-RB* to *OB-RA* (Lee *et al.*, 1996). Therefore, the leptin protein in $LepR^{db}/LepR^{db}$ mice is unable to transduce a signal through the long isoform of the receptor or to activate the STAT pathway, indicating that the $LepR^{db}$ leptin receptor mutation is responsible for the inefficient signalling and hence the obese $LepR^{db}/LepR^{db}$ phenotype (Ghilardi *et al.*, 1996). Interestingly, two $LepR^{db}/LepR^{db}$ mutants ($LepR^{db3J}/LepR^{db3J}$ and $LepR^{dbPas}/LepR^{dbPas}$) lack the soluble receptor in addition to the functional leptin receptor (Li *et al.*, 1998).

A human equivalent of the $LepR^{db}/LepR^{db}$ mutation was recognised when the genetics of three severely obese sisters of Kabilian origin were analysed (Clement *et al.*, 1998). The genetic analyses revealed that a homozygous splice site mutation in the *OB-R* gene

results in the loss of the transmembrane and intracellular domains of the leptin receptor (Chapter 1.1.4), giving a receptor without signalling capacity.

1.2.1.3 The Zucker obese (*LepR^fa^/LepR^fa^*) mutant

A mutation in the Zucker obese ($LepR^{fa}/LepR^{fa}$) rat causes the amino acid substitution glutamine to proline at position 269 of the leptin receptor (Chua *et al.*, 1996; Phillips *et al.*, 1996; White *et al.*, 1997). The $LepR^{fa}$ mutation is a mis-sense mutation of the extracellular domains of the *OB-RA* and *OB-RB* with a relative insensitivity to leptin. *OB-RB* is detected in brown adipose tissue of the $LepR^{fa}/LepR^{fa}$ mutant mouse, this is because the $LepR^{fa}$ mutation alters intracellular trafficking, not the expression of the receptor (Siegrist Kaiser *et al.*, 1997; Yamashita *et al.*, 1998). The $LepR^{fa}$ mutation and the $LepR^{db}$ mutation are believed to be homologues of each other as rodents with the mutations have identical behavioural and metabolic phenotypes and because both map to syntenic homologous chromosomal regions (Truett *et al.*, 1991; Chua *et al.*, 1996). The $LepR^{fa}$ mutation results in hyperphagia, weight gain, adiposity, low brown fat thermogenesis and obesity, with associated high levels of insulin and corticosterone (Yarnell *et al.*, 1998; Hufnagel *et al.*, 1999). In agreement, treatment of normal rats with leptin results in an up-regulation of insulin and corticosterone (van Dijk *et al.*, 1997).

The mutant receptor has a 10-fold lower binding of leptin at the cell surface (Chua *et al.*, 1996). The receptor distribution on the cell is not altered by high leptin levels or diminished leptin receptor density in the obese mutants (Chua *et al.*, 1996), indicating a possible defect in leptin-leptin receptor feedback regulation. Transfection of the *OB-RB* gene to $LepR^{fa}/LepR^{fa}$ rat islets and the addition of leptin causes a decrease in the islet fat by 87 per cent and blocks the formation of triglycerides (Wang *et al.*, 1998), indicating that the absence of a functional receptor is the main cause of the obesity

phenotype.

1.2.2 Leptin function in anabolic and catabolic pathways

Energy homeostasis is a complex process that minimises the impact of short-term fluctuations of food intake on fat mass. Integral parts of this system are hormones released in proportion to body adiposity, including leptin, to interact with the central nervous system. The two major systems responsible for energy balance are anabolism, the system that promotes weight gain, and catabolism, the system that promotes weight loss (Figure 1.9). Hormones regulated by adipose tissue inhibit anabolic pathways, but stimulate catabolic pathways (Figure 1.9). For further reading, the reader is directed to a comprehensive review of hypothalmic appetite control and regulation (Kalra *et al.*, 1999).

1.2.2.1 Leptin and anabolic/orexigenic hormones

The orexigenic peptide, neuropeptide Y (NPY), is a protein that plays a dual function in feeding and reproduction (Aubert, 1996; Stephens, 1996). NPY is expressed in the arcuate nucleus of the hypothalamus and its release is increased in various unfavourable metabolic circumstances (Stephens *et al.*, 1995). Central administration of antibodies or antisense oligonucleotides against NPY cause a block in the normal onset of feeding (Stephens, 1996), while an increase in NPY results in an inhibitory effect on the gonadotrophin axis; inhibiting sexual maturation and reproductive function in times of food restriction and/or an increase in energy expenditure (Gruaz *et al.*, 1993; Pierroz *et al.*, 1995; Pierroz *et al.*, 1996).

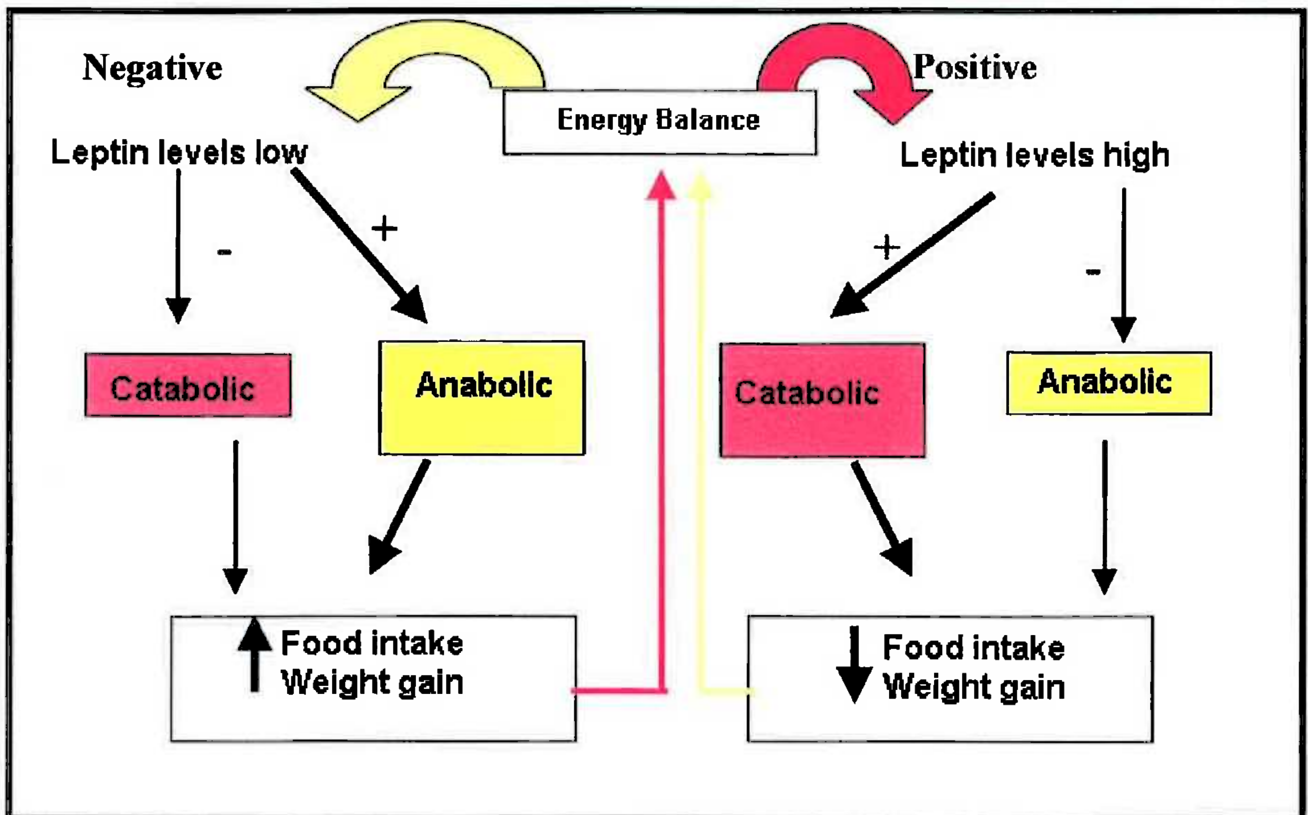


Figure 1.9 The mechanism of body weight regulation. Energy homeostasis is maintained by a combination of anabolic and catabolic pathways. During times of negative energy balance, the adipose tissue contracts and decreases its release of leptin, thereby stimulating the anabolic pathways and suppressing catabolic pathways, in order to increase levels of food intake and stimulate weight gain. In contrast, in times of positive energy balance, the adipose mass expands to release high levels of leptin, thereby stimulating catabolic pathways to reduce food intake and encourage weight loss. Adapted from Woods *et al.*, 1998.

NPY, in synergy with gonadotrophin releasing hormone (GnRH), stimulates the follicle stimulating hormone (FSH) and luteinising hormone (LH) secretory responses *in vivo* (Figure 1.10) (Bauer-Dantoin *et al.*, 1993; Bauer-Dantoin *et al.*, 1993). One of the responses of the pre-ovulatory progesterone surge is an up-regulation of pituitary sensitivity to the actions of NPY on the reproductive system (Bauer-Dantoin *et al.*, 1993; Bauer-Dantoin *et al.*, 1993). The di-oestrus increase in oestrogen and the pro-oestrus surge of progesterone result in an increase in NPY gene expression on the afternoon of pro-oestrus; NPY is able to stimulate GnRH in female mice (Parent *et al.*, 2000), resulting in the stimulus for the pre-ovulatory LH surge. Therefore, an absence of NPY, such as in times of low food availability, would disrupt the oestrous cycle.

Neurons containing NPY mRNA in the arcuate nucleus also express *OB-R* mRNA (Mercer *et al.*, 1996), suggesting that NPY mRNA may be directly responsive to circulating leptin (Figure 1.11). The full-length leptin receptor negatively correlates with NPY expression in the arcuate nucleus (Bennett *et al.*, 1999), a region where leptin has been found to suppress NPY expression directly (Stephens *et al.*, 1995). Leptin-treated NPY-deficient mice have decreased food intake and lose weight (Erickson *et al.*, 1996). In contrast, Y5 NPY receptor deficient mice grow normally while they are young with normal responses to leptin, but develop mild onset obesity with age (Marsh *et al.*, 1998). Therefore, NPY and leptin interact to regulate the reproductive system (Figure 1.10).

Other anabolic peptides, agouti gene-related protein (AGRP), galanin (GAL), orexins and melanin-concentrating hormone (MCH) are important contributors to the anabolic pathway (Figure 1.11). AGRP is a 132 amino acid protein normally expressed in the adrenal and arcuate nucleus (Ollmann *et al.*, 1997). AGRP is 25 per cent identical to agouti signalling protein (ASP), a protein over-expressed in agouti mice, and ASP and

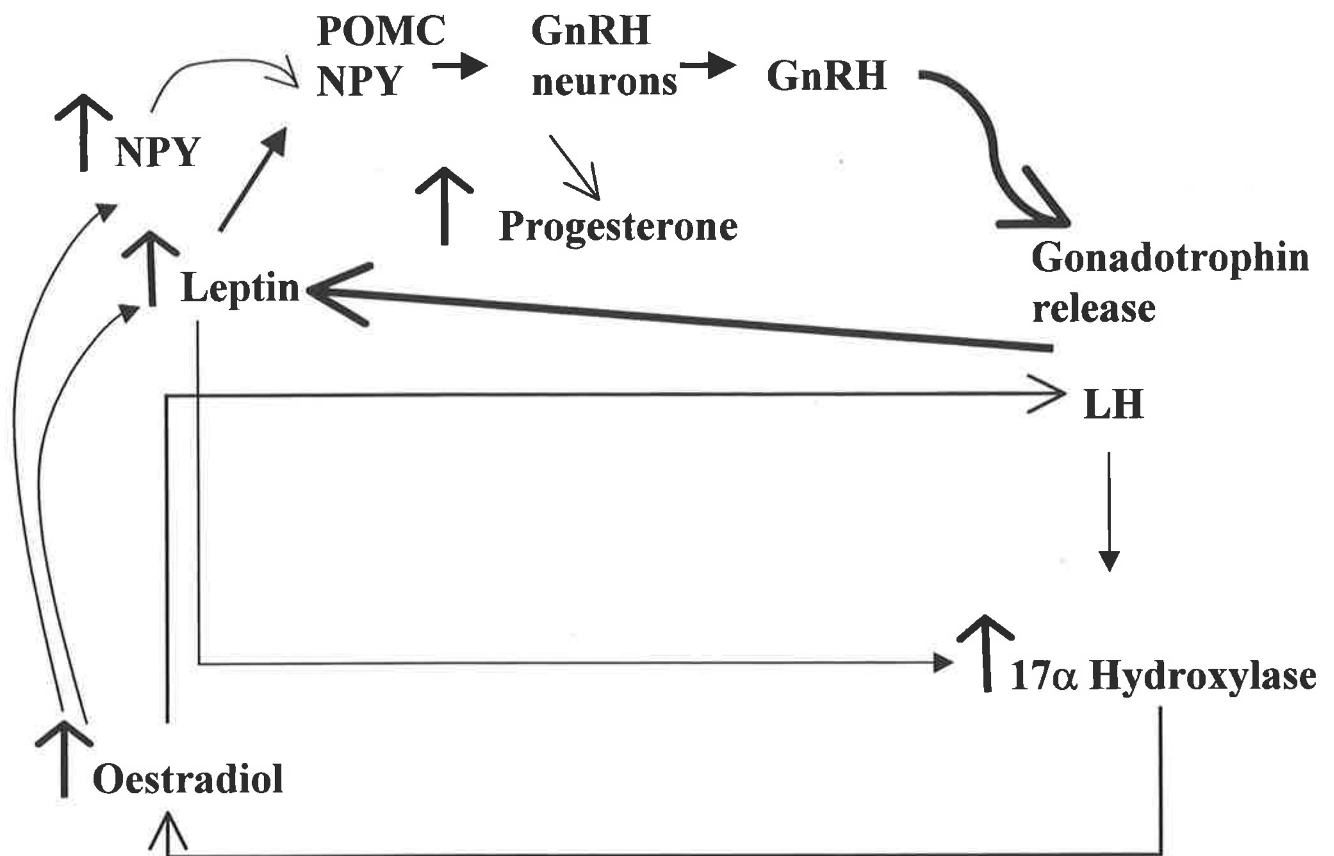


Figure 1.10 A proposed role for neuropeptide Y (NPY) and leptin in stimulating GnRH release. The release of estradiol at di-oestrous and the release of progesterone at pro-oestrous are known to be important hormones in the oestrus cycle. Leptin interacts with steroid hormones and NPY to stimulate the release of GnRH, and therefore has been postulated to have an important role in oestrus cyclicity.

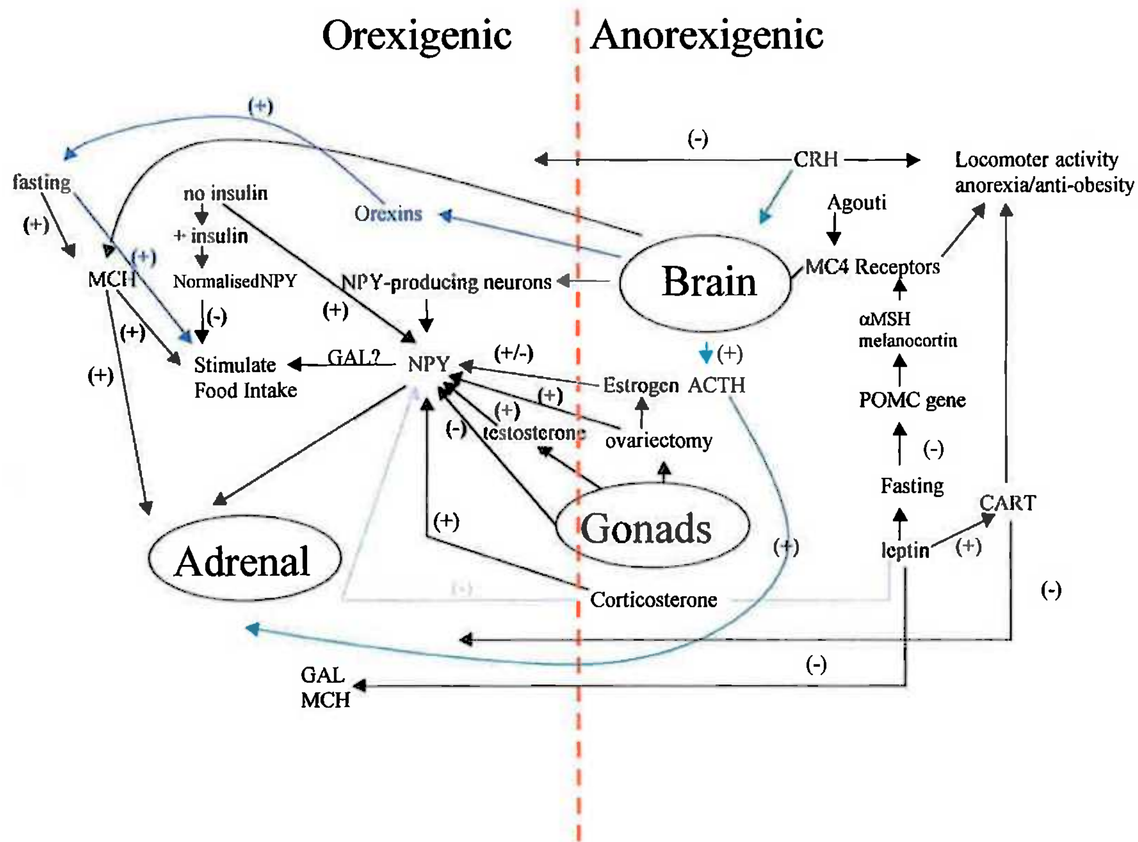


Figure 1.11 Schematic diagram showing the interaction between hormones and neuropeptides in the appetite control pathway. Orexigenic and anorexigenic peptides interact to control food intake. The orexigenic or anabolic peptides, neuropeptide Y (NPY), insulin, galanin (GAL), melanin concentrating hormone (MCH) and orexins interact to bring about an increase in food intake. The anorexigenic or catabolic peptides, corticotrophin release hormone (CRH), melanocortin, αMSH, agouti protein and cocaine and amphetamine-regulated transcript (CART) induce fasting and anti-obesity behaviour. Some of these hormones also interact with the adrenal and gonadal axes. The solid orange line divides the two processes.

AGRP over-expressing mice are obese (Ollmann *et al.*, 1997). AGRP is over-expressed in Lep^{ob}/Lep^{ob} and $LepR^{db}/LepR^{db}$ mice and is an antagonist of MC4 and other melanocortin receptors (Leibel *et al.*, 1997). The melanocortin receptors are known to have roles in energy maintenance with MC4-deficient mice being obese (Houseknecht and Portocarrero, 1998). These receptors are an integral part in the regulation of leptin satiety, as MC4 receptor antagonists block the satiety effects of leptin (Seeley *et al.*, 1997). AGRP is co-expressed with NPY mRNA in the arcuate nucleus and over-expressed in the hypothalamus of leptin-deficient Lep^{ob}/Lep^{ob} mice, resulting in a phenotype of obesity (Ollmann *et al.*, 1997). AGRP increases during low food intake (Ebihara *et al.*, 1999) and intracerebroventricular injections of AGRP cause hyperphagia (Rossi *et al.*, 1998; Ebihara *et al.*, 1999). GAL is an appetite stimulating protein. It has been shown to stimulate feeding in satiated rats (Kyrkouli *et al.*, 1990). Orexins are recently discovered hyperphagic neuropeptides. Expression of these proteins increases with fasting (Peyron *et al.*, 1998). MCH is a functional antagonist of the catabolic protein α-melanocyte-stimulating hormone (Chapter 1.2.2.2). Lep^{ob}/Lep^{ob} mice have high levels of MCH, but with leptin administration, independent of food intake, the expression of MCH mRNA is reduced (Houseknecht and Portocarrero, 1998).

1.2.2.2 Leptin and catabolic/anorexigenic hormones

The catabolic pathways promote negative energy balance by decreasing food intake and encouraging weight loss (Figure 1.11). Hormones such as α-melanocyte-stimulating hormone (α-MSH), corticotropin-releasing hormone (CRH) and cocaine and amphetamine-regulated transcript (CART) may be classified as catabolic hormones. Melanocortins, regulators of glucocorticoid production (Chapter 1.2.3.4), are the products of pro-opiomelanocortin (POMC). POMC-expressing neurons in the

hypothalamus express *OB-RB* and leptin treatment stimulates POMC expression (Thornton *et al.*, 1997). POMC is linked to the regulation of food intake and gonadotrophin secretion, indicating that some effects of leptin and NPY may be signalled through POMC (Cheung *et al.*, 1997; Thornton *et al.*, 1997). Fasting reduces POMC mRNA in the arcuate nucleus and Lep^{ob}/Lep^{ob} mice also have low levels of POMC mRNA, with leptin treatment reversing this (Schwartz *et al.*, 1997). Treatment with CRH reduces food intake and body weight and leptin treatment increases gene expression of CRH (Woods *et al.*, 1998). CART neurons are a target of NPY and leptin (Koylu *et al.*, 1997; Kristensen *et al.*, 1998). CART treatment of rats inhibits the feeding effects of NPY, while treatment with antibodies to CART stimulates feeding behaviour (Kristensen *et al.*, 1998). Lep^{ob}/Lep^{ob} mice have low levels of CART mRNA and these levels are normalised upon treatment with leptin (Kristensen *et al.*, 1998). Leptin requires CART peptide to stimulate the pre-pubertal GnRH pulse generator in female mice hypothalamus, *in vitro* (Lebrethon *et al.*, 2000; Lebrethon *et al.*, 2000). Female mice treated with leptin and CART have an increased GnRH pulse amplitude, regardless of the stage of the oestrous cycle, while immunoneutralising CART reduces GnRH pulse amplitude (Parent *et al.*, 2000). Together, these peptides regulate feeding behaviour and reproduction in conjunction with leptin.

1.2.3 Factors important in leptin regulation

There are several factors involved in the induction and suppression of leptin gene expression. These include feeding, fasting, insulin, glucose, thiazolidinediones and glucocorticoids (Table 1.1).

Table 1.1 The effects of certain agents or actions to induce (+) or suppress (-) leptin expression in rodents or humans. Adapted from Auwerx and Staels, 1998.

Action/Agent	Response	Species
Feeding	+	**Rodent & Human**
Fasting	-	**Rodent & Human**
Glucocorticoids	+	**Rodent & Human**
Insulin	+	**Rodent & Human**
Thiazolidinediones	-	**Rodent**
Cytokines	+	**Rodent**
Obesity	+	**Rodent & Human**

1.2.3.1 Leptin and insulin

The relationship between insulin and leptin is of importance as both hormones are integral parts of the anabolic-catabolic pathway. Insulin secretion, but not that of leptin, is stimulated acutely in response to meals (Sivitz *et al.*, 1996). Functional and short isoforms of the leptin receptors have been located on rat pancreatic β-cells (Kieffer *et al.*, 1996; Islam *et al.*, 1997; Tanizawa *et al.*, 1997; Poitout *et al.*, 1998; Morton *et al.*, 1999), with leptin treatment resulting in an inhibition of glucose stimulated β-cell insulin secretion (Emilsson *et al.*, 1997; Kieffer *et al.*, 1997). Leptin treatment of rat adipocytes *in vitro* results in an impairment of several insulin stimulated actions, including glucose transport, glycogen synthase and lipogenesis (Muller *et al.*, 1997). Isolated rat adipocytes treated with leptin also experience an inhibition of a glucose-stimulated insulin secretion and insulin mRNA production (Kulkarni *et al.*, 1997; Pallett *et al.*, 1997; Tanizawa *et al.*, 1997; Poitout *et al.*, 1998). Despite the inhibitory actions of leptin on pancreatic β-cells and adipocytes, leptin also induces a proliferative response from rat insulinoma-derived pancreatic β-cells (Islam *et al.*, 1997). Both acute and chronic administration of insulin to rodents, *in vivo* and *in vitro*, increases adipose tissue *OB* mRNA (Cusin *et al.*, 1995; Saladin *et al.*, 1995; Slieker *et al.*, 1996; Zheng *et al.*, 1996). However, rats exposed to circulating glucose and insulin have reduced *OB* mRNA in epididymal fat pads (Sivitz *et al.*, 1996).

Islets in the human pancreas possess the long isoform of the leptin receptor (Kulkarni *et al.*, 1997). Leptin treatment of human islets causes an inhibition of insulin secretion in the presence of high levels of glucose (Fehmann *et al.*, 1997; Kulkarni *et al.*, 1997; Seufert *et al.*, 1999). Physiologically, in normal non-diabetic humans, there is a positive correlation between fasting insulin and leptin, in multiple linear regression analyses

(Bloomgarden, 1997), and leptin concentration in humans increases several hours after insulin release (Dagogo Jack *et al.*, 1996). In contrast to rodents, insulin does not acutely regulate leptin mRNA expression or leptin secretion in humans (Vidal *et al.*, 1996). However, chronic insulin secretion (hyperglycaemic clamp experiments) is able to regulate *OB* mRNA expression and leptin secretion and chronic *in vitro* human adipocyte culture (48 h) in the presence of insulin appears to induce *OB* mRNA and leptin synthesis (Kolaczynski *et al.*, 1996). The effect on leptin production indicates that a long duration (chronic treatment) of the clamp or culture may increase lipid stores in adipocytes, thereby increasing leptin synthesis and release. However, the ability of hyperinsulinaemic clamps to induce leptin is controversial as some studies (Dagogo Jack *et al.*, 1996; Kolaczynski *et al.*, 1996; Larsson and Ahren, 1996) have not been able to repeat results of others in the same time periods, indicating that the lag between leptin and insulin may be greater in humans than rodents. This suggests that acute insulin administration does not affect leptin levels while an effect of chronic insulin administration does exist, *in vivo* and *in vitro*.

The differences in the physiology of adipose tissue between rodents and humans may account for some of the differences observed in response to acute and chronic insulin treatment. The rhythmicity of adipose tissue *OB* mRNA in rodents as compared with humans may also account for this variation of leptin secretion. Rodents experience a diurnal variation of leptin secretion, as do humans (Chapter 1.2.5.4), however rodents are nocturnal foragers and their leptin levels are at a maximum at the initiation of eating behaviour (Saladin *et al.*, 1995). In humans, the leptin nocturnal rise does not coincide with feeding (Sinha *et al.*, 1996).

1.2.3.2 Leptin regulation in states of fasting and food intake

Leptin levels do not respond to normal meals, however, they are responsive to fasting and subsequent re-feeding. Fasting decreases and re-feeding or high dietary fat content increases leptin mRNA in rat adipose tissue and protein levels in rodents (Becker *et al.*, 1995; Frederich *et al.*, 1995; Saladin *et al.*, 1995; Masuzaki *et al.*, 1996). Furthermore, a day of overfeeding results in a 40 per cent increase in plasma leptin while body weight is unaffected. An increase in plasma leptin in rodents can be inhibited by fasting, and this inhibition can be reversed upon re-feeding or an indication of re-feeding (such as a single injection of insulin), resulting in an increase in *OB* mRNA, both *in vivo* and in *in vitro* rat adipocyte culture (Saladin *et al.*, 1995). This links to the fact that acute and chronic insulin administration, both *in vivo* and *in vitro*, lead to an increase in adipose tissue *OB* mRNA in rodents (Cusin *et al.*, 1995; Saladin *et al.*, 1995).

The mechanisms that regulate leptin expression in response to feeding are less clear in humans than rodents. In humans, the basal or stimulated leptin levels or leptin gene expression do not change after food intake (Andersen *et al.*, 1997; Korbonits *et al.*, 1997; Niskanen *et al.*, 1997; Joannic *et al.*, 1998), while serum insulin and glucose concentrations increase in a transient manner (Considine *et al.*, 1996). However 6 days of fasting reduces serum leptin by 40 per cent (Andersen *et al.*, 1997). Short term fasts or dieting still result in a basal decline in leptin in both lean and obese subjects and re-feeding restores the leptin levels to basal concentrations (Horn *et al.*, 1996; Sinha *et al.*, 1996; Sinha *et al.*, 1996; Sinha *et al.*, 1996). In humans, plasma leptin levels are not affected by the dietary fat content per se (Havel *et al.*, 1996), but they are in part regulated by insulin in women of normal weight (Carmina *et al.*, 1999). There is a relationship between fasting insulin, leptin and *OB* mRNA in humans (Considine *et al.*, 1996; Hickey *et al.*, 1996; Larsson and Ahren, 1996). Leptin levels are up-regulated in

response to chronic insulin, *in vivo* and *in vitro*, in human adipocytes (Chapter 1.2.3.1) (Andersen *et al.*, 1997). Insulin clamps applied whilst fasting are able to increase serum leptin levels significantly, thus insulin affects leptin levels dependent on nutrition (Andersen *et al.*, 1997). Fasting results in a greater decline in serum leptin levels in lean humans when compared to obese humans (Korbonits *et al.*, 1997) with a significant correlation between insulin and leptin (Boden *et al.*, 1996; Jenkins *et al.*, 1997). However, leptin and insulin decrease during caloric restriction, with the leptin decreasing more, and the insulin decreasing less, in women than in men (Dubuc *et al.*, 1998). The reduction of leptin secretion during fasting inhibits insulin, resulting in impaired glucose uptake and metabolism in adipocytes, and an increase in lipolysis (Dubuc *et al.*, 1998). A 7 day energy restriction results in a significant decline in serum leptin indicating that glucose and lipid metabolism may be involved in leptin regulation since factors that impact on uptake of glucose by adipocytes, such as glucose and insulin, may suppress leptin (Dubuc *et al.*, 1998; Mueller *et al.*, 1998). This indicates that leptin may not be a regulator or suppresser of body fat as initially hypothesised, but instead may be a body fuel stress indicator or a long-term adiposity-related signal.

1.2.3.3 Leptin and glucose metabolism

Leptin appears to be able to regulate glucose metabolism and insulin action. Skeletal muscle, adipose tissue and liver are significant insulin-responsive organs in glucose metabolism and leptin receptors are expressed in each of these organs (Kakuma *et al.*, 2000; Szanto and Kahn, 2000). Rat skeletal muscle treated with glucosamine results in an increase in *OB* gene expression and leptin protein levels (Wang *et al.*, 1998). In *in vivo* experiments, chronic leptin treatment of lean mice results in an impairment of glucose uptake, however, *in vitro* leptin treatment of muscle has a stimulatory effect on insulin action (Harris, 1998). In *vitro* culture of the C2/C12 muscle cell-line with leptin

results in increased glucose uptake and glycogen synthesis (Berti *et al.*, 1997). However, several other studies report that glucose uptake in adipocytes or muscle is not increased with leptin treatment (Muoio *et al.*, 1997; Ranganathan *et al.*, 1998; Zierath *et al.*, 1998). Acute leptin treatment may regulate whole body glucose metabolism centrally, since intraventricular leptin treatment increases glucose utilisation in brown adipose tissue (BAT) (Siegrist Kaiser *et al.*, 1997) and both intraventricular and intracerebroventricular treatment of leptin increases glucose turnover, glucose oxidation and glucose uptake into muscle and BAT (Kamohara *et al.*, 1997). Human hepatic tissue and HepG2 cells possess only the short isoform of the leptin receptor (Cohen *et al.*, 1996), however attenuation of tyrosine phosphorylation of the insulin receptor substrate-1 in human hepatic cells has been reported in response to leptin treatment (Cohen *et al.*, 1996; Szanto and Kahn, 2000).

1.2.3.4 Glucocorticoids and thiazolidinediones

Glucocorticoid response elements exist in both mouse and human leptin gene promoter regions (Gong *et al.*, 1996; De Vos *et al.*, 1998). Glucocorticoids are hormones secreted by the adrenal cortex and are potent regulators of leptin expression in rats and humans (De Vos *et al.*, 1995; Murakami *et al.*, 1995; Slieker *et al.*, 1996; Pralong *et al.*, 1998). They can increase leptin mRNA and protein in rodents, *in vivo* and in rat adipocyte culture (De Vos *et al.*, 1995; Murakami *et al.*, 1995; Slieker *et al.*, 1996; De Vos *et al.*, 1998). Glucocorticoids are endogenous antagonists of leptin and insulin in the control of normal body energy homeostasis (Woods *et al.*, 1998) and induce serum leptin levels despite a reduction in body weight and food consumption in rats and humans (De Vos *et al.*, 1995; Berneis *et al.*, 1996). The presence of the functional leptin receptor in the rat and human adrenal gland indicates the potential for leptin to act directly on adrenal tissue (Cao *et al.*, 1997; Pralong *et al.*, 1998). Leptin treatment of human and rat adrenal

tissue inhibits cortisol secretion, further implicating leptin in modulating the activity of the hypothalmic-pituitary-adrenal axes (Pralong *et al.*, 1998). In the ovary, leptin treatment of rat and human granulosa cells, in the presence of glucocorticoids, inhibits the production of progesterone and progesterone precursors and metabolites (Barkan *et al.*, 1999).

Thiazolidinediones, insulin-sensitising agents, such as troglitazone, bind to and activate the nuclear hormone receptor, peroxisome proliferator activated receptor γ (PPARγ). PPARγ domains are located on the promoter of the *OB* gene (Hollenberg *et al.*, 1997). Thiazolidinediones induce weight gain in rodents through their stimulatory actions on PPARγ, an important initiator in adipocyte differentiation and adipogenesis. The PPARγ activators decrease leptin gene transcription by a direct effect on the leptin promoter, thereby reducing leptin mRNA and protein levels *in vivo* (De Vos *et al.*, 1996; Zhang *et al.*, 1996) and in *in vitro* adipocyte culture (Kallen and Lazar, 1996). The decrease in leptin levels in rodents after thiazolidinedione treatment occurs in spite of an increase in weight and adipose tissue mass and increased food intake (Zhang *et al.*, 1996). PPARγ stimulation favours adipocyte differentiation, decreases leptin levels and increases food intake; all of which promote adipocyte storage of fat (De Vos *et al.*, 1996; Kallen and Lazar, 1996; Hollenberg *et al.*, 1997). In *in vitro* human adipocyte culture, troglitazone decreases leptin mRNA by 40 per cent, although this result is not replicated *in vivo* (Nolan *et al.*, 1996). A recent study has also shown that a genetic variation in PPARγ gene results in varied serum leptin levels (Meirhaeghe *et al.*, 1998). PPARγ mRNA has been localised in granulosa cells in the human ovary, and troglitazone treatment directly inhibited aromatase activity of human granulosa cells (Mu *et al.*, 2000).

1.2.3.5 Leptin and other factors in the anabolic-catabolic system

Some studies indicate that leptin and cortisol interact in a negative feedback loop, where leptin inhibits cortisol synthesis by adrenal cells while cortisol stimulates leptin expression (Bornstein *et al.*, 1997). Another method of leptin control on metabolism may be by stimulating various uncoupling proteins (UCPs). UCPs place breaks in energy production thereby increasing metabolic rate (for a more detailed review of UCPs, see (Gura, 1998)). The expression and activation of the UCPs in rodents is regulated partially by NPY, which also regulates sympathetic stimulation of brown adipose tissue, together resulting in reduced energy expenditure. Leptin increases the specific expression of UCP-1 in BAT and UCP-2 and -3 in peripheral tissues (Scarpace *et al.*, 1997; Zhou *et al.*, 1997; Liu *et al.*, 1998), possibly by inhibiting NPY. In addition, leptin also stimulates fatty acid oxidation in skeletal muscle (Muoio *et al.*, 1997), a potential response of UCP activation or expression.

1.2.4 Obesity in humans

Obesity is an increasingly prevalent and complex health problem throughout the world. It is associated with type II diabetes, hypertension, hyperlipidaemia, female infertility and certain cancers, and therefore a solution for obesity is required urgently. With the genetically obese Lep^{ob}/Lep^{ob} mouse (Chapter 1.2.1.1) as a model for human obesity, it was initially proposed that mutations in the leptin protein in obese humans might be the cause of obesity. However, genetic studies (Maffei *et al.*, 1996) have shown that mutations in the *OB* gene are not the cause of obesity. Leptin molecules from both obese and non-obese subjects are identical in retention time when analysed using HPLC (high pressure liquid chromatography), indicating that there are no major differences

between the protein from the different sources (McGregor *et al.*, 1996). Therefore, the leptin protein is similar in lean and obese humans, except for two extremely rare cases. In the first of these, a homozygous frameshift mutation causes the deletion of a single guanine nucleotide in the leptin gene. In the second, there is a homozygous mis-sense mutation in the leptin gene; both result in little or no functional leptin and give a phenotype of obesity (Campfield *et al.*, 1998). It was then hypothesised that mutations in leptin receptors may be the cause of defective leptin signalling. However, the finding that there are no differences in immunoreactivity of *OB-R* between lean, obese and diabetic subjects (Couce *et al.*, 1997), indicated that receptors are similar regardless of the obesity phenotype. Similarly, four amino acid variants (located in coding exons 2, 4, and 12 - codons 109, 204, 223, 656) of the human leptin receptor are found in both lean and obese subjects, indicating that it is unlikely that leptin receptor mutations cause obesity (Echwald *et al.*, 1997).

Leptin levels correlate to the body mass index (BMI = weight/(height)^2, kg.m^{-2}) in both rodents and humans (Maffei *et al.*, 1995; McGregor *et al.*, 1996). Obese individuals suffer from a phenomenon known as 'leptin resistance', a situation where there is inadequate leptin signalling (Figure 1.12) (Hamilton *et al.*, 1995; Considine *et al.*, 1996). Leptin resistance appears to be the final result of an amalgamation of problems in obese humans. Firstly, the average serum leptin concentration in obese individuals is triple that of normal subjects, with obese human *OB* mRNA being twice that of normal subjects in their subcutaneous adipocytes (Hamilton *et al.*, 1995; Considine *et al.*, 1996; Geldszus *et al.*, 1996). The high levels of leptin being released into circulation appear to decrease the sensitivity of leptin receptors. Secondly, leptin in the circulation of lean individuals is twice as likely to be bound to a leptin receptor (60-98 per cent bound leptin) than in obese individuals (Sinha *et al.*, 1996; Sinha *et al.*, 1996; Sinha *et al.*, 1996). The high levels of free leptin in the circulation of obese individuals allows leptin

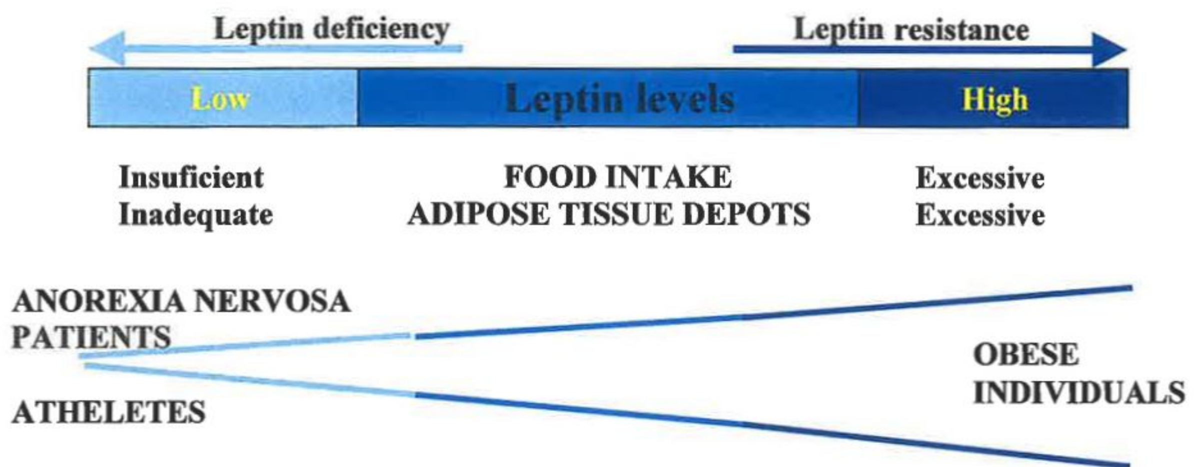


Figure 1.12 Leptin deficiency and resistance in humans. Obese humans tend to have increased energy intake and high leptin levels associated with 'leptin resistance'. Humans with low adiposity tend to experience leptin deficiency.

to bind easily to functional leptin receptors, causing a further state of resistance. Thirdly, free leptin in the circulation of obese humans and rodents is a reflection of the amount of long to short receptor available for leptin binding and also contributes to leptin resistance (Bennett *et al.*, 1998; Roth *et al.*, 1998). Finally, obesity is associated with a decrease in leptin transport across the blood brain barrier in both rats and humans (Burguera *et al.*, 2000), so leptin resistance may be the result of a transport defect at the level of the blood brain barrier (Houseknecht and Portocarrero, 1998). Circulating leptin is transported across the blood brain and blood-CSF barrier to the CNS via a saturable transport system, in order to reach its target, the hypothalamus (Chapter 1.1.5). The efficiency with which leptin enters the brain is reduced with increasing concentrations of leptin (Banks *et al.*, 1996; Caro *et al.*, 1996). Although obese subjects have high levels of serum leptin, the amount of free circulating leptin in the CSF is greatly reduced in comparison to lean subjects (with only 5 per cent free leptin in lean subjects), possibly due to saturation of the leptin-CSF transport system (Stephens and Caro, 1998). Inadequate leptin entering the brain may result in an insufficient control of food intake and energy expenditure. This would lead to an increase in exposure to peripheral tissues while the CNS would only be exposed to moderate/low levels of leptin (Caro *et al.*, 1996; Schwartz *et al.*, 1996).

1.2.4.1 Weight changes in humans

Due to the absence of an appropriate rodent correlate for human obesity, a majority of weight loss studies have been carried out in obese humans.

Baseline leptin levels do not predict weight change or weight loss in moderately obese (Haffner *et al.*, 1996) or normal weight (Niskanen *et al.*, 1997) humans. In fact, changes in leptin levels correlate with changes in body fat mass, more than changes in body

weight (Niskanen *et al.*, 1997). Weight loss as a result of food restriction in obese humans (and mice), regardless of sex, decreases the level of serum leptin (Rosenbaum *et al.*, 1997). In addition, the level of *OB* mRNA content of adipocytes also decreases, but these two levels soon rise to those prior to weight loss when the lowered body weight is maintained (Hamilton *et al.*, 1995; Maffei *et al.*, 1995; Considine *et al.*, 1996; Geldszus *et al.*, 1996). In contrast, a ten per cent increase in body weight results in a 300 per cent rise in serum leptin (Considine *et al.*, 1996).

Studies have assessed the effect of exogenous leptin treatment on weight loss in obese humans. The results from these studies indicate that there is a modest decrease in body weight and body fat with leptin treatment (Heymsfield *et al.*, 1999). Administration of leptin for a short term was beneficial at the beginning of a weight loss program, however this weight loss was not maintainable. Similar studies in rodents have shown that leptin treatment also causes a loss in body weight and fat, which is not repleted until several weeks of treatment termination (Chehab *et al.*, 1996; Chen *et al.*, 1996).

1.2.5 Leptin involvement in biological processes

1.2.5.1 Energy expenditure, thermogenesis and angiogenesis

Leptin treatment can increase energy expenditure, locomoter activity and body temperature in genetically obese (Pellymounter *et al.*, 1995) and normal mice (Luheshi *et al.*, 1999). Exercise in the form of voluntary wheel running reduces leptin levels (Zachwieja *et al.*, 1997) and leptin mRNA (Zheng *et al.*, 1996) in rodents. Leptin levels do not correlate to resting and non-resting energy expenditure in humans in the manner that they do with weight loss and weight gain (Rosenbaum *et al.*, 1997). However leptin levels do correlate with rates of energy expenditure in exercising humans (Tuominen *et al.*, 1997). Exogenous leptin treatment appears to increase sympathetic activity and

alters metabolism by increasing sympathetic nervous system outflow (Haynes *et al.*, 1997), thereby promoting weight loss (Halaas *et al.*, 1995). When leptin is injected into Lep^{ob}/Lep^{ob} mice, an increase in oxygen consumption, locomoter activity and body temperature is observed (Pellymounter *et al.*, 1995). These effects may be mediated in part by mitochondrial uncoupling proteins (UCPs) (Chapter 1.2.3.5) (Liu *et al.*, 1998). UCPs prevent the generation of ATP, so that energy from carbohydrate oxidation is released as heat by brown adipose tissue (Flier and Lowell, 1997; Hossner, 1998). Leptin can therefore affect thermogenesis and the rate of heat loss from brown adipocytes. There is also a strong correlation between leptin levels and the size of subcutaneous fat depots which regulate body heat loss (Caprio *et al.*, 1996). Furthermore, acute exposure of mice to cold suppresses leptin mRNA expression in white adipose tissue (Trayhurn *et al.*, 1995).

Leptin treatment of lean C57BL/6J mice results in an increase in lipid oxidation and adipose tissue vascularity (Sarmiento *et al.*, 1997). Leptin addition to normal rat corneas also results in vigorous neovascularisation (Sierrahonigmann *et al.*, 1998). Leptin treatment of mouse adipose tissue induces new fenestrated blood vessels (Cao *et al.*, 2001). Leptin is able to initiate vascular permeability when mice are treated intradermally and leptin stimulates angiogenesis in synergy with two potent stimulators of angiogenesis, fibroblast growth factor-2 and vascular endothelial growth factor (VEGF) (Bouloumie *et al.*, 1998; Cao *et al.*, 2001). The functional leptin receptor is expressed in human vasculature and in primary cultures of human endothelial cells (Bouloumie *et al.*, 1998; Sierrahonigmann *et al.*, 1998). Leptin secreted into the bloodstream has paracrine effects on local endothelial cells resulting in an increase in fatty acid oxidation and an angiogenic response (Sierrahonigmann *et al.*, 1998). An angiogenic response from leptin may be responsible for the increase in energy expenditure and hence the loss of body weight (Sierrahonigmann *et al.*, 1998). Leptin

treatment of human umbilical venous endothelial cells (HUVECs) and porcine aortic endothelial cells *in vitro*, evokes tyrosine phosphorylation of several endothelial proteins (Bouloumie *et al.*, 1998). HUVECs treated with leptin, proliferate and are positively stimulated by leptin in *in vitro* angiogenesis assays and neovascularisation in an *in vivo* model of angiogenesis (Bouloumie *et al.*, 1998). This implicates leptin action in energy expenditure regulation and angiogenesis.

1.2.5.2 Leptin removal and turnover

Long term fasting does not affect *OB* mRNA expression, but decreases serum leptin levels, therefore factors other than adiposity and total fat mass are important for leptin production and turnover (Andersen *et al.*, 1997). Leptin transcription rate and *OB* mRNA stability appear unchanged even after fasting, but the decrease in leptin after long term fasting may be due to post transcriptional events including decreased translation, increased clearance or decreased half-life (Andersen *et al.*, 1997). Leptin is extracted intact by the kidney, with only minute amounts detected in rat urine (Cumin *et al.*, 1996) and none detected in human urine (Meyer *et al.*, 1997; Garibotto *et al.*, 1998; Wiesholzer *et al.*, 1998; Jensen *et al.*, 1999). Both splanchnic organs and the kidney remove leptin from circulation, while peripheral organs add leptin into circulation (Garibotto *et al.*, 1998). In cases of acute renal failure, elevated leptin levels are observed, which are normalised with peritoneal dialysis (Kim *et al.*, 1999).

Leptin has a half-life of approximately 1.6 hours in the circulation of normal lean rats (Cumin *et al.*, 1996), while a half-life of 9.4 ± 3 minutes has also been reported (Zeng *et al.*, 1997). In humans, leptin has a half life of only 24.9 ± 4.4 minutes (Klein *et al.*, 1996; Gavrilova *et al.*, 1997). Leptin clearance in humans is dependent upon renal plasma flow but not on glomerular filtration rate, with renal plasma clearance being

about 50 per cent of the glomerular filtration rate (Esler *et al.*, 1998; Garibotto *et al.*, 1998). Although leptin is cleared out of circulation by renal filtration, the binding of leptin to the soluble receptor (*OB-RE*) decreases the rate of renal clearance of leptin and hence increases peptide half-life in lean and obese humans (Diamond *et al.*, 1997; Gavrilova *et al.*, 1997). There does not appear to be a difference in the clearance rate between obese and lean humans (Klein *et al.*, 1996).

1.2.5.3 Leptin, hematopoeiesis and the immune system

Hematopoiesis is a process of cell turnover and the responses to pathogenic and non-pathogenic stimuli to replenish and increase blood and immune cells. This process is responsible for the turnover of erythroid, myeloid and lymphoid cell populations through the life-time of an animal. A haematopoietic role for leptin has also been proposed as leptin is expressed in haematopoietic stroma and provides a proliferative signal to haematopoietic stem cell populations (Bennett *et al.*, 1996). In addition, the leptin receptor gene was independently identified as a member of the hemopoietin family in rats and humans (Bennett *et al.*, 1996; Cioffi *et al.*, 1996; Gainsford *et al.*, 1996), indicating a clear role of leptin in hematopoeisis.

The leptin receptor is expressed in murine and human haematopoietic cell lineages and is expressed in low levels in CD4-expressing T cells in $LepR^{db}/LepR^{db}$ mice and humans, indicating that leptin may be involved in lymphopoeisis and macrophage function (Bennett *et al.*, 1996; Gainsford *et al.*, 1996; Ghilardi and Skoda, 1997; Martin-Romero *et al.*, 2000). Normal macrophages express signalling competent leptin receptors, however macrophages from Lep^{ob}/Lep^{ob} mice are abnormal in their phenotype, with abnormalities including having reduced UCP-2 mRNA production (Lee *et al.*, 1999). Leptin is able to enhance phagocytosis and cytokine production in normal

macrophages in response to parasites (Gainsford *et al.*, 1996). Leptin receptors are also expressed on monocytes, lymphocytes and polymorphonuclear cells in human umbilical cord blood (Garcia *et al.*, 1999). Leptin treatment of human monocytes or T-lymphocytes stimulates dose-dependent proliferation of these cells and induces the production of cytokines in the presence of co-stimulators (Santos-Alvarez *et al.*, 1999; Martin-Romero *et al.*, 2000). Leptin has been shown to stimulate erythroid and myeloid development (Mikhail *et al.*, 1997) and acts on human marrow stromal cells to enhance their differentiation (Thomas *et al.*, 1999). Low numbers of leptin receptors are present on early granulocytes and monocytes, with higher numbers present on a subset of lymphocyte-like cells (Gainsford *et al.*, 1996; Garcia *et al.*, 1999). Leptin is able to regulate the proliferation of naïve and memory T cells and can further influence the production of cytokines by these cells (Lord *et al.*, 1998). These *in vitro* studies have demonstrated that leptin has a relationship with the immune system. *In vivo* studies also indicate a role of leptin in a normally functioning immune system. In normal male and female adolescents, the log of serum leptin concentrations correlates with circulating leukocyte count and erythrocyte count (Hirose *et al.*, 1998).

The products of leukocytes in an inflammatory reaction, namely cytokines, are also responsive to leptin. Endotoxin and pro-inflammatory cytokine administration to rodents over-rides the normal reduction in *OB* mRNA and serum leptin due to fasting and instead increases mRNA and leptin protein in conjunction with reduced food intake (Grunfeld *et al.*, 1996; Sarraf *et al.*, 1997). When endotoxin, TNFα or IL-1 are administered into hamsters, *OB* mRNA increases and an increase in body temperature and anorexia are observed (Grunfeld *et al.*, 1996). Increased body temperature and anorexia result in larger adipocyte cells (Hamilton *et al.*, 1995), signalling leptin expression to increase via stretch receptors (Li and Nicklas, 1995). It has also been hypothesised that the effects of leptin on food intake and thermogenesis are mediated by

IL-1 (Luheshi *et al.*, 1999). Many reviews have detailed the involvement of leptin in hematopoiesis (Pighetti *et al.*, 1999; Matarese, 2000).

1.2.5.4 Diurnal variation in circulating leptin

Plasma leptin levels are modulated by both a diurnal variation and food intake in rats (Saladin *et al.*, 1995; Pickavance *et al.*, 1998) and a diurnal variation and sleep in humans (Simon *et al.*, 1998). The diurnal pattern of leptin is independent of the activity of the pituitary-gonadal axis (Kousta *et al.*, 1998; Palmert *et al.*, 1998), but appears to be closely linked with body temperature and melatonin concentrations (Cagnacci *et al.*, 1992; Simon *et al.*, 1998).

There is a linear relationship between daytime and night time serum leptin levels in rodents (Saladin *et al.*, 1995) and humans, with a 3 fold increase in serum leptin between 8pm and 8am (Matkovic *et al.*, 1997; Simon *et al.*, 1998). Leptin is at its lowest in the light cycle, increasing soon after rats start eating at 8pm and reaching a maximum point between midnight and 4am (Saladin *et al.*, 1995). This indicates that rhythmicity of the *OB* mRNA expression is linked directly and entirely to food intake and not time of day in rats as fasting prevents the cyclic variation and decreases *OB* mRNA and re-feeding restores *OB* mRNA to fed animal levels (Saladin *et al.*, 1995). Leptin treated mice eat less food during the dark phase than their ad-libitum fed counterparts (Cheung *et al.*, 1997). Circadian rhythmicity shows that excessive diurnal feeding may contribute adversely to body weight regulation in the obese Zucker rat, as animals fed ad-libitum (as compared with animals fed only at night) gain 23 per cent more weight over 60 days (Mistlberger *et al.*, 1998).

There is a strong correlation between relative total body fat and the average daytime

serum leptin level in humans (Matkovic *et al.*, 1997). Nocturnal leptin concentrations are responsive to changes in body mass, either body fat gain or loss. The change in total body fat over a six month interval is a powerful determinant of increases in the nocturnal leptin concentrations (Matkovic *et al.*, 1997). The diurnal variation of leptin is compromised in humans with anorexia nervosa in response to the decreased food intake (Stoving *et al.*, 1998). The nocturnal rise in leptin in humans is hypothesised to be related to appetite suppression during sleep, and may be linked with the prevention of obesity (Sinha *et al.*, 1996; Sinha *et al.*, 1996; Matkovic *et al.*, 1997; Kousta *et al.*, 1998). Preventing a nocturnal rise in leptin in rodents or humans over a long period of time has implications for the development of obesity, through the inadequate suppression of night-time appetite (Matkovic *et al.*, 1997; Mistlberger *et al.*, 1998). However, the circadian rhythm of leptin is retained in upper-body and lower-body obese individuals even after a decline in leptin resulting from weight loss (Langendonk *et al.*, 1998).

1.3 The female reproductive system

The following section reviews some background information on ovarian biology (Chapter 1.3.1) and the documented involvement of leptin in ovarian compartments and the reproductive system (Chapter 1.3.2).

1.3.1 The ovary: folliculogenesis, luteolysis and ovulation

The ovarian cycle is a product of two distinct phases separated by ovulation: the follicular and luteal phases. The follicular phase (folliculogenesis) stimulates the growth and development of follicles for the development of a meiotically competent oocyte for

ovulation (reviewed by Hillier, 1985). Folliculogenesis involves the proliferation and differentiation of granulosa cells into oestrogen-producing cells with the ability to respond to FSH (reviewed by Hillier, 1985). In return, FSH stimulates the differentiation of granulosa cells by inducing $P450_{arom}$ activity and LH responsiveness (reviewed by Fink, 1988). Due to activation of their LH receptors, theca interna cells transform into theca interstitial cells and production of progesterone and also of androgens increases (reviewed by Findlay, 1991). $P450_{17\alpha}$ (17α-hydroxylase) in the theca converts progesterone to androgens, such as androstenedione, thereby providing granulosa cells with precursors to oestradiol (reviewed by Hillier, 1985). Prior to ovulation, oestradiol increases dramatically, resulting in an increase in LH release (reviewed by Hillier, 1985). The pre-ovulatory LH surge is a stimulus for increased progesterone production and ovulation (reviewed by Fink, 1988). Nearing the time of ovulation, the follicle is surrounded by a collagenous network that strengthens the follicle wall (reviewed by Tsafriri and Dekel, 1994). The LH surge stimulates increased blood flow to the ovary, promoting vascularity and enzymatic degradation of the basal membrane and extra cellular matrix surrounding the pre-ovulatory follicle (reviewed by Tsafriri and Dekel, 1994). In addition, many immune cells, their products and blood-borne factors, also contribute to oocyte release, including leukocytes, collagenases, plasminogen activator and prostaglandins (reviewed by Brannstrom, 1988). Prior to ovulation and following follicular rupture, the follicle transforms into the corpus luteum with theca interna and granulosa cells transforming into luteal cells (reviewed by Tsafriri and Dekel, 1994). Luteal cells provide the large amount of progesterone required for ovulation. The luteal phase (luteolysis) follows for the destruction of the corpus luteum (corpus albicans) when progesterone is no longer required. In the case where pregnancy does not occur, luteolysis signifies the beginning of folliculogenesis again (reviewed by Tsafriri and Dekel, 1994).

In rodents, the oestrous cycle is divided into five main stages, which occur over a total of 4 days: pro-oestrus, oestrus, met-oestrus-I, met-oestrus-II and di-oestrus (Butcher *et al.*, 1974; Kalra and Kalra, 1974). The endocrine milieu of these stages is outlined in Table 1.2. In humans, the menstrual cycle occurs over a time period of 27-34 days and is divided into the follicular and luteal phases.

1.3.2 Leptin and the reproductive system

1.3.2.1 Leptin and leptin receptors in the ovary

Many researchers have investigated the role of leptin in energy homeostasis. However, it is now apparent that leptin also has an important role in reproductive physiology. The leptin protein has been localised in granulosa and theca cells in the rat ovary (Kim *et al.*, 1999) and granulosa cells, cumulus cells and mature oocytes in the human ovary (Antczak *et al.*, 1997; Cioffi *et al.*, 1997). Ovarian *OB* gene expression in the rat is undetectable (Kim *et al.*, 1999), while ovarian *OB* gene expression is controversial in the human ovary as it is detectable by some (Cioffi *et al.*, 1997) and undetectable by other authors (Billig *et al.*, 1997; Karlsson *et al.*, 1997).

The long and short isoforms of the leptin receptor are expressed at high levels in compartments of the ovary. Leptin receptor gene expression has been identified in the rat ovary (Zamorano *et al.*, 1997) using a specific RT-PCR technique that has identified the presence of *OB-RA* in theca cells and *OB-RA* and *OB-RB* in granulosa cells (Zachow *et al.*, 1999). Leptin receptors have been localised in the rat ovary in interstitial cells and ova of preantral follicles (Kim *et al.*, 1999). Leptin receptor mRNA is expressed in human meiotically mature oocytes, cumulus, granulosa, theca and interstitial cells of pre-ovulatory and dominant follicles and throughout antral follicle development (Cioffi *et al.*, 1996; Billig *et al.*, 1997; Cioffi *et al.*, 1997; Karlsson *et al.*, 1997; Agarwal *et al.*,

Table 1.2 The secretion patterns of the steroid hormones, oestradiol and progesterone, and gonadotrophins follicle stimulating hormone (FSH) and luteinising hormone (LH) in the 4-dayoestrouscycle of the rat. Symbols (+ and -) are used to indicate an up-regulation (++) or down-regulation (-) of the circulating levels of hormones at specific times of the oestrous cycle, a single + indicates the basal state (Kalra and Kalra, 1974; Butcher *et al.*, 1974).

	Met-oestrus	Di-oestrus	Pro-oestrus	Oestrus
Progesterone	2300++	1200+	1200+ 1600 until 2300+++	1200+
Oestradiol	0800+ 1200+++ 2300++	0800 until 1800+++	0800+++++ 1400-(due to LH)	+
LH	+	+	1400+ 1600, 1800, 2300 ++	+
FSH	+	+	1500++ 2400+++	1200+

1999). The receptor is detectable on the surface of human metaphase II stage oocytes (Cioffi *et al.*, 1997). Leptin receptors have also been identified on the surface of bovine granulosa (Spicer and Francisco, 1997) and theca cells (Spicer and Francisco, 1998).

1.3.2.2 Leptin interaction with the ovary

In rat and bovine granulosa cells, FSH and insulin or insulin-like growth factor I (IGF-I) stimulate oestradiol and progesterone production in a dose dependent manner (Spicer and Echternkamp, 1995). Insulin stimulates granulosa cell proliferation from small and large bovine follicles (Spicer and Francisco, 1997) and IGF-I works in synergy with FSH to stimulate granulosa cell steroidogenesis of oestradiol in dominant follicles (Zachow and Magoffin, 1997). IGF-I may enhance the ability of granulosa cells to respond to FSH in small follicles, as FSH alone is ineffective and IGF-I deficient mice are infertile (Spicer and Echternkamp, 1995; Zachow and Magoffin, 1997; Accili *et al.*, 1999).

Several lines of evidence indicate that leptin is able to inhibit the positive actions of many hormones important in the selection of dominant follicles (such as IGF-I and TGF-β, FSH and insulin) and steroidogenesis. 1) Treatment of rat and human granulosa cells with leptin (0.1-100 ng/ml) can block the stimulatory effects of IGF-I on FSH induced oestradiol but not progesterone production, without altering the effects of FSH alone (Zachow and Magoffin, 1997; Agarwal *et al.*, 1999). 2) Leptin is able to suppress the stimulatory effects of TGF-β on FSH-dependent oestrone and oestradiol levels in cultured rat granulosa cells (Zachow *et al.*, 1999). Leptin abolishes the otherwise stimulatory effects of FSH and TGF-β on aromatase mRNA expression and decreases aromatase activity (Zachow *et al.*, 1999). 3) Treatment with leptin can suppress FSH

and dexamethasone-induced pregnenolone, progesterone and 20α-hydroxy-4-pregnen-3-one production in rat granulosa cells, a result partly caused by the reduction of an enzyme in the $P450_{scc}$ system (adrenodoxin) (Barkan *et al.*, 1999). 4) In cultured human granulosa cells, leptin (100 ng/ml) inhibits LH (0.1 ng/ml) stimulated oestradiol production (Karlsson *et al.*, 1997). 5) Similarly, leptin (10-300 ng/ml) treatment of small and large bovine follicles inhibits the insulin-induced granulosa cell oestradiol and progesterone production, without affecting granulosa cell proliferation (Spicer and Francisco, 1997). Possibly, leptin may antagonise the stimulatory effects of insulin. Much like the inhibitory actions of leptin on stimulated granulosa cells, it appears that leptin also inhibits the actions of otherwise stimulatory hormones on thecal cells. 6) Leptin may affect rat theca cells, however the presence of the functional leptin receptor on normal rat theca cells has not been reported (Zachow *et al.*, 1999). Hypophysectomised rats do not possess functional leptin receptors on theca cells however an effect of leptin on theca cells of other species has been reported. 7) In particular, leptin treatment of human theca cells inhibits the IGF-I augmentation of LH-induced androstenedione production (Agarwal *et al.*, 1999). 8) Similarly, leptin blocks the insulin augmented progesterone and androstenedione production of bovine thecal cells (Spicer and Francisco, 1998).

In contrast to the inhibitory actions of leptin on granulosa and theca cell steroidogenesis, leptin appears to have no effect on proliferation of bovine granulosa cells (Spicer and Francisco, 1997) while it is able to enhance insulin-induced proliferation of bovine theca cells (Spicer and Francisco, 1998). In addition, leptin also has a stimulatory effect in human luteinised granulosa cells. Leptin stimulates oestrogen production, aromatase mRNA and protein levels, aromatase activity in the presence or absence of FSH and/or IGF-I, without affecting progesterone levels in human luteinised granulosa cells (Kitawaki *et al.*, 1999). Leptin treatment of human luteinised granulosa

cells also inhibits the stimulatory effects of insulin on HCG-induced progesterone production (Brannian *et al.*, 1999).

1.3.2.3 Leptin, gonadal steroids and ovariectomy

Ovariectomy is the surgical removal of the ovaries. Ovariectomy in rats results in an increase in food intake and body weight (Yoneda *et al.*, 1998), a decrease in adipose tissue *OB* gene expression (Shimizu *et al.*, 1997; Bennett *et al.*, 1998; Yoneda *et al.*, 1998; Machinal *et al.*, 1999) but there is no effect on serum leptin levels (Yoneda *et al.*, 1998). Oestradiol administration into ovariectomised female rats reverses the effects of ovariectomy on food intake and body weight (Yoneda *et al.*, 1998; Wu-Peng *et al.*, 1999) and elevates *OB* mRNA in rat adipocytes and *in vivo* (Murakami *et al.*, 1995; Slieker *et al.*, 1996; Shimizu *et al.*, 1997; Bennett *et al.*, 1998; Yoneda *et al.*, 1998; Machinal *et al.*, 1999). Leptin levels in oestradiol-treated ovariectomised rats are controversial, as some studies indicate that leptin levels remain unchanged (Wu-Peng *et al.*, 1999), while another study indicates an increase in leptin secretion (Kristensen *et al.*, 1999). Oestradiol treatment of rat adipocytes, *in vitro,* results in an increase in leptin release into the culture medium (Murakami *et al.*, 1995; Slieker *et al.*, 1996). Therefore, *OB* gene expression appears to be sensitive to oestradiol. This may be partly due to the fact that oestradiol treatment of adipose cells stimulates adipose cell proliferation (Roneari and Van, 1978).

Although sex difference does not influence the expression of the *OB-R* in the brain, transcripts encoding long and short isoforms of the leptin receptor are differentially sensitive to oestrogen (Bennett *et al.*, 1998). Oestradiol treatment decreases *OB-R* gene expression in the arcuate nucleus and ovariectomy increases expression in all areas except the piriform cortex (Bennett *et al.*, 1998).

Testosterone administration into gonadectomised female rats causes an increase in body weight and a decrease in *OB* gene expression, while food intake and serum leptin levels remain unchanged (Wu-Peng *et al.*, 1999). The strong correlation between serum concentrations of leptin and testosterone in male monkeys suggests that the secretion of these two hormones may be causally linked (Urbanski and Pau, 1998). In humans, treatment of male or female adipose tissue *in vitro* with testosterone is unable to affect leptin secretion (Pineiro *et al.*, 1999; Kristensen *et al.*, 2000). However, treatment with dihydrotestosterone (DHT) or dehydroepiandrosterone sulphate (DHEA-S) is controversial, with studies (Kristensen *et al.*, 1999; Kristensen *et al.*, 2000) indicating that leptin secretion is unaffected by androgens in rat or human adipose tissue, while another study claims a decrease in leptin secretion (Pineiro *et al.*, 1999). When human adipose tissue is cultured with stanozolol (a non-aromatisable androgen) or androstenedione, a decrease in leptin secretion is observed (Pineiro *et al.*, 1999). In contrast, when pre-pubertal rats are treated with leptin, there is an increase in testosterone production in the ovary (Cannady *et al.*, 2000). Therefore, while the interactions between leptin and oestrogen have been investigated, the research on leptin, androgens and testosterone is limited. However, the available literature does indicate that leptin is affected by testosterone treatment and vice versa, while the interactions between leptin and other androgens are controversial.

1.3.2.4 Leptin interactions with gonadotrophins and leptin pulsatility

Leptin is able to influence the secretion of gonadotrophins. An absence of leptin in the *Lep*^{ob}/*Lep*^{ob} mouse is associated with low levels of gonadotrophins (Barash *et al.*, 1996). Treatment of *Lep*^{ob}/*Lep*^{ob} mice with leptin increases the secretion of LH in females and FSH in males, while changing aspects of ovarian and uterine histology in *Lep*^{ob}/*Lep*^{ob}

females (Barash *et al.*, 1996). Leptin also stimulates FSH and LH secretion in cultures of rat pituitaries (Yu *et al.*, 1997). Leptin antiserum administration into rat brains, *in vivo* reduced LH pulsatility and stopped oestrous cyclicity (Carro *et al.*, 1997; Yu *et al.*, 1997). Leptin treatment of Lep^{ob}/Lep^{ob} mice restores oestrous cyclicity and ovulation (Chehab *et al.*, 1996; Mounzih *et al.*, 1997). In times of severe food restriction, sexual maturation in the female is inhibited by inhibiting gonadotrophin releasing hormone (GnRH) secretion in rodents and other animals (Aubert, 1996; Ahima *et al.*, 1997). In fasted rats and rhesus macaque monkeys, leptin treatment increases the LH pulses to levels seen in normally fed animals (Finn *et al.*, 1998; Nagatani *et al.*, 1998). Treatment of ovariectomised rats with oestradiol and leptin stimulates the release of LH (Yu *et al.*, 1997; Walczewska *et al.*, 1999). Leptin may therefore protect against the inhibitions of gonadotrophin secretion linked with fasting, although it may not increase the levels of gonadotrophins directly (Barash *et al.*, 1996; Lado-Abeal *et al.*, 1999).

Leptin pulses in humans have been recorded as 32 pulses in a 24 hour period, with pulses lasting approximately 40-45 min in length (Licinio *et al.*, 1998). At night, as leptin levels rise to their peak due to the diurnal variation in leptin secretion (Chapter 1.2.5.4), the pulsatility profiles of LH change and become synchronous with those of leptin (Licinio *et al.*, 1998). LH pulses are fewer, last for a longer time with higher amplitude and larger area during the day (Licinio *et al.*, 1998). At night, leptin and oestradiol exhibit stronger pattern correlations than during the day, thus it has been hypothesised that an oestradiol surge may precede the leptin surge, followed by an increase in pulse amplitude and release of LH (Licinio *et al.*, 1998). In support of the above hypothesis, there is a clear correlation between the release of leptin and LH with a lag of 42-84 minutes in humans (Licinio *et al.*, 1998).

1.3.2.5 Importance of leptin in the oestrous and menstrual cycle

Although the actions of leptin on the reproductive cycle are not clearly understood, it is thought that leptin is an important hormone in the human menstrual cycle. The mechanisms by which leptin interacts with, and influences, gonadotrophin secretion are important (Chapter 1.3.2.3, Chapter 1.3.2.4). In the oestrous cycle, GnRH acts mainly as the trigger for gonadotrophin release. Although leptin receptors have been located on immortalised GnRH-secreting neurons (Magni *et al.*, 1999), it is believed that normal rat GnRH-releasing neurons do not express high levels of the functional leptin receptor (Hakansson *et al.*, 1998). It has therefore been hypothesised that leptin does not directly impact on GnRH. Instead, it is believed that leptin exerts its effects on the gonadotrophin axis by impacting on the hypothalmic peptides, POMC and NPY (Section 1.2.2.1), which in turn, modulate GnRH neurons to affect GnRH release (Figure 1.10) (Cunningham *et al.*, 1999). The di-oestrus increase in oestradiol causes an increase in NPY and is followed by a progesterone surge in pro-oestrus (Freeman, 1988). NPY may enhance GnRH binding to its receptor by increasing NPY gene expression in the arcuate nucleus on the afternoon of pro-oestrus (before the pre-ovulatory LH surge which triggers ovulation) (Bauer-Dantoin *et al.*, 1992; Bauer-Dantoin *et al.*, 1992). In line with these observations, the addition of leptin to rat median eminence-arcuate nuclear explants increases GnRH release and stimulates GnRH secretions (Yu *et al.*, 1997). Both high concentrations of leptin and the absence of leptin cause a decrease in GnRH and gonadotrophin release (Yu *et al.*, 1997).

Plasma leptin levels increase abruptly after the pre-ovulatory LH surge on the day of pro-oestrus (Licinio *et al.*, 1998), and could be used as information for the timing of the oestrous cycle (Roosendaal, 1998). LH stimulates a dose related increase in 17α-hydroxylase mRNA in theca interstitial cells (Magoffin and Weitsman, 1993). Both LH

and oestradiol appear to be partially regulated by leptin action (Chapter 1.3.2.3, Chapter 1.2.3.4), therefore, the LH surge may result in an increase in leptin, possibly affecting oestradiol concentrations by influencing 17α-hydroxylase expression (Magoffin and Weitsman, 1993). In support of this, it has been shown that an injection of leptin into Lep^{ob}/Lep^{ob} mice causes an up-regulation of side chain cleavage and 17α-hydroxylase mRNA levels in the ovary (Zamorano *et al.*, 1997). Since leptin signals metabolic fuel availability to glucose sensitive regions of the brain, which influence GnRH secretion (Yu *et al.*, 1997; Schneider *et al.*, 1998), this may be a mechanism for leptin to act on the cycle.

Circulating leptin levels in women with normal menstrual cycles associate with the menstrual phase, with peak leptin levels recorded during the luteal phase coinciding with maximal progesterone and oestradiol levels (Hardie *et al.*, 1997; Lukaszuk *et al.*, 1998; Messinis *et al.*, 1998). Oestradiol can potentiate leptin release *in vitro* (Slieker *et al.*, 1996), therefore the leptin rise during the luteal phase may be a response to the rise in oestradiol in the peri-ovulatory phase (Hardie *et al.*, 1997). Also, the response to intracerebroventricular leptin and oestradiol benzoate treatment in the ovariectomised rat results in an increase in LH, mediated by GnRH release (Licinio *et al.*, 1998). In other studies, leptin levels are higher in pre-ovulatory and mid-luteal phases rather than in the two mid-follicular phases with cyclic variations of leptin parallel to those of circulating oestradiol (Hardie *et al.*, 1997; Geisthovel *et al.*, 1998; Messinis *et al.*, 1998). The ultradian fluctuations between leptin and both LH and oestradiol show synchrony in humans (Licinio *et al.*, 1998). Women with normal spontaneous menstrual cycles and those treated with FSH have similar leptin levels at the start of the cycle (Messinis *et al.*, 1998). However, leptin levels decline gradually up to day 7 in the spontaneous cycling women and then increase gradually to the day of the LH onset, at which time the leptin levels reach their peak (Messinis *et al.*, 1998). Leptin

concentrations correlate positively with oestradiol concentrations during the second half of the follicular phase in the spontaneous cycles and during the first half in the FSH-treated cycles, while a negative relationship between oestradiol and leptin is present in the first half of the follicular phase (Messinis *et al.*, 1998). Oestradiol and progesterone are believed to participate in the control of leptin production during the human menstrual cycle, as ovariectomised women experience a significant reduction in leptin concentration (Messinis *et al.*, 1999). In Lep^{ob}/Lep^{ob} mice, leptin treatment increases the pulses of LH and FSH frequency (Barash *et al.*, 1996), however unlike humans, leptin levels do not fluctuate across the oestrous cycle of the rodent, the leptin levels remaining relatively constant throughout the mouse and rat oestrous cycle (Chehab *et al.*, 1997; Bennett *et al.*, 1999).

1.4 Developmental roles of leptin in the reproductive system

The following section discusses the involvement of leptin in normal physiological systems. This section discusses the variations in leptin levels in males and females (Chapter 1.4.1) and the developmental role of leptin in the reproductive system and like processes, namely puberty (Chapter 1.4.2), pregnancy (Chapter 1.4.3) and the fetus (Chapter 1.4.4).

1.4.1 Sexual dimorphism of leptin: levels in males and females

A sexual dimorphism of leptin is apparent in humans at all ages but not in rodents, with plasma leptin in male and female rodents being similar even after adjustment for fat mass (Chung *et al.*, 1998). Girls have higher levels of leptin than boys in obese and non-obese cases and a closer relationship between adiposity and serum leptin is evident in

females than males (Lahlou *et al.*, 1997). Androgens suppress plasma leptin levels and this may explain the lower levels of circulating leptin in boys than girls. Leptin expression can be detected in boys in late puberty and adolescence (Chapter 1.4.2) (Blum *et al.*, 1997). This is mimicked to a certain extent in pre- and post-menopausal women, who experience higher leptin concentrations pre-menopause, when there are lower levels of androgens, and lower concentrations of leptin post-menopause, possibly due to the suppressive effect of circulating androgens (Rosenbaum *et al.*, 1996). In elderly subjects, leptin is inversely related to testosterone in men while no correlations are found between leptin and androstenedione in either sex (Janssen *et al.*, 1998). Adipocytes bind androgens and express androgen receptors, thus it is possible that androgens can have direct effects on leptin expression. Serum leptin in hypogonadal men is 3-fold elevated at baseline and normalised with the administration of testosterone (Jockenhovel *et al.*, 1997). The elevated leptin levels in hypogonadal men correlate to the androgen/oestrogen ratio, indicating the major influence of androgens and sex steroids on leptin levels (Jockenhovel *et al.*, 1997). This may also support the anabolic action of testosterone which reduces lean body mass but not adipose tissue (Young *et al.*, 1993; Bhasin *et al.*, 1996).

A sexual dimorphism clearly exists when *OB* gene expression is considered in humans (Rosenbaum *et al.*, 1997). *OB* gene expression in females is 75 per cent above that of males at all levels of body fat and body mass index, with all CSF leptin levels being higher in females than in males (Lonnqvist *et al.*, 1995; Schwartz *et al.*, 1996; Zimmet *et al.*, 1996). If the per cent body fat is greater than 25 per cent, serum leptin levels rise 3.2 times more rapidly in females than in males (Kennedy *et al.*, 1997). This may be attributed to a higher level of leptin transport into or lower level of leptin transport out of the CSF in females (Schwartz *et al.*, 1996). Baseline levels of leptin in females are still 55 per cent above those of males even after weight loss (Chapter 1.2.4.1),

indicating that the topography of fat tissues may influence serum leptin concentration (Niskanen *et al.*, 1997). A difference in fat distribution might also contribute to the gender differences observed, as central/peripheral obesity, the type often observed in males, does not influence the amount of leptin in obese females (Wickelgren, 1998). Oestradiol is not expected to be the factor responsible for the gender difference, as post-menopausal women treated with oestrogen do not experience a change in plasma leptin (Hickey *et al.*, 1998).

1.4.2 Leptin and pubertal maturation

Sexual maturation is dependent upon normal progression of growth in humans, however this does not apply to rats, as growth hormone (GH) deficient rats with a retarded growth pattern still reach sexual maturity (Aubert, 1996). Leptin appears to be a metabolic signal that regulates GH secretion, as leptin antiserum decreases spontaneous GH secretion in otherwise normal rats (Carro *et al.*, 1997). However, reproduction in both human and rat females is dependent upon nutritional status. This follows as rats possess only small fat stores which are vital at the time of conception and are necessary for energy to give birth to a large litter and for lactation (Aubert, 1996). Interestingly, rats fed a high fat diet are fatter, weigh less and have an earlier onset of oestrus than carbohydrate fed rats (reviewed by Frisch, 1980). In female rats, 700 pg/ml is required as a minimal circulating leptin concentration for reproductive function to continue (Kiess *et al.*, 1998). Leptin concentrations in mice with or without vaginal opening are similar (Ahima *et al.*, 1997). Normal pre-pubertal female rats injected with leptin, gain weight at a slower rate than controls due to the thinning effects of leptin, however, the reproductive tracts of the leptin treated rats mature earlier and these rats reproduce earlier (Chehab *et al.*, 1997). Exogenous leptin administration therefore induces an earlier onset of classical pubertal parameters in mice and rats (vaginal opening, oestrus,

cycling, ovarian weight, ovulatory index (corpora lutea/ovarian section), uterine weight and uterine cross-sectional area), while these pubertal parameters are absent in pair-fed (food restricted) controls (Ahima *et al.*, 1997; Chehab *et al.*, 1997; Cheung *et al.*, 1997). When rats are food restricted (70 per cent of ad-libitum feed intake), the delay in sexual maturation is partially reversible with leptin treatment (Cheung *et al.*, 1997). In a similar study (Gruaz *et al.*, 1998), rats were fed 36 per cent of ad-libitum fed rats until day 53 (of the experiment), at which time, leptin or control infusions began. Animals given leptin experience vaginal opening, while control animals remain in an arrested state. In contrast to the studies of Ahima (Ahima *et al.*, 1997) and Chehab (Chehab *et al.*, 1997), a study has reported that these results are not repeatable (Cunningham *et al.*, 1999). Nevertheless, Ahima (Ahima *et al.*, 1997) and Chehab (Chehab *et al.*, 1997) do confirm that leptin may not be the factor required for the initiation of puberty, but rather, it may be necessary during pubertal development. Leptin is the first peripheral hormone to show an acceleration in reproductive maturation in normal rodents. These facts, in addition to the conditions to which rodents are subjected in their normal habitat, indicates that leptin is the signal to indicate an adequate energy source for reproductive actions to continue or begin (Ahima *et al.*, 1997; Cheung *et al.*, 1997; Kiess *et al.*, 1998). The ability of leptin to accelerate puberty onset in immature leptin-overexpressing mice (Yura *et al.*, 2000) and improve reproductive tissue parameters has the potential for improving breeding programs of endangered species (Hossner, 1998) and the improvement of reproductive tissue parameters has applications for infertile obese humans.

In humans, there is evidence that a specific ratio of fat to lean mass is required for progression into puberty and female reproductive ability (reviewed by Frisch, 1980). Body fat distribution is linked with early pubertal endocrine activity. An elevation of the body mass index at age 18 is an adequate predictor of irregular menstrual cycles and

risk for ovulatory infertility, indicating a link between nutritional status and fertility (Rich-Edwards *et al.*, 1994). Likewise, rats fed a high fat diet reach first oestrus earlier than animals fed a low fat diet (Frisch *et al.*, 1975). Body fat mass is negatively related to the rate of pubertal development toward menarche, the increase in adrenal androgen production that shows a hormonal change in puberty (de Ridder *et al.*, 1992). A minimum of 17 per cent of body fat is required for menarche and 22 per cent body fat is required to sustain and maintain regular menses for girls older than 16 years (reviewed by Magoffin and Huang, 1998).

Leptin is bound in the circulation to the soluble leptin receptor (Chapter 1.1.4.3). In humans, the amount of bound leptin is low at birth, high in pre-pubertal years, followed by a fall through puberty and then stable levels are found through adult life (Quinton *et al.*, 1999). The fall in bound leptin is linked with puberty and reflects a decline in soluble leptin receptor, thereby releasing leptin to bind to leptin receptors (Quinton *et al.*, 1999). Boys and girls show a strong correlation between leptin concentration and BMI, and per cent body fat with age and pubertal development (Blum *et al.*, 1997; Ahmed *et al.*, 1999). Similar relationships are observed between both boys and girls in early pubertal stages (Tanner stages 1 & 2) (Blum *et al.*, 1997; Ahmed *et al.*, 1999). Circulating leptin concentrations increase in girls with age but decrease in boys leading into puberty (Tanner stage 2) (Blum *et al.*, 1997; Palmert *et al.*, 1998; Ahmed *et al.*, 1999). A 50 per cent rise in circulating leptin 3-6 months prior to the initial rise in testosterone before puberty occurs in boys. An inverse correlation between plasma leptin and testosterone concentration in boys, explains 10.5 per cent in the variation of leptin levels seen between boys and girls (Blum *et al.*, 1997), while oestrogen has no effect on leptin in an early childhood model (Palmert *et al.*, 1998). Leptin levels in boys then fall during the period of initial rise in testosterone and return to near baseline values following the initiation of puberty (Mantzoros *et al.*, 1997). These values remain

stable for 2 years, despite a constant increase in BMI (Mantzoros *et al.*, 1997). Adolescent girls differ from boys in height and weight at menarche, however, they tend to have similar per cent body fat (reviewed by Frisch, 1994). Therefore, leptin may contribute to the initiation of puberty by indicating the attainment of long-term energy stores required for reproduction (Chehab, 1997), and may be responsible for LH surges due to its diurnal variation (Chapter 1.2.5.4) (Mantzoros *et al.*, 1997). However, leptin does not play a role in initiating adrenarche (Mantzoros *et al.*, 1997).

Leptin levels near and at the time of puberty have also been investigated in primates. Nocturnal leptin levels increase significantly prior to the onset of puberty in male agonadal monkeys (Suter *et al.*, 2000). However, these findings are in contrast to previous research, which indicated that the timing of the onset of puberty in male monkeys is not triggered by rising circulating leptin concentrations (Plant and Durrant, 1997; Urbanski and Pau, 1998).

1.4.3 Leptin levels in pregnancy

High levels of leptin are found in the circulation during pregnancy. It is likely that high serum leptin is maintained due to the requirement for adequate maternal fat stores to maintain pregnancy, thereby further extending the role of leptin in reproduction. Rodents experience an increase in serum leptin levels (30 fold) during pregnancy (Gavrilova *et al.*, 1997), which decrease just prior to parturition (Chien *et al.*, 1997). Although the murine placenta produces high amounts of leptin (Hoggard *et al.*, 1997), the hyperleptinaemia observed during late pregnancy is attributed to the over expression of the soluble leptin receptor (*OB-RE*) by the placenta, which prolongs the stability and prevents the degradation of leptin (Gavrilova *et al.*, 1997). This overproduction of the *OB-RE* is the cause of the increase in leptin rather than increased production of leptin by

the murine placenta or an increase in leptin synthesis by maternal fat (Gavrilova *et al.*, 1997). Leptin resistance (Chapter 1.2.4) may be switched 'on' during pregnancy (Barinaga, 1996) in order to secure abundant food intake throughout the pregnancy. Following pregnancy, the resistance is switched 'off' and normal satiety is restored.

Leptin levels are 2-3 fold higher in pregnant (Cioffi *et al.*, 1997) than non-pregnant women matched for age and BMI (Butte *et al.*, 1997; Masuzaki *et al.*, 1997; Schubring *et al.*, 1997) and are similar to leptin levels in non-pregnant obese women. During pregnancy, plasma leptin increases ten per cent above the levels in non-pregnant women in the first trimester; in the second trimester, they reach 160 per cent of the level of the first trimester and remain high until birth (Hardie *et al.*, 1997; Masuzaki *et al.*, 1997). However, one hour after delivery, the leptin concentration declines and postpartum leptin levels are normalised to values below the first trimester (Hardie *et al.*, 1997; Masuzaki *et al.*, 1997). Between the end of pregnancy and 3 months postpartum, a mean 6 per cent decrease in fat mass is associated with a mean 61 per cent decrease in leptin (possibly partly due to a decline in insulin) returning leptin levels to those prior to pregnancy (Butte *et al.*, 1997; Hartmann *et al.*, 1997; Schubring *et al.*, 1997). In humans, high leptin levels during pregnancy are attributed to leptin overproduction by the human placenta and the over-production of *OB-RE* (Lewandowski *et al.*, 1999). Leptin originates from the placental trophoblasts and amnion cells from the uteri of pregnant women and adipose tissue (Masuzaki *et al.*, 1997). At 8 weeks gestation, leptin is made in human placental chorionic villi, cytotrophoblasts and syncytiotrophoblasts, at levels similar to adipose tissue, and detected in amnion cells and amniotic fluid in second and third trimesters of pregnancy (Barinaga, 1996; Senaris *et al.*, 1997). Hence, leptin in circulation is not only derived in adipose tissue, but is also a placentally derived hormone in humans; it may therefore be important for normal pregnancy (Masuzaki *et al.*, 1997; Senaris *et al.*, 1997; Lepercq *et al.*, 1998). The high levels of

leptin during pregnancy are most likely a mechanism to counter appetite satiating and metabolic effects to facilitate maternal weight gain. As energy expenditure is not correlated to fat free mass and fat mass, other factors besides fat mass must also modulate leptin in reproducing women (Butte *et al.*, 1997).

1.4.4 Leptin in the fetus

Leptin, *OB-R* and *OB-RB* mRNAs are highly expressed in the 14.5 day post-coitus mouse fetus, in fetal cartilage/bone and hair follicles (Hoggard *et al.*, 1997). *OB-R* protein and mRNA is also localised in the lung, the leptomeninges and choroid plexus (Hoggard *et al.*, 1997). Therefore, leptin may have roles in fetal development. Leptin levels in human amniotic fluid are derived from the mother and correlate with levels in maternal serum but not with fetal blood (Schubring *et al.*, 1997). Leptin concentrations (6 hours after birth) are related to gestational age and adipose tissue in infants; however, 48 hours after birth, leptin levels in all infants are similar (Harigaya *et al.*, 1997). The umbilical cord serum leptin concentration derived from fetal or placental tissues correlate to the amount of adipose tissue in new-born human infants, but not the adiposity or the serum leptin concentration in the mother (Harigaya *et al.*, 1997; Hartmann *et al.*, 1997; Schubring *et al.*, 1997). The correlation of leptin to fat stores at such an early age suggests that leptin may be important in the growth and development of the fetus and the infant and that there may be a feedback loop between the supply of leptin and adipose tissue status during gestation.

1.5 Body weight and reproductive dysfunction

A link between nutritional status and fertility is evident in undernourished and obese

females. Starvation stops oestrous cyclicity in rodents (reviewed by Frisch, 1980). Leptin treatment of starved female mice results in the resumption of oestrous cycles (Chehab *et al.*, 1996). Humans with anorexia nervosa exhibit a linear relationship between body fat and leptin, indicating that the correlation of leptin with body fat is maintained even in extreme undernutritional circumstances (Grinspoon *et al.*, 1996; Stoving *et al.*, 1998). A loss of body weight below the ideal (loss of 1/3 body fat), or excessive fatness, results in amenorrhea (reviewed by Frisch, 1980). In non-obese subjects, plasma leptin correlates with the amount of plasma sex steroids: DHEAS, oestradiol and testosterone and the amount of body fat (Paolisso *et al.*, 1998). Much like obese patients, anorexia patients have a disruption in LH release, pulsatility and starvation induced GnRH release with low vaginal maturation scores (reviewed by Frisch, 1980). However, serum oestradiol levels are low and do not correlate with leptin levels and are consistent with serum leptin levels seen in early follicular stages (Grinspoon *et al.*, 1996). Upon refeeding, vaginal maturation scores improve and LH pulsatility and GnRH release are restored, but the resumption of the menstrual cycle requires a minimum of 90 per cent of the ideal body weight (reviewed by Frisch, 1980) to increase leptin levels and normalise reproductive endocrine function (Kennedy *et al.*, 1997).

A suitable correlate or model of human obesity has not been found in rodents, however, the link between obesity and ovarian dysfunction has been well established in humans (Rich-Edwards *et al.*, 1994; Clark *et al.*, 1995). Much work has been done on the quantification of leptin in obese individuals. The high levels of leptin in the circulation of obese humans indicate a potential inhibitory role of leptin in ovulation. Body fat distribution is known to relate to androgen/oestrogen balance and fat tissue is an important steroid hormone site with high levels of aromatase and 17β-hydroxysteroid dehydrogenase activity (reviewed by Frisch, 1980; Pasquali, 1989 #998]. Women that

are obese tend to have menstrual irregularities along with high testosterone, insulin and leptin levels (Weiss *et al.*, 1994; Geldszus *et al.*, 1996). High levels of insulin promote androgen production in ovarian tissue causing anovulation (Clark *et al.*, 1995) and obese infertile women have an approximate 30 per cent higher plasma androstenedione and testosterone compared with normal women (Bates and Whitworth, 1982). An increase in androstenedione results in an increase in oestrone, due to aromatase activity. Oestrone in turn may cause increased gonadotrophin releasing hormone pulsatility. This would result in an increase in LH, due to the increase in 17β-hydroxysteroid-dehydrogenase from excess androstenedione in adipose tissue or ovaries. Together, this endocrine environment may lead to anovulation (Unzer *et al.*, 1995).

Weight loss in obese, infertile women results in the lowering of serum leptin and the normalisation of plasma androstenedione and testosterone, a response which promotes an improvement in reproductive endocrine parameters (including LH and FSH concentrations), ovulation and conception (Bates and Whitworth, 1982; Pasquali *et al.*, 1989; Guzick *et al.*, 1994; Clark *et al.*, 1995). Thus, weight loss tends to increase oestrogens and decrease plasma testosterone, glucose stimulated insulin and progesterone without large changes in plasma oestradiol (Pasquali *et al.*, 1989). For the present study it was recognised as important to investigate the connection between high leptin levels and ovulation inhibition. It was also regarded as important to investigate the relationship between leptin and the ovary throughout the oestrous cycle in order to understand the role of leptin in normal ovarian processes, to understand the sensitivity of the leptin-leptin receptor relationship throughout a normal reproductive cycle.

1.6 Hypothesis and aims of the present study

1.6.1 Hypothesis

High leptin levels inhibit ovulation and ovarian follicular development. Endocrine parameters across the oestrous cycle alter the ovarian sensitivity to leptin.

1.6.2 Aims

At the onset of this study, the effects of acute leptin administration on ovulation and the ovary were undefined. The administration of active recombinant leptin *in vivo* and *in vitro* provides a means to establish the effects of acute leptin treatment on the rat ovary. The aims of this thesis are:

1. To investigate the effects of acute leptin administration on the ovulation process, *in vivo* and *in vitro*.
2. To investigate the effects of leptin on ovarian follicles and ovarian leukocyte biology at the time of ovulation, using histopathology and immunohistochemistry.
3. To investigate the effects of acute leptin administration on proliferation and development of ovarian compartments *in vitro*.
4. To investigate the effects of physiological levels of leptin on the expression of the functional leptin receptor, throughout the oestrous cycle.

Chapter 2

Materials and methods

2.1 General

2.1.1 Chemicals and solutions

All chemicals were of analytical grade and, unless otherwise stated, were purchased from Ajax Chemicals, BDH Laboratory Supplies or Sigma Chemical Company. TCM 199 media was purchased from Gibco. Buffers and solutions were prepared using milli Q water or milli U water (Millipore Corporation); mains water purified by reverse osmosis followed by deionisation, resulting in resistivity > 18.2 MΩ.cm. Saline (0.9 % NaCl) was purchased from Baxter Healthcare. The recipes, sterilisation and storage for all solutions, buffers and utensils are described in Appendix 1, along with some technical procedures. The product title is listed throughout the text and the company from which it was purchased is listed in parentheses (and where applicable, model number) after the product in the text. The company addresses (including state and country) are listed in alphabetical order in Appendix 2.

2.1.2 Biological materials

Recombinant human leptin was produced using a previously described protocol (Appendix 1) (Chehab *et al.*, 1996) and kindly provided by Dr Denis Magoffin for *in vivo* experimentation or purchased from DSL scientific for *in vitro* studies. Human insulin (Actrapid) was purchased from Nova, while IGF-I was purchased from Gro*Pep* Pty Ltd. and recombinant human FSH was donated by Organon. Both Equine chorionic gonadotrophin (eCG-Folligon®) and human chorionic gonadotrophin (hCG-Pregnyl®) were purchased from Intervet and Organon, respectively. BSA (fraction V) was

purchased from Boehringer Mannheim, unless stated otherwise. Ovine LH (oLH-26no. AFP-5551B, specific activity of 2.3 U/mg) was kindly provided by NIADDK. Penicillin and streptomycin sulfate were purchased from CSL Biosciences. Androstenedione, gentamycin and 3-isobutyl-1-methyl xanthine (IBMX) were purchased from Sigma-Aldrich.

2.2 General procedures and protocol

2.2.1 Rats

Female Sprague-Dawley rats (*Rattus rattus*) were purchased from The University of Adelaide Animal House and housed in a viral free holding facility at The Queen Elizabeth Hospital Animal House. Dry food (Ridley Agriproducts, formula 8215) and 0.015 % hydrochloric acid, (UV)-treated mains water were supplied *ad libitum* (unless otherwise stated) with a 14 hour light and 10 hour dark cycle maintained within the enclosure. All rats were virgins prior to the onset of any experiment. All experiments were approved by The University of Adelaide Animal Ethics Committee and The Queen Elizabeth Hospital Animal Ethics Committee and were performed under the ethics approval numbers M/42/98 and N-15-98.

2.2.2 Anaesthetics

Animals were anaesthetised using two procedures. If the outcome of the surgical procedure was not death, an inspired gas mixture of 3 % halothane (Zeneca) in nitrous oxide and oxygen dispensed through a Midget-3 Anaesthetic Apparatus from CIG,

followed by 1 % halothane in nitrous oxide and oxygen for anaesthetic maintenance was used. Following the surgical procedure, animals were placed under a heat lamp until recovery. If the animals were to be sacrificed immediately after the surgical procedure, a combination (1:1) of the anaesthetic Ketamil (100 mg/ml ketamine) (Troy Laboratories Pty Ltd) and the muscle relaxant Rompun (20mg/ml xylazine) (Bayer AG) was used and the animals sacrificed using cervical dislocation.

2.2.3 Gonadotrophin priming in the immature rat

In experiments requiring timed follicular development without ovulation, immature rats (aged 25-27 days) were primed subcutaneously at 1200 h with 5 or 10 IU eCG for 46 hours (Figure 2.1A). The eCG is a preparation with predominantly FSH activity, to induce synchronous follicle development. In experiments requiring timed follicular development with ovulation as the final result (Chapter 2.2.4), immature rats (aged 21-28 days) were primed subcutaneously at 0900 h by infusion of 8 IU rhFSH via Alzet® mini-osmotic pumps (Alza Corporation) releasing 0.5 µl per hour (Figure 2.1B). The minipumps were implanted under anaesthesia (Chapter 2.2.2) (Armstrong and Opavsky, 1988; van Cappellen *et al.*, 1995), the skin was closed with sterile autoclip® surgical wound clips (Becton-Dickinson) and the rats were placed under a heat lamp until normal activity resumed. The FSH dose used was based on preliminary dose-response studies in which concentrations of 4, 8 and 12 IU FSH were used to stimulate follicle growth. 8 IU FSH administered over the course of the experiment produced a mild super-ovulatory response (approximately 14 ovulations per ovary) with ovulation rates only slightly greater than those of naturally cyclic adult rats of this strain (Armstrong and Opavsky,

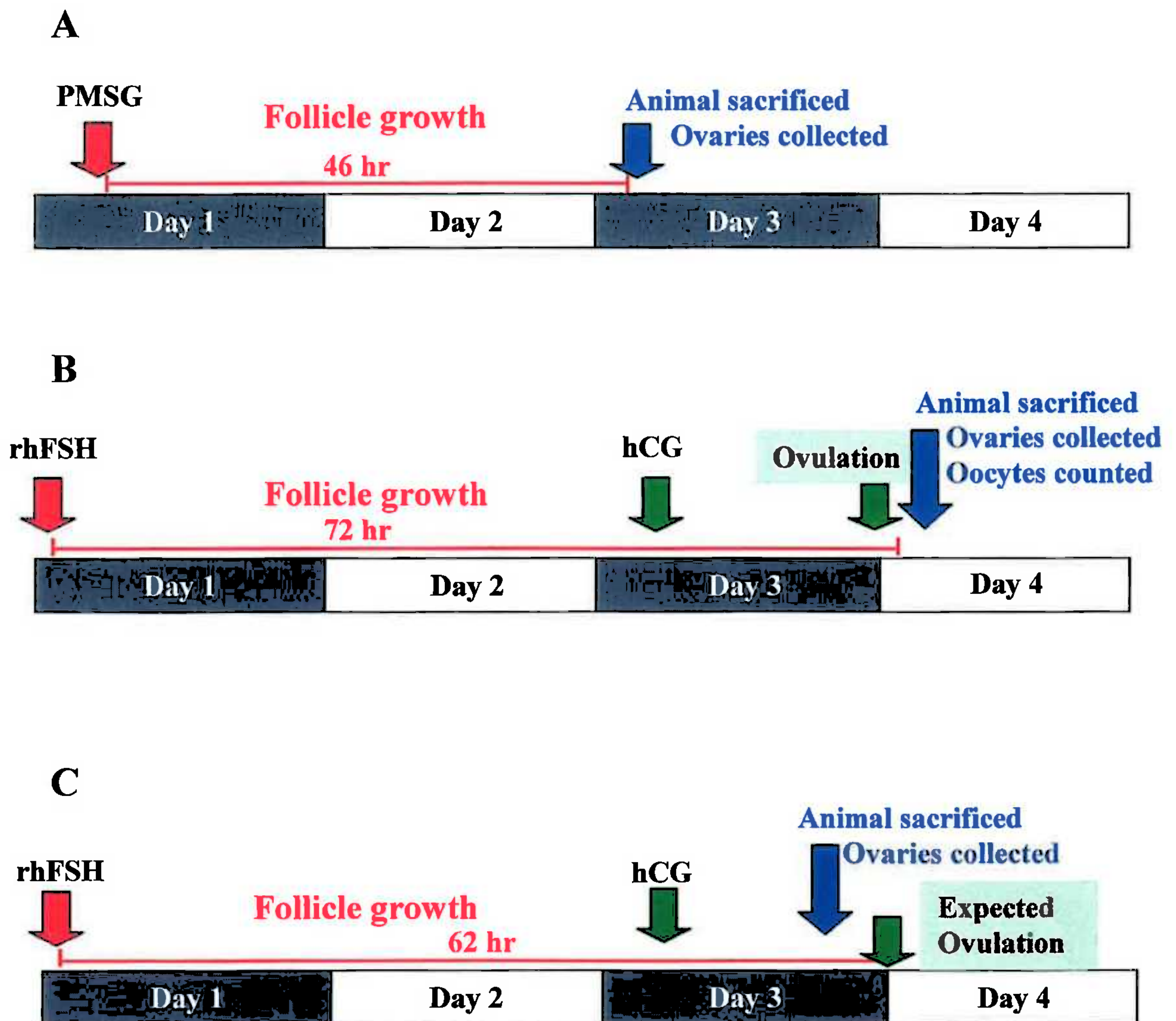


Figure 2.1 Priming protocols for follicular growth and synchronous ovulation in immature rats. A) In experiments requiring follicle development, immature rats were primed with 5 or 10 IU PMSG subcutaneously. Animals were sacrificed 46 hours post PMSG administration. B) In experiments where rats were required to ovulate, minipumps containing 8 IU rhFSH were implanted subcutaneously at 0900 h on day 1. 10 IU hCG was administered ip 50 hours post minipump insertion to initiate ovulation, which occurred approximately 12 hours post hCG. C) In experiments where tissue collection was required prior to ovulation, the procedure of (B) was followed except animals were sacrificed at 2300 h on the day of ovulation.

1988; van Cappellen *et al.*, 1995), 4 IU and 12 IU FSH resulted in approximately 7 and 24 ovulations, respectively.

2.2.4 Synchronised ovulation in the immature rat

Ovulation was induced using 10 IU human chorionic gonadotrophin (hCG), injected intra-peritoneally approximately 50 hours from the time of rhFSH administration (Runner and Gates, 1954) (Figure 2.1B). Ovulation occurred approximately 12 hours after hCG treatment.

2.2.5 Oocyte collection and counting

On the morning following ovulation, animals were sacrificed using ketamine/xylazine anaesthesia (Chapter 2.2.2) and cervical dislocation. The oocytes were collected according to the method described by Hogan *et al* (1986). Briefly, the ampulla region was isolated from the ovarian tissue and dissected free of the uterus and fat (in a petri dish containing saline) with the aid of a dissecting microscope (Wild, M3Z). Oocytes were released from the swollen ampulla of the oviduct by tearing the oviduct using a 30 gauge needle (Becton Dickinson). The ovulated oocytes were removed and counted from both left and right ovaries.

2.2.6 Tissue collection for histological analyses and storage

For tissue collection post-ovulation, animals were sacrificed 21 h following hCG administration (Figure 2.1B). For tissue collection prior to ovulation, animals were sacrificed at 2300 h on the day of ovulation (Figure 2.1C). Ovaries were weighed and frozen in Jung tissue freezing media (Leica Instruments) using isopentane (BDH) and liquid nitrogen and stored at –80°C.

2.2.7 Serum and plasma collection

Blood was collected by cardiac puncture on rats following anaesthesia (Chapter 2.2.2). A 26 gauge needle (Becton Dickinson) was inserted into the heart and blood collected in a syringe. Blood was transferred to an EDTA coated tube (Terumo Corporation, T-MQ Capiject) or a lithium heparin separator tube (Vacuette Greiner Labortechnik) and centrifuged (Eppendorf, 5415C) at 13 000 g for 10 minutes at 4°C. The upper clear layer (serum) was stored at –80°C prior to assaying. Normal rat serum required for immunohistochemical procedures (Chapter 2.3.3) was extracted in the same manner.

2.2.8 Oestrous cycle tracking by vaginal smear

Oestrous cycles of 33-35 day-old female Sprague Dawley rats, weighing 80 g, were followed by daily vaginal smearing between 0900 - 1100 hr. The rats were housed 4 per cage (45 x 30 cm^2). The vagina was flushed twice with approximately 30 µl sterile saline and the sample was viewed as an unstained wet preparation on a microscope slide

(Menzel) with a coverslip (Menzel) under an inverted Olympus microscope (Olympus Optical Co Ltd, CK2). The stage of oestrus was determined according to Bronson (1966) by two analysers, one an independent observer using a blind protocol (Table 2.1).

2.3 Immunohistochemistry

2.3.1 Tissue collection and sectioning

Fresh tissue was collected from rats and frozen (Chapter 2.2.6) prior to being serially sectioned (6 μm thick) using a Leica cryostat (Leica Instruments). Sections were collected every 130 μm on Superfrost® microscope slides (Menzel). The sections were air dried for 4 hours prior to being stored in desiccant at –80°C, for a maximum of 3 months.

2.3.2 Immunoreactive antibodies

Two monoclonal antibodies were purchased from commercial companies. The mouse anti-rat MCA149A and MCA341 (ED1) primary antibodies (Serotec) recognise antigens on the surface of neutrophilic granulocytes and monocytes/macrophages, respectively. The signal was amplified using a secondary sheep-anti-mouse Ig horse-radish peroxidase linked fragment (Amersham).

Table 2.1 Rat oestrous cycle. Vaginal smear content was viewed under an inverted microscope and the proportions of epithelial, cornified and leukocyte cells were used to determine the stage of the cycle. Adapted from Bronson *et al.*, 1966 and Kalra and Kalra, 1974.

Stage	Smear	Activity in Reproductive Tissues
Pro-oestrus	**E / ECL / EC**	LH surge, Oestrogen & Progesterone max
Oestrus	**EC / C+**	Ovulation occurs
Met-oestrus I	**C++**	Tissue remodelling begins
Met-oestrus II	**EC++L++**	Corpus Luteum Regressing, high progesterone
Di-oestrus	**EL, + mucous**	Quiescence, leading into new follicle growth in Pro-oestrus

E = epithelial cells, C = cornified epithelial cells, L = leukocytes, + indicates many cells

2.3.3 Immunohistochemistry protocol

Fresh frozen sections (Chapter 2.3.1) were air dried overnight or for a minimum of 4 hours prior to being rinsed 3 times for 5 minutes in cold PBS (Gibco BRL, Dulbecco). Slides were fixed in acetone (Rhone-Poulenc Lab Products) for 10 minutes at 4°C. The tissue section was circled with a PAP grease pen (Dako Corporation, S 2002) to make a hydrophobic barrier between the section and the remainder of the slide. The slides were then dipped in 1 % BSA (Sigma Chemical Co., A-7030) in PBS (PBS/BSA) to reduce non-specific binding. All incubations were performed in a humidified chamber at 4°C. Primary antibody was diluted in PBS-BSA in the presence of 10 % normal rat serum (PBS/BSA/NRS). Sections were incubated in the diluted primary antibody (1/800 dilution for MCA149A and 1/400 dilution for MCA341) mixture for a minimum of 18 hours. Slides were then rinsed in PBS and then dipped in PBS/BSA prior to a 3 hour incubation with the secondary antibody (diluted 1/200 in PBS/BSA/NRS). Bound antibody was visualised by incubating the slides with 0.22 μm filtered Sigma Fast™ diaminobenzidine (DAB) (Sigma Chemical Co.) for 10 minutes. Slides were rinsed in PBS to stop the DAB reaction and counter-stained with haematoxylin (Chapter 2.3.5). DAB was neutralised in 4 % sodium hypochlorite solution (Ajax Chemicals, 484) prior to disposal. Positive control tissue (liver, lung) and negative controls (where primary antibody incubation was omitted) were included for qualitative comparisons.

2.3.4 Haematoxylin and eosin stain

For simple morphological studies, tissue sections were stained with haematoxylin (Surgipath Medical Industries) and eosin (Sigma Diagnostics). Sections were incubated for 60 seconds in haematoxylin, then rinsed in milli-U water, acid-alcohol dipped (0.5 % HCl/70% ethanol) for 5 seconds and then re-rinsed in fresh milli-U water. Blue counterstain was enhanced by washing slides in tap water (alkaline and rich in calcium) for 2 minutes. Slides were dipped in eosin for 10 seconds and dehydrated for 5 minutes in 95 % ethanol and followed by 2 sequential washes of absolute alcohol. The slides were cleared by washing twice in xylene substitute (Shandon Scientific, 99900506) for 5 minutes, air dried overnight, mounted in DPX mountant (BDH Laboratory Supplies, 36029 4H) and covered with coverslips (Menzel).

2.3.5 Haematoxylin counterstain

For immunohistochemically stained slides, tissue sections were counter-stained with haematoxylin prior to dehydration, clearing and mounting as described in Chapter 2.3.4.

2.3.6 Follicle size determination & quantification of DAB stain by video image analysis

To determine the size of follicles and to measure the level of antibody binding, a video image analysis system was used. The system consisted of an Olympus BH-2 microscope bearing a Panasonic wv-CP610/A colour video camera (Olympus Optical Co. Ltd.)

connected to an IBM-compatible computer with Video Pro 32 software (Leading Edge). To determine the size of follicles, tissues were stained with haematoxylin and eosin (Chapter 2.3.4) and a line was drawn to make a diameter through the centre of the follicle. The numbers of follicles in each of the five antral follicle classes were counted, as per Osman (1985). Briefly, antral follicles were classified on the basis of mean diameters and placed into one of the five following classes: 275-350 μm (class 1); 351-400 μm (class 2); 401-450 μm (class 3); 451-575 μm (class 4); or ≥576 μm (class 5). Class 5 follicles are pre-ovulatory follicles and are destined to ovulate. To quantify DAB staining, the microscope, video camera and light intensity were adjusted and calibrated at the beginning of each session. Two observers using a blind protocol estimated the density of neutrophils and monocytes/macrophages in the theca of pre-ovulatory follicles and stroma of the ovary. The density of the leukocytes in the thecal layer of pre-ovulatory follicles was determined using the video image analysis (VIA) software set up as described in Petrovska (1996). This system determines the positive DAB (brown) stained cells against the total (haematoxylin + DAB (blue + brown)) stain and expresses this value as a percentage. Thecal data is expressed as the average of 3 random thecal areas per pre-ovulatory follicle. Stroma data is expressed as the sum of 6 random stromal areas per ovarian section.

2.4 Leptin treatment of gonadotrophin-primed immature rats

2.4.1 Gonadotrophin priming

Follicle growth was induced in immature rats using rhFSH via Alzet® mini-osmotic pumps (Chapter 2.2.3) or saline (control) on experimental day 1 (Figure 2.2A).

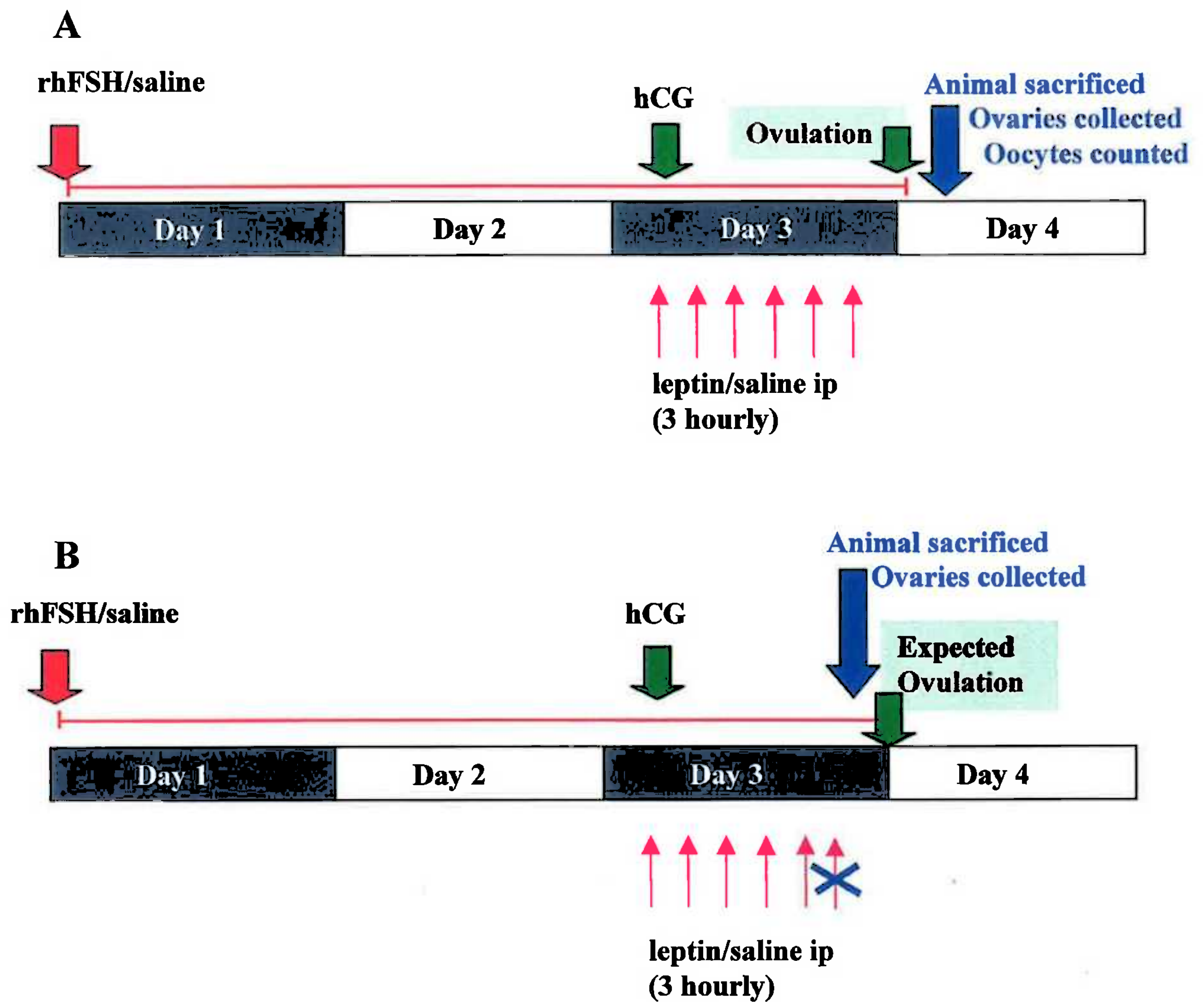


Figure 2.2 Protocol for leptin treatment in immature gonadotrophin-primed rats. Follicle growth was induced in immature rats using rhFSH as described in Figure 2.1. In one group, saline was inserted in place of rhFSH, for control purposes. On experimental day 3, at 0800 h, rhFSH treated and untreated (control) rats were injected ip with either leptin or saline, and thereafter at 3 hourly intervals. At 1200 h on day 3, 10 IU hCG was administered ip to all groups to induce ovulation. A) Animals were injected for a total of 15 hours. B) The final leptin/saline injection was omitted to obtain pre-ovulatory ovaries. Animals were only injected for a total of 12 hours.

2.4.2 Leptin treatment protocol

On experimental day 3 at 0800 h, rhFSH stimulated and unstimulated rats were injected intraperitoneally with either leptin (30 μg in 200 μl) or 200 μl saline only, and thereafter at 3 hourly intervals for 15 hours (Figure 2.2A). There were a total of 5 groups of animals: 1) FSH/saline - in which animals received an 8 IU FSH minipump and saline injections on day 3. 2) FSH/leptin - in which animals received an 8 IU FSH minipump and leptin injections on day 3. 3) saline/saline - in which animals received a saline minipump and saline injections on day 3. 4) saline/leptin - in which animals received a saline minipump and leptin injections on day 3 and 5) pair fed - in which animals were treated with FSH/saline as in group 1 but were fed the same amount of food as that consumed by the FSH/leptin treated animals (group 2). At 1200 h on day 3, 10 IU hCG was administered intraperitoneally to all groups to induce ovulation. In all groups, body weight and food consumption were measured daily. The dose of leptin administered was based on studies by others (Barash *et al.*, 1996; Chehab *et al.*, 1996; Mounzih *et al.*, 1997; Cheung *et al.*, 1999) that used similar concentrations of leptin. The final dose used over the course of the day was determined using pharmodynamic observations to give an expected concentration of leptin in the high physiological range. For experiments requiring pre-ovulatory ovaries, the final injection of leptin or saline was omitted (Figure 2.2B). Plasma was collected from these pre-ovulatory animals using heart puncture (Chapter 2.2.7) to establish the levels of steroids and rh-leptin in circulation (Chapter 2.8).

2.4.3 Ovarian histological analyses

Four pairs of ovaries (five for the pair fed group) were selected for ovarian morphological analyses on the basis of mean representative ovulations for each treatment group. Each pair of ovaries was serially sectioned as described (Chapter 2.3.1) and stained with haematoxylin and eosin (Chapter 2.3.4). The numbers of follicles in each of the five antral follicle classes were counted (Chapter 2.3.6).

2.4.4 Leukocyte distribution in leptin treated ovaries

Three to four pairs of ovaries were selected for analysis of leukocyte distribution in leptin treated ovaries. Ovaries were collected and sectioned (Chapter 2.3.1) and immunoreactivity for leukocytes determined using antibodies (Chapter 2.3.2) and the protocol outlined in Chapter 2.3.3. The results were analysed using the procedure in Chapter 2.3.6.

2.5 *In vitro* perfusion of the ovary

2.5.1 Surgical isolation of the ovary

Rats received a subcutaneous injection of eCG (20 IU) at 1200 hr on experimental day 1 to promote the growth and maturation of a first generation of antral follicles. On the morning of day 3, animals were anaesthetised (Chapter 2.2.2) with ketamine/xylazine (67/14 mg/Kg BW) and the right ovary with its vasculature was isolated using surgical procedures described in detail previously (Brannstrom *et al.*, 1987; Brannstrom, 1993a).

Two experienced technicians (Acknowledgements) performed the surgery. The left ovary is unsuitable for use in perfusion systems as the left ovarian artery branches from the renal vessel and not the aorta.

2.5.2 Perfusion apparatus

The ovary was placed in a 30-ml recirculating system, filled with perfusion medium M199 (supplemented with 4 % BSA, 50 mg/ml gentamycin, 0.026 M sodium bicarbonate and 0.021 U/ml insulin). Temperature was maintained at 37°C by circulating heated water through jackets of the perfusion apparatus. Pressure was maintained at 80 mm Hg resulting in an average flow of 1.25 ml of media per minute through perfused ovaries. The media was continuously re-circulated and oxygenated with 95 % O_2 and 5 % CO_2. Following connection, ovaries were perfused for one hour to allow metabolic stabilisation of the tissue before the treatments of ovine LH alone (0.1µg/ml) or ovine LH with leptin (1µg/ml) were added to the perfusion media.

2.5.3 Perfusion sample collection & *in vitro* ovulation rate

Samples (1 ml) of the circulating medium were taken at time 0, 1, 2, 3, 4, 8, and 22 h following commencement of treatments and stored at –20°C for subsequent steroid hormone analyses. The perfused ovaries were removed from the perfusion system at 22 h post treatment and the surface rinsed with saline to dislodge any adhering oocytes before the number of oocytes found in the perfusion chamber were counted.

2.5.4 Scoring criteria for perfused Ovaries

Two criteria were used to ensure that only properly perfused ovaries were included in the results: firstly, progesterone levels in the first 3 h of the perfusion met specific criteria (Brannstrom and Janson, 1989) and secondly, ovaries were examined post perfusion with haematoxylin to determine if vasculature of the ovary was intact.

2.6 Collection and *in vitro* culture of ovarian tissue

2.6.1 Isolation of whole follicles

Follicle growth was induced in immature Sprague Dawley rats (25-28 days old, weighing 60-80 g) by a subcutaneous injection of 10 IU eCG (Chapter 2.2.3) at 1200h for 4h follicle culture on day -2 and at 0000h for 12h follicle culture on the night of day -2. Rats were sacrificed 46-48h later using ketamine/xylazine anaesthesia and cervical dislocation (Chapter 2.2.2). Both ovaries were dissected aseptically free of the ovarian fat, bursa and mesentery tissue in hepes buffered TCM 199/0.1 % BSA media on a 37°C warming tray.

2.6.2 Follicle culture and meiotic oocyte stage determination

For whole follicle culture, the largest follicles (≥ 576 μm) were isolated with the aid of a dissecting microscope, graticule and fine needles. Healthy looking follicles of this size were presumed to have escaped atresia. Layers of stromal tissue were allowed to remain on the surface of the follicle, as this promotes follicle health. Rat follicles were cultured

as described by Tornell *et al.*, 1995. Follicles were dissected with the aid of a stereomicroscope and fine forceps. Approximately 45 pre-ovulatory follicles were obtained from 6 ovaries and these were collected and pooled. Aliquots of bicarbonate buffered TCM 199/0.1 % BSA media were added to clean rubber-capped vacutainer® (no additive) tubes from Becton Dickinson. Five follicles were cultured in 1 ml of bicarbonated TCM199/0.1 % BSA media containing treatments for 4 or 12-13 hr. Follicles were cultured in the presence or absence of leptin (300ng/ml) in combination with FSH (500 mIU), IGF-I (50ng/ml) or LH (100ng/ml). Each tube was purged with a 95 % O_2 /5 % CO_2 mixture of gas (BOC Gases, 28863D), until media colour matched 5 % carbonated media, and incubated in a slow gyrating water bath (Grant Instruments) set at 37°C. Preliminary follicle culture (4h) experiments incubated with various concentrations of leptin, in the presence of LH or FSH and IGF-I, indicated a similar response to meiotic maturation and steroid synthesis at all concentrations of leptin tested. Therefore, the final dose of leptin chosen was in the high physiological range in order to evaluate the response of follicles to high leptin concentrations. Following incubation, the follicles were recovered and conditioned media was collected and frozen at -20°C until further analyses could be performed.

To assess meiotic maturation of oocytes, cultured follicles were placed in a petri dish containing Hepes buffered TCM 199/0.1 % BSA media containing 100μM IBMX, opened with a 30 gauge needle to release the oocyte/cumulus cell complex and the oocyte denuded of cumulus cells. Meiotic competence of oocytes was assessed by an independent observer using contrast microscopy by denoting the presence (GV) or absence of a germinal vesicle (GVB), where GVB indicates that the germinal vesicle has undergone germinal vesicle breakdown. Oocytes with intact germinal vesicles were

considered immature and those with germinal vesicle breakdown considered to have resumed meiosis. Polar bodies were seen in some oocytes and the results of these were considered to be equivalent to an observation of germinal vesicle breakdown.

2.6.3 Granulosa cell proliferation assay

For granulosa cell culture, immature Sprague Dawley rats (25-28 days old, weighing 60-80 g) were primed with 5 IU eCG. Primed ovaries (Chapter 2.2.3) were collected in cold Hepes-TCM199 media and cleaned free of blood. Granulosa cells were released from ovaries using a procedure similar to that described previously (Armstrong *et al.*, 1996), in which follicles were ruptured using a 30-gauge needle and granulosa cells were aspirated using a fine mouth pipette to isolate sheets of granulosa cells into single cell populations. Granulosa cells were collected in cold Hepes-TCM199 media and centrifuged for 5 minutes at 2000 rpm. The media was discarded and granulosa cells were resuspended in fresh Hepes-TCM199 media, followed by centrifugation for 5 minutes at 2000 rpm. The media was again discarded and the granulosa cells resuspended in sufficient bicarbonated-TCM 199 media to result in a granulosa cell count of 1×10^5 cells per well. Cell viability was determined by adding 20 μl of 4 % trypan blue to an equal volume of granulosa cells and counted using a haemocytometer (Neubauer Assistant). Culture was initiated if cell viability was approximately 50 %. Granulosa cell cultures were plated in 96-well Falcon® dishes (Becton Dickinson) in a total of 125 μl for 18 hours, followed by a 6 hr pulse of 0.8 μCi ^{3}H-thymidine (20 Ci/mmole) (ICN) as an indicator of granulosa cell DNA synthesis. Granulosa cell culture was performed in an incubator (Forma Scientific, 3029) with atmospheric

conditions of 39°C, 96 % humidity in 5 % CO_2 in air. DNA synthesis was assessed as previously described (Gilchrist *et al.*, 2000). Data is expressed as proliferation-relative to the control (cpm/1000 viable cells).

2.6.4 Granulosa cell culture

A previously described procedure (Zachow and Magoffin, 1997) was followed exactly, except cells were cultured in TCM 199/0.1 % BSA. This study was carried out in order to act as a control for the remaining studies and to validate the biological activity of all hormones used in the *in vitro* experiments. Granulosa cells were obtained from immature rats and approximately 60,000 cells plated/well. Designated wells were challenged with androstenedione (0.1 μM) in the presence of leptin (0.1-600 ng/ml), FSH (100 mIU) and/or IGF-I (50 ng/ml) and cultured for 48h. Granulosa cell culture was performed in an incubator (Forma Scientific, 3029) with atmospheric conditions of 39°C, 96 % humidity in 5 % CO_2 in air. Steroid synthesis was assessed as described in Chapter 2.8.

2.7 Leptin receptor transcript quantitation

2.7.1 Animals

Female Sprague-Dawley rats aged 28 days and weighing approximately 80 g were allowed 5 days to acclimatise to their new surroundings and then vaginal smears performed (between 0900 and 1100 hours) for three consecutive oestrous cycles (Chapter 2.2.8). Only animals with three 4-day consecutive oestrous cycles were

included in the study. Given that leptin protein circulates in a diurnal pattern in rodents (Mistlberger *et al.*, 1998; Pickavance *et al.*, 1998) and rodents are nocturnal foragers, all animals were anaesthetised and sacrificed by cervical dislocation (Chapter 2.2.2) between 1100 and 1300 hours, a time not associated with eating in rats.

2.7.2 Ribonuclease (RNase) free conditions during tissue collection

RNase free conditions were maintained during the collection of tissues and preparation of solutions and materials for total cellular RNA isolation. Precautions included aseptic technique, disposable gloves, disposable plastic petri dishes and solutions treated with diethyl pyrocarbonate water and sterilisation by autoclave for 20 minutes at 121°C. In order to minimise RNase contamination, all utensils or equipment used was treated as described in Appendix 1.

2.7.3 Tissue collection and isolation of total cellular DNA and RNA

Ovaries were collected in diethyl pyrocarbonate treated water and the ampulla region, any ovulated oocytes, ovarian fat and bursa were removed. Left and right ovaries were weighed and frozen separately using liquid nitrogen and stored at –80°C. RNA and DNA were isolated using Tri Reagent - the RNA, DNA, and protein isolation reagent, in accordance with the manufacturers protocol (1995), (Molecular Research Center Inc). RNA and DNA were quantified using a fluorometer (Turner Designs) using RiboGreen™ RNA Quantitation Reagent and PicoGreen® dsDNA Quantitation Reagent Kits (Molecular Probes), respectively.

2.7.4 Reverse transcription of RNA

A master mix containing 1x PCR buffer II (Perkin Elmer), 50 nmol of each dNTP (Gibco BRL), 250 ng Oligo $(dT)_{12-18}$ (Promega), 50 U RNasin® Ribonuclease inhibitor (Promega), 250 nmol $MgCl_2$ (Perkin Elmer), 500 U M-MLV Reverse Transcriptase (Gibco BRL) and 2 µl of RNA were added to a final reaction volume of 50 µl. Reactions were added to a 96 well plate (Perkin Elmer) and incubated for 30 minutes at 37°C and 5 minutes at 95°C in a thermal cycler (Perkin Elmer) and then immediately stored at -20°C until the PCR reaction. Both negative (no RNA in the RT reaction) and positive (Ambion Inc., RT-check™) controls were included in the RT reaction. Total genomic DNA from ovarian tissue was quantified to control for tissue to tissue variation.

2.7.5 Generation of standard *OB-RB* & *OB-RA* DNA by PCR

Previously used primers (Zachow *et al.*, 1999), synthesised by Gibco BRL, were used to amplify both the long and short isoforms of the leptin receptor. A common forward primer 5'-ATGAAGTGGCTTAGAATCCCTTCG-3' for both *OB-RB* and *OB-RA* detection was used. The reverse primers for the long and short isoforms are 5'-ATATCACTGATTCTGCATGCT-3' and 5'-TACTTCAAAGAGTGTCCGCTC-3', respectively. A PCR master mix containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 400nM of each *OB-RB* primer or 200 nM of each *OB-RA* primer, 2.5 U of Taq Polymerase (Perkin Elmer) and 2 µl of the above generated cDNA (to a final volume of 50 µl) was placed in a thermal cycler and a two step PCR protocol (denature

94°C and anneal 55°C for 30 seconds each) of 40 cycles was applied. A single band of the expected size (346 bp for *OB-RA* and 375 bp for *OB-RB*) was visualised using 2 % agarose gel electrophoresis and confirmed by enzymatic digestion and direct sequence, the gel bands excised and the DNA purified using a QIAprep gel extraction kit (QIAgen). Both DNA fragments were inserted into TOPO TA cloning vectors (Invitrogen) and transformed into TOP10F' cells and plated on LB agar plates with 50 µg/ml ampicillin, according to manufacturers instructions (1999). Positive clones (white colonies) were considered to have an insert in the TOPO TA cloning vector and these colonies were cultured overnight at 37°C in LB media with 50 µg/ml ampicillin. Vector-insert DNA was extracted using QIAprep spin miniprep kits (QIAgen) and the presence of the insert was identified by EcoRV restriction enzyme digestion (Gibco BRL) and visualised using gel electrophoresis. Vector-inserts were linearised using KpnI (Promega) for *OB-RB* and HindIII (Gibco BRL) for *OB-RA*. The concentration of DNA was quantified and dilutions prepared for the synthesis of standards for real time PCR.

2.7.6 Real time RT-PCR of ovary *OB-RB* and *OB-RA*

A common hybridisation probe (Operon) was constructed, 5'-CCTTGTGCCCAGGAACAATTCAAGG-3', with the reporter fluorochrome (6-carboxyfluorescein [6-FAM]) at the 5' end and a quencher fluorochrome (6-carboxy-tetramethyl-rhodamine [TAMRA]) at the 3' end. The probe had a Tm approximately 15°C above the PCR primers and had 4 additional base pairs at the 3' end to promote a hairpin structure of the hybridisation oligonucleotide to minimise background fluorescence. The PCR master mix consisted of all products purchased from Gibco BRL: 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 µM dNTP each, 400 nM primers for *OB-RB*

detection and 200 nM primers for *OB-RA* detection and 2.5 units Platinum Taq DNA polymerase. Ten µl of the RT reaction was added to the appropriate wells, in duplicate. 500 nM of the hybridisation oligonucleotide was added to each well of the 96 well I-cycler plate (Bio-Rad) under low intensity light conditions. Real time RT-PCR was carried out using the two step procedure described above and data recorded by the Bio-Rad I-cycler program (Bio-Rad).

2.8 Assays

2.8.1 Leptin

Levels of rh-leptin in plasma samples collected from animals immediately prior to ovulation (Chapter 2.4.2) were analysed using a human leptin ELISA kit from DSL Scientific. This assay does not cross-react with rat leptin. The minimum detection limit of this assay is 0.05 ng/ml and the intra-assay CV is 2.9 %. Leptin levels in rat plasma in the real time RT-PCR study were analysed using a rat Active™ murine leptin ELISA kit (DSL-10-24100) from DSL scientific, which is sensitive for both rat and mouse plasma leptin. The minimum detection limit of this assay is 0.04 ng/ml with an intra-assay CV of less than 6 %. To quantify the low levels of serum leptin, the option of using 100 µl in the assay was used.

2.8.2 Progesterone

Perfusion media progesterone concentrations were analysed by Johnson & Johnson Vitros ECI Chemiluminescent immunoassay system (Orthoclinical Diagnostics). The

inter-assay coefficients of variation was calculated as < 8 % with sensitivities of 0.3 pmol/L. For analysis of *in vitro* culture media, serum and plasma progesterone concentrations, a RIA (DSL Inc, DSL-3400) was used unless otherwise stated, in which case, the Johnson & Johnson assay system was used and validated for rat plasma using a hexane/ethyl acetate extraction. The minimum detection limits of the progesterone assay is 0.3 nmol/L and the intra-assay CV is less than 7 %. Samples for progesterone analysis for the *in vivo* studies were assayed in duplicate.

2.8.3 Oestradiol

Perfusion media oestradiol concentrations were analysed by Johnson & Johnson Vitros ECI Chemiluminescent immunoassay system (Orthoclinical Diagnostics) was used. The inter-assay coefficients of variation was calculated as < 6 % with sensitivities of 10 pmol/L. For analysis of *in vitro* culture media, serum and plasma oestradiol concentrations, a RIA (DSL Inc, DSL-4400) was used unless otherwise stated, in which case, the Johnson & Johnson assay system was validated for rat plasma using a hexane/ethyl acetate extraction. The minimum detection limits of the oestradiol assay was 17.39 pmol/L, with intra-assay CV of less than 7 %. Samples for oestradiol analysis for the *in vivo* studies were assayed in duplicate

2.8.4 Prostaglandin E_2

The levels of prostaglandin E_2 were quantified with a prostaglandin E_2 [^{125}I] assay system (RPA 530) purchased from Amersham Pharmacia Biotech and conducted

according to the manufacturer's protocol. The Intra-assay CVs of this assay are less than 6 %, with a sensitivity of 0.8pg/ml.

2.8.5 Interleukin-1β

The IL-1β ELISA (RPN 2743) kit was obtained from Amersham Pharmacia Biotech and conducted according to the manufacturer's protocol. The sensitivitity of IL-1β ELISA is 5 pg/ml and intra-assay CV is < 9 %.

2.9 Data analyses

Data are presented as mean ± SEM. Graphpad Instat version 2.04a (Graphpad Software) was used to analyse all data (ANOVA with post hoc tests). Statistical significance was assessed at $*P < 0.05$. $**P < 0.01$ and $***P < 0.001$. The numbers of samples or animals used are presented throughout the text. The details of data analysis and P values are presented within each chapter.

2.10 Photography and image capture

Digital images of immunohistochemistry were captured in JPEG file format (.jpg) using the video image analysis system (Chapter 2.3.6). Digital photographs of electrophoresed gels were captured on Electronic Documentation and Analysis System 120 (Eastman Kodak Co.) in the BIP file format (.bip) format.

Chapter 3

The effect of acute exogenous leptin administration on ovulation.

3.1 Introduction

The association of extremes of body mass and reproductive dysfunction has been recognised in many species (Frisch *et al.*, 1975; Rich-Edwards *et al.*, 1994). These two fields have recently been linked by the hormone leptin, which is synthesised and secreted from adipose tissue (Chapter 1.1.3). With regard to humans, there is a strong positive correlation between serum leptin and percent body fat. For some obese individuals, obesity translates into impaired fertility, indicating that the leptin protein and the ovary may be linked. Several lines of evidence suggest that leptin may be functional in reproductive tissues (Chapter 1.3.2.1). In line with this, some studies have reported the inhibitory effects of leptin treatment in the ovary in many species (Chapter 1.3.2.2) (Spicer and Francisco, 1997; Zachow and Magoffin, 1997; Spicer and Francisco, 1998).

Leptin appears to be an important link between obesity and infertility. The hypothesis of the studies presented within this chapter is that excessive circulating leptin may be a contributing factor to infertility observed in obese women (Chapter 1.5). The present study therefore investigates the effects of high systemic leptin on ovulation, both *in vivo,* using immature gonadotrophin-primed rats, and *in vitro,* using the perfused rat ovary model (Chapter 2.4). We also set out to establish if the levels of the steroid hormones, progesterone and oestradiol, in either model were affected by leptin treatment.

3.2 The effects of leptin on food consumption and body weight

To investigate the effects of leptin administration on the amount of food consumed and changes in body weight, food and body weight were measured daily at 0900 hrs (Chapter

2.4). As leptin treatment affects the amount of food consumed and body metabolism (Chapter 1.1.5), a preliminary experiment was designed to establish the average amount of food consumed by leptin treated rats. The amount of food eaten by leptin treated rats was given to a control group, the pair fed group, to establish if the effects observed on the ovary were due to a decrease in food consumption or due to the leptin treatment.

Food consumption prior to leptin or saline administration was similar between treatment groups (Figure 3.1A). Leptin treatment caused the amount of food consumed by gonadotrophin-primed, leptin treated rats to decline significantly ($p < 0.001$) compared to gonadotrophin-primed, saline treated controls. Pair fed controls were given the same amount of food consumed by the leptin-treated animals. Animals that were not treated with leptin or the pair feeding treatment had similar food consumption (Figure 3.1B), indicating that gonadotrophin stimulation was not a factor affecting appetite.

All groups gained weight prior to the saline or leptin treatment given on day 3 (Figure 3.2A). Both gonadotrophin-stimulated and un-stimulated, saline treated animals gained weight normally and were not significantly different to each other (Figure 3.2A). The day after leptin or saline intraperitoneal injections, leptin-treated animals consumed less food than control animals, and as a result, lost weight or gained less weight between days 3 and 4 (Figure 3.2B). Body weight of pair fed animals was not significantly different from those of the gonadotrophin primed-leptin treated group (Figure 3.2B). However, both gonadotrophin stimulated and un-stimulated leptin-treated animals experienced body weight loss. The results clearly show that leptin has an effect on body weight, an observation that has also been previously documented (Zhang *et al.*, 1994; Halaas *et al.*, 1995).

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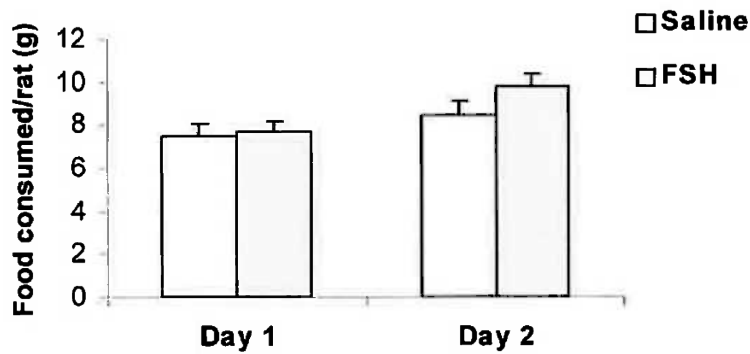

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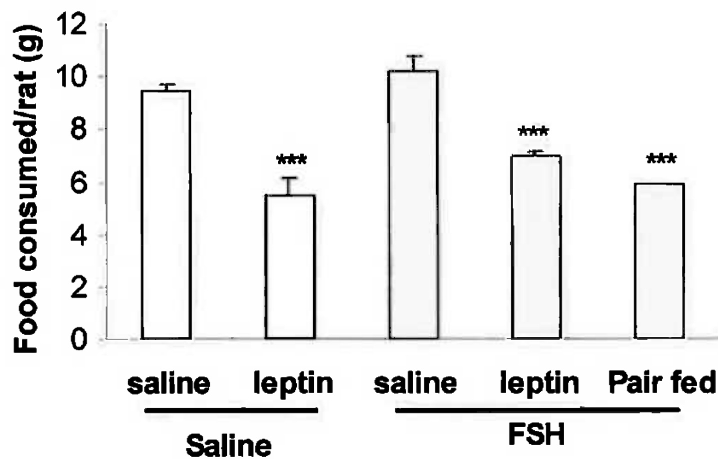

Figure 3.1 Amount of food consumed (g) by un-stimulated (saline) and gonadotrophin stimulated (FSH) Sprague-Dawley rats treated with leptin, saline or pair feeding. Alzet® minipumps containing FSH or saline were surgically implanted on day 1 of the experiment. On Day 3, the animals were primed with hCG and given regular injections of leptin or saline. Pair fed animals were given the amount of food consumed by gonadotrophin primed leptin treated rats. A) Food consumed prior to leptin or saline treatment; B) food consumed post leptin or saline treatment. ***significantly different from each other as assessed by ANOVA and the Tukey Kramer Multiple Comparisons Test, where $P < 0.001$. Data are presented as mean ± SEM, n≥12.

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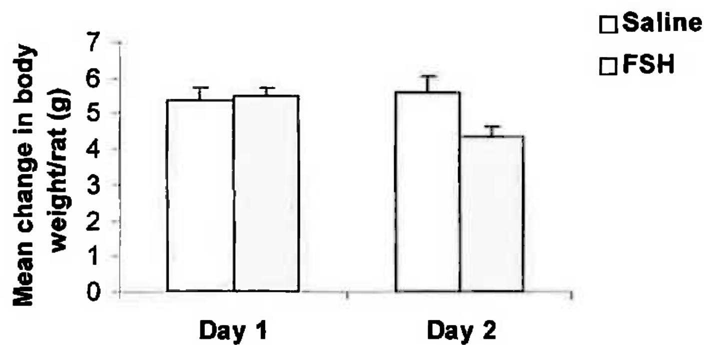

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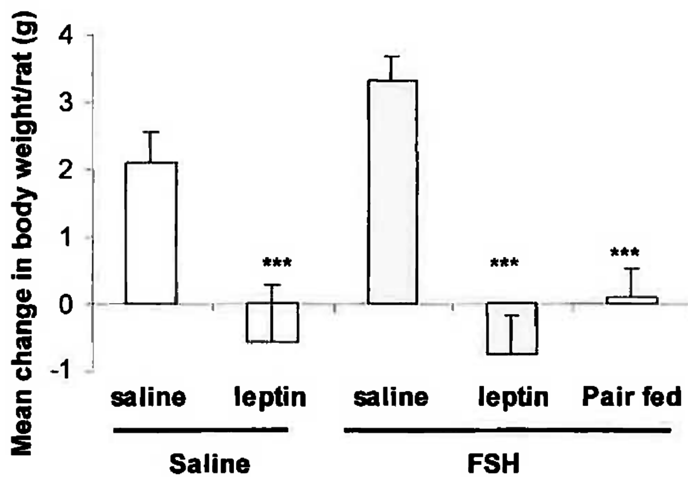

Figure 3.2 Mean change in body weight in un-stimulated (saline) and gonadotrophin stimulated (FSH) Sprague-Dawley rats following treatment of leptin, saline or pair feeding. Rats were given minipumps containing FSH or saline on day 1 (of 4) of the experiment. On day 3, the animals were primed with hCG and given regular injections of leptin or saline. Pair fed animals were given the same amount of food consumed by FSH/Leptin treated animals. A) Mean change in body weight on days 1 and 2 - prior to leptin or saline treatment; B) mean change in body weight - post leptin or saline treatment. *** significantly different from gonadotrophin primed saline treated rats, as assessed by ANOVA and the Tukey Kramer Multiple Comparisons Test, $P < 0.001$. Data are presented as mean ± SEM, $n \geq 12$.

3.3 The effect of leptin on ovulation

The total number of oocytes ovulated *in vivo* from left and right ovaries were examined from the ampullae of rats at 22 h post hCG (Chapter 2.2.5). Counting of oocytes revealed that animals primed with gonadotrophins (FSH) and treated with saline ovulated the highest number of oocytes (15.9 ± 2.0) and animals that did not receive any gonadotrophins (saline/saline) ovulated the least number of oocytes (3.7 ± 1.0) (Figure 3.3A). The number of oocytes ovulated by pair fed animals (14.5 ± 1.7) was not significantly different from those of the FSH/saline treated group, while leptin treatment of FSH-stimulated animals caused a significant reduction in ovulation (5.3 ± 1.6) ($p < 0.001$). The number of oocytes ovulated in unstimulated animals following leptin treatment (2.3 ± 0.9) were not significantly different to saline treated animals. Both of these groups ovulated significantly fewer oocytes than animals receiving either gonadotrophins with saline or pair fed treatments ($p < 0.001$). The number of oocytes ovulated from animals receiving gonadotrophins and leptin and those unstimulated animals receiving saline or leptin were not significantly different.

Ovaries from gonadotrophin stimulated animals were used for the *in vitro* study. Ovaries were surgically isolated and perfused in the presence of LH and leptin or leptin alone (Chapter 2.5). The reduction in ovulation rate observed *in vivo* was reflected *in vitro* (Figure 3.3B). Ovaries perfused with both LH and leptin ovulated significantly fewer oocytes (1.3 ± 0.6 per ovary) than ovaries perfused with LH alone (5.7 ± 1.6) ($p < 0.05$).

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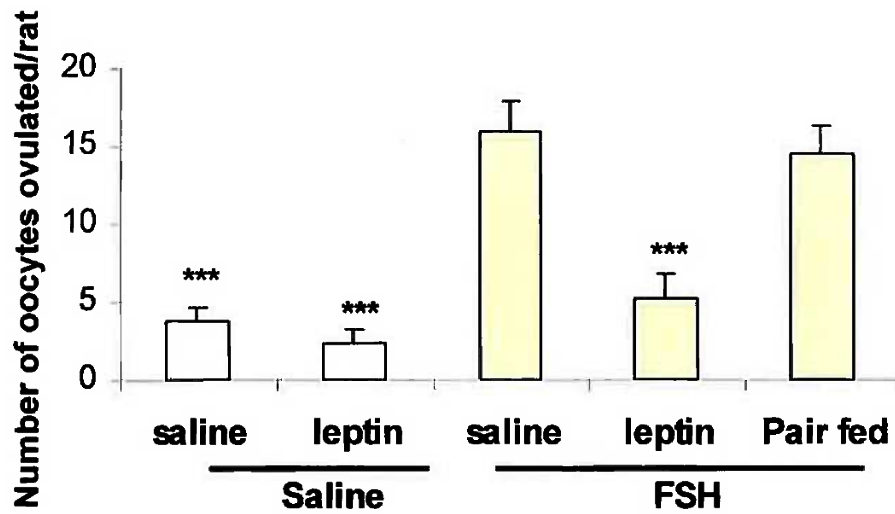

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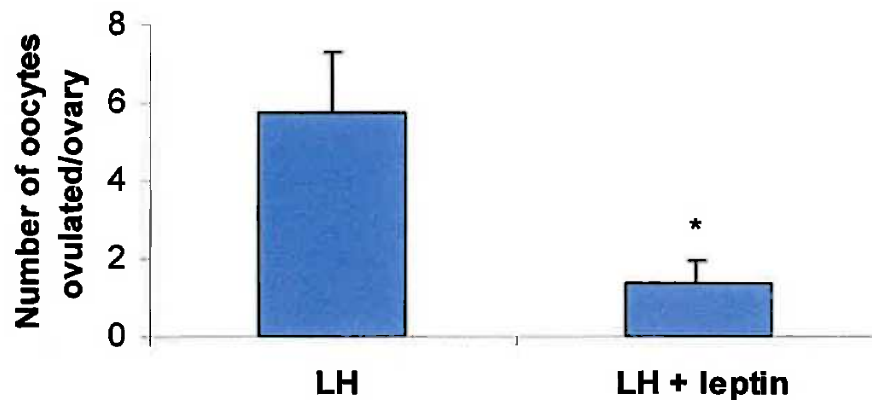

Figure 3.3 Number of oocytes ovulated *in vivo* and *in vitro* in the presence and absence of leptin. A) Rats were given minipumps containing FSH or saline on day 1 of the experiment. On day 3, the animals were primed with hCG and given regular injections of leptin or saline. Pair fed animals were given the same amount of food consumed by FSH/Leptin treated animals. On day 4, animals were sacrificed and the number of oocytes in the ampulla region counted. *** significantly different from gonadotrophin stimulated saline treated rats, as assessed by ANOVA and the Tukey Kramer Multiple Comparisons Test, $P < 0.001$. Data are presented as mean ± SEM, n = 12. B) Ovulations from *in vitro* perfused ovaries. * significantly different from LH treated rats, as assessed by a two-tailed students T-test, $P < 0.05$. Data are presented as mean ± SEM, n = 6-7.

3.4 The effect of leptin on steroid hormones

Plasma was collected immediately prior to ovulation in gonadotrophin stimulated rats (Chapter 2.2.7). Steroid hormone analyses (Chapter 2.8) of rat plasma revealed that leptin treatment did not significantly affect the progesterone levels at that time (Figure 3.4A). Plasma oestradiol concentrations were below the detection limit of the assay.

In the *in vitro* perfusion study (Chapter 2.5), media samples were collected at 1, 2, 3, 4, 8 and 22 hrs, post LH or LH and leptin addition. LH addition resulted in the stimulation of oestradiol to a maximal level within 8 h (Figure 3.4B), after which, levels plateaued for the remainder of the perfusion period. No significant difference between oestradiol levels of the two treatment groups was observed. Perfused ovaries treated with LH or LH and leptin showed an immediate increase in the level of circulating progesterone in response to LH addition (Figure 3.4C). The levels of progesterone then declined after this initial peak, as previously described (Brannstrom and Janson, 1989). No significant difference was seen in circulating progesterone levels between treatment groups at any time during the perfusion period.

3.5 The effect of leptin on ovarian weight

All animals treated with gonadotrophins had large follicles, well developed into the graffian stage of follicular growth on the surface of the ovaries. Ovarian weights of the FSH-treated animals were significantly different from those animals receiving saline treatment ($p < 0.05$), however leptin treatment on its own did not affect ovarian weights (Figure 3.5). Therefore, ovarian weight appears to be unaffected by leptin treatment.

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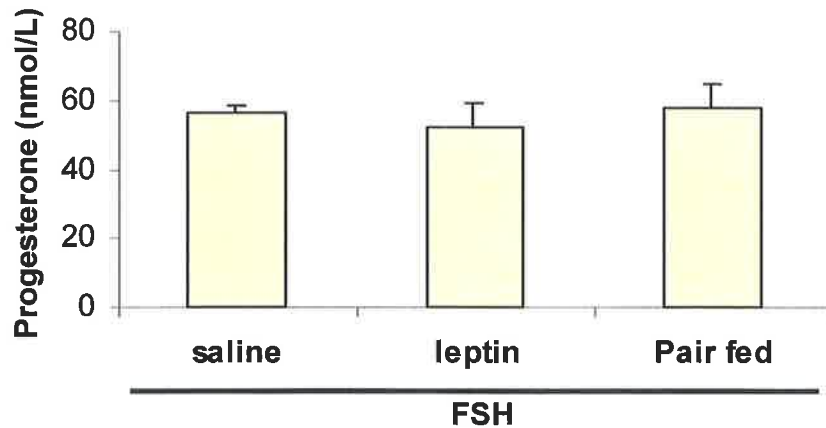


B **C**

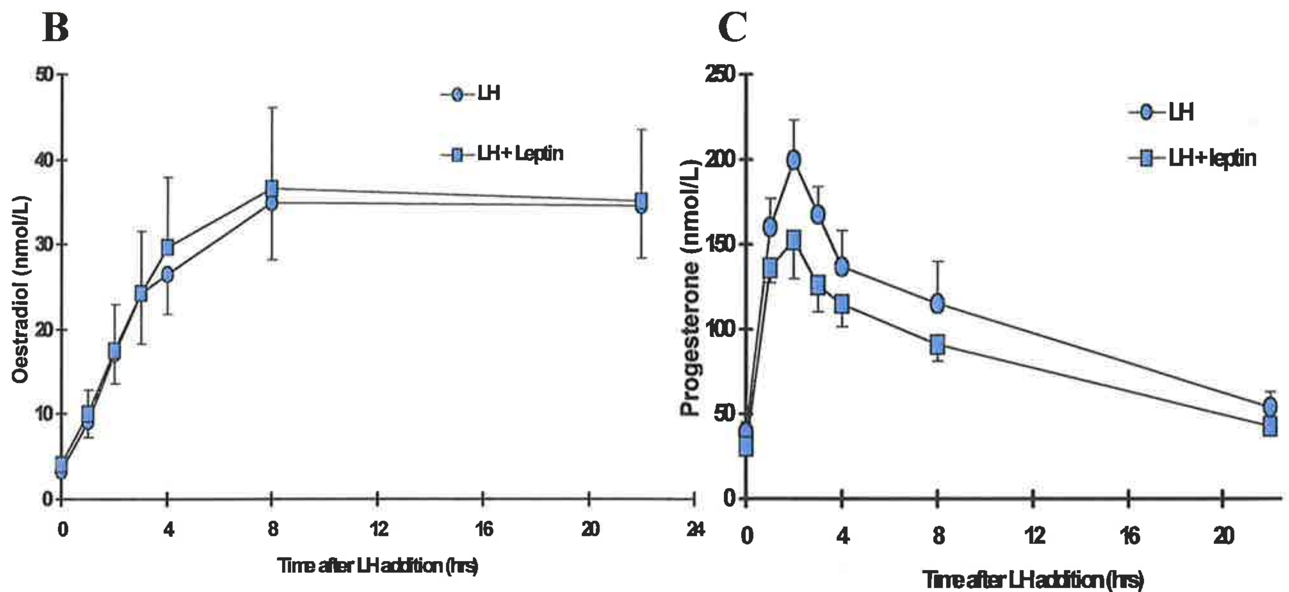


Figure 3.4 Steroid production *in vivo* and *in vitro* following leptin treatment. A) Rats were given minipumps containing FSH or saline on day 1 of the experiment. On day 3, the animals were primed with hCG and given regular injections of leptin or saline. Pair fed animals were given the same amount of food consumed by FSH/Leptin treated animals. Animals were sacrificed immediately prior to ovulation and blood collected for plasma progesterone analysis. Data are presented as mean ± SEM, n = 6. B) Oestradiol and C) progesterone production from *in vitro* perfused ovaries. Data are presented as mean ± SEM, n = 6-7.

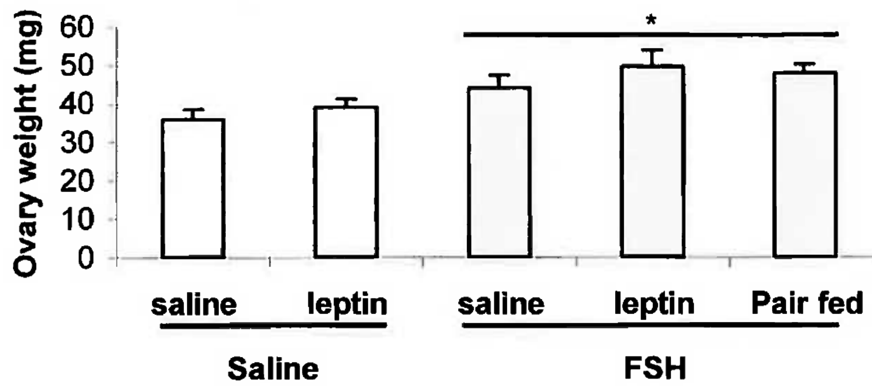


Figure 3.5 Ovarian weights (mg) of un-stimulated and gonadotrophin stimulated Sprague-Dawley rats following treatment of leptin, saline or pair feeding. Rats were given minipumps containing FSH or saline on day 1 (of 4) of the experiment. On day 3, the animals were primed with hCG and given regular injections of leptin or saline. Pair fed animals were given the same amount of food consumed by FSH/Leptin treated animals. On day 4, animals were sacrificed and both ovaries isolated and weighed. Data are presented as mean ± SEM, $n \geq 9$. * significantly different from un-stimulated (saline minipump) rats, as assessed by the Tukey-Kramer Multiple Comparisons Test and ANOVA, $P < 0.05$.

3.6 Plasma leptin levels

To confirm that leptin was being metabolised, plasma samples taken immediately prior to ovulation were analysed for leptin levels (Chapter 2.8). Analyses revealed that plasma from FSH/leptin, FSH/saline and pair fed animals had leptin levels below the detection level of the assay (data not shown). This suggests that all leptin injected into the gonadotrophin primed leptin treated animals was cleared before the time of ovulation, consistent with the half-life of the protein in circulation (Chapter 1.2.5.2).

3.7 Conclusions and discussion

Numerous studies have investigated the levels of plasma leptin in obese individuals, however, there has been limited research in the field of *in vivo* leptin treatment on the ovary. Previous publications have concentrated on the effect of leptin in leptin-deficient (Lep^{ob}/Lep^{ob}) mice or in ovarian dispersates from normal animals. However, some studies (Spicer and Francisco, 1997; Zachow and Magoffin, 1997) have shown how leptin may interact with the ovary *in vitro*. This chapter discusses the first study to demonstrate conclusively the impact of acute leptin treatment on ovulation in immature, gonadotrophin-primed rats and in the whole-perfused ovary.

The expression of the long form of the leptin receptor has been detected in granulosa, theca, interstitial and cumulus oophorus cells (Billig *et al.*, 1997; Cioffi *et al.*, 1997; Karlsson *et al.*, 1997; Agarwal *et al.*, 1999) of the human ovary. The use of the *in vitro* perfusion system clearly demonstrates that leptin as a cytokine exerts a direct and specific effect on the ovary and on the ovulation process. Leptin injected either systemically *in vivo* or into

the perfusate led to a significant reduction in ovulation rates. In the *in vivo* study, we used immature animals primed with gonadotropins. This design allowed us to reduce or eliminate the effects of leptin on the hypothalamic-pituitary axis (Dyer *et al.*, 1997) by over-riding endogenous levels of gonadotropins with exogenous FSH. Leptin was able to inhibit ovulation in these gonadotropin-stimulated ovaries, suggesting that its primary action was on the ovary and not on organ systems outside the hypothalamic/pituitary/ovarian axes.

As reported in other studies, leptin treatment in our experiments resulted in weight loss. The loss in weight was most likely due to leptin treated animals consuming less food when compared with saline treated rats. Pair feeding also caused rats to gain less weight when compared with animals injected with saline instead of leptin. Leptin treatment is known to cause a loss in body weight by increasing metabolic activity, stimulating satiation earlier or causing a loss in appetite. Studies have shown that leptin interacts with regions of the brain that are responsive to feeding. Leptin also causes a decline and inhibition of neuropeptide Y (NPY), a peptide which induces feeding. Together, these findings may explain the decrease in food intake and body weight loss.

Given that leptin treatment causes a decline in body weight and food consumption (Considine and Caro, 1997), a set of pair fed animals were introduced to compensate for the effects of weight loss and altered acute nutrition. The use of this group clearly demonstrates that leptin directly, as opposed to indirectly through a decrease in body weight, is responsible for the reduction in ovulation seen in these experiments. Ovarian weight was not affected by leptin treatment or by pair feeding, while, as expected, animals treated with gonadotropins had significantly higher ovarian weights when compared with saline-treated

controls. The un-stimulated saline treated animals provide a suitable control to support the well-documented effects of gonadotrophin stimulation on the ovary. Concentrations of leptin measured in the plasma of animals immediately prior to ovulation were undetectable, in agreement with the short half-life (Chapter 1.2.5.2) of the leptin protein (Cumin *et al.*, 1996; Klein *et al.*, 1996; Gavrilova *et al.*, 1997). Biological activity was clearly present however, shown by the effect on body weight and decreased food consumption.

The value of the *in vitro* perfused ovary system with a variety of gonadotropins and cytokines (Brannstrom *et al.*, 1995; Bonello *et al.*, 1996) has been studied extensively. CG priming of animals, followed by surgical removal of their ovaries, selective cannulation and injection of LH allows for investigation of factors acting directly on the ovary to influence the process of ovulation. The injection of leptin at the same time as LH clearly shows that this protein was able to inhibit the ovulatory process in the isolated ovary, thereby supporting the suggestion that leptin has a direct endocrine action on the ovary. In both *in vivo* and *in vitro* studies, there were no changes seen in steroid secretion. This finding is consistent with previous research in rat ovarian granulosa cells, where in the absence of growth factor augmentation, leptin is unable to affect steroid production (Zachow and Magoffin, 1997; Barkan *et al.*, 1999). In the presence of high levels of IGF-I, the sensitising effects of FSH enhance progesterone and oestradiol production of rat granulosa cells (Agarwal *et al.*, 1999). However, high levels of leptin have been postulated to block the stimulatory effects of IGF-I on rat granulosa cell oestradiol, but not progesterone, production without altering the effects of FSH alone (Agarwal *et al.*, 1999).

Leptin has prominent effects on the reproductive axis and is able to reverse the sterility observed in the leptin-deficient obese (*Lep*^{ob}/*Lep*^{ob}) mouse (Chehab *et al.*, 1996; Mounzih

et al., 1997). It has been shown that serum leptin levels proportionally correlate with body mass index and percentage body fat in women (Maffei *et al.*, 1995; Considine *et al.*, 1996). Furthermore, obesity has also been linked to reproductive dysfunction. The leptin levels found in grossly obese women range to 100 ng/ml (Considine *et al.*, 1996). The leptin levels used in this study could be expected to exceed the physiologically high leptin levels observed in obese women (Considine *et al.*, 1996; Niskanen *et al.*, 1997). Our data and other reports (Spicer and Francisco, 1997; Zachow and Magoffin, 1997) indicate that elevated leptin concentrations are able to exert a direct inhibitory effect on ovarian function. This research therefore provides a new dimension to studies on leptin and reproduction by indicating that acute administration of leptin, both *in vivo* and *in vitro*, can impair the ovulatory process. This may have clinical correlates in the observation that overweight women (with high leptin) are more prone to ovulatory disorders that cannot be entirely explained by the hypothalamic-pituitary axis. However, it is important to acknowledge that the ovulatory dysfunction experienced by most obese women may be due to the insensitivity to endogenous leptin (Chapter 1.2.4) and the chronically high levels of leptin may not mirror the effects seen by acute exogenous leptin administration.

In summary, this study has investigated the effects of high leptin levels both *in vivo* and *in vitro*. These results extend previous reports on an involvement of leptin in female reproduction. Both *in vivo* and *in vitro*, leptin treatment resulted in significant decreases in the number of oocytes ovulated, without any apparent effect on steroid levels. While leptin treatment decreased appetite and body weight, pair feeding of animals did not alter the results, indicating that leptin alone affects ovulation. Our evidence suggests a direct effect of leptin on the ovary, independent of alteration in the amount of gonadotropins and other circulating growth factors. This study shows that high

exogenous leptin levels can directly influence the ovary when natural FSH/LH cycling is over-ridden by FSH/LH administration.

Chapter 4

The *in vivo* effects of leptin on ovarian morphology and leukocyte distribution

4.1 Introduction

The release of oocytes from follicular rupture in mammalian ovulation has been likened to an inflammatory reaction (Espey, 1994) (Figure 4.1). This hypothesis is supported by many findings. Firstly, potent non-steroidal anti-inflammatory drugs (eg indomethacin) are able to inhibit ovulation if administered in the first 80 per cent of the ovulatory process (Parr, 1974). Secondly, steroidogenic activity may contribute markedly in the process of ovulation. The increase in gonadotrophins (LH) at the time of ovulation initiates a hyperaemic response, causing an increase in blood flow to the ovary. These biochemical events result in an increase in vasodilation (Tanaka *et al.*, 1989) and collagenolysis (Parr, 1974; Butler *et al.*, 1991), cell proliferation, tissue remodelling, and other common changes in inflamed tissue. Vasoactive agents, such as bradykinin, histamine and interleukins have been measured in ovulating ovaries, with kinin increasing 10-fold at the time of follicular rupture. Histamine and interleukins decline at the time of ovulation, possibly from dissipation from the local area. This local decline in the ovary is characteristic of inflamed tissue (Espey and Lipner, 1994). Thirdly, eicosanoids are also important in ovulation. These mediate proteolytic degradation of the thecal connective tissue of the follicle wall by three main inflammatory enzymes: glandular kallikrein, tissue-type plasminogen activator, and interstitial collagenase. These enzymes all rise substantially during ovulation. Finally, leukocytes have been implicated as important cells in the ovulatory process (Norman *et al.*, 1997; Van der Hoek *et al.*, 2000). Leukocytes increase in the vascular compartment at the time of ovulation (Brannstrom *et al.*, 1993b). Evidence from rodent experiments suggests that neutrophils and macrophages are actively recruited into the pre-ovulatory follicle following the LH surge, via chemotaxis. This results in an 8-fold increase in the density of thecal neutrophils and a 5-fold increase in the density of macrophages (Herriot *et al.*, 1986;

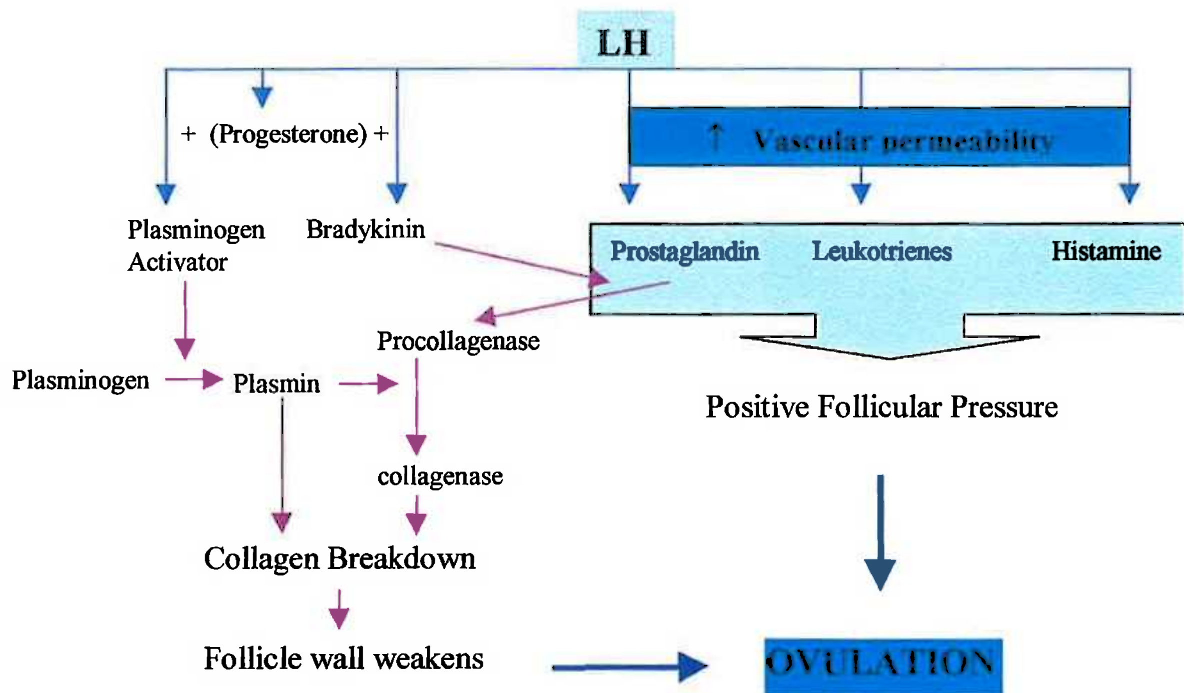


Figure 4.1 Schematic diagram of changes in the follicle at ovulation. Luteinising hormone (LH) initiates an increase in vascular permeability and collagen breakdown of the follicle wall. The increased vascularity promotes an increase in intrafollicular pressure, which eventually results in the rupture of the follicle and release of the follicular fluid and cumulus-enclosed oocyte. Adapted from Brannstrom and Norman, 1993c.

Brannstrom *et al.*, 1993b). Leukocyte supplementation of *in vitro* perfused rat ovaries results in an increase in ovulation rate (Hellberg *et al.*, 1991). Furthermore, *in vivo* administration of a neutrophil-depleting antibody causes a reduction in the ovulation rate in the rat (Brannstrom *et al.*, 1995) and macrophage depletion results in a decrease in ovulation rate in the mouse (Van der Hoek *et al.*, 2000). The mechanism by which neutrophils and macrophages contribute to the ovulatory process may be linked with the local cytokine network of macrophage secretory products (Nathan, 1987; Ben-Rafael and Orvieto, 1992; Brannstrom and Norman, 1993c), including degradative enzymes, in the pre-ovulatory follicle that lead to follicle wall breakdown and subsequent oocyte release (Brannstrom and Norman, 1993c; Norman *et al.*, 1997).

It is now established that leptin is capable of interactions with the immune system (Chapter 1.2.5.3). *In vitro* studies have shown that leptin can stimulate human monocyte proliferation by inducing tumour necrosis factor-α and interleukin-6 production (Santos-Alvarez *et al.*, 1999) and leptin increases Th1 and suppresses Th2 cytokine production in $CD4^+$ T cells from mice (Lord *et al.*, 1998). Furthermore, the effects of leptin on food intake and body temperature are believed to be mediated by the leukocyte-secreted cytokine interleukin-1 (Luheshi *et al.*, 1999).

The studies described in the previous chapter show that acute leptin treatment has an inhibitory effect on ovulation. Nutritional restriction is able to interfere with ovarian follicular development due to its effects in synthesis/release of hormones, thereby potentially compromising ovulation and steroidogenesis (Wodzicka and Tomaszewska, 1974; McClure and Saunders, 1985). We therefore hypothesised that the disruption to the ovulatory process we observed following leptin administration may be caused by a

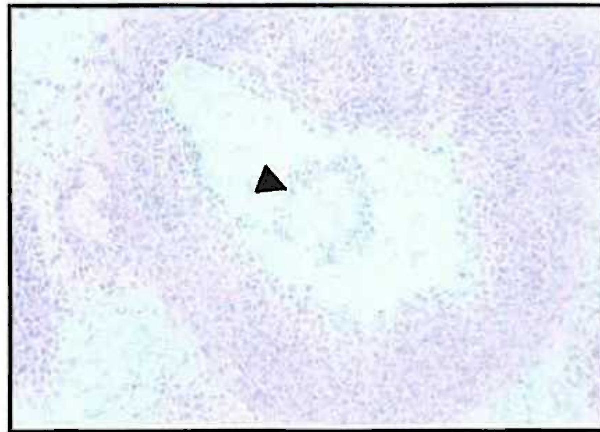
reduction in the development of antral follicles into pre-ovulatory follicles or a reduction in the numbers of leukocytes infiltrating the pre-ovulatory follicle. Any inhibition of leukocyte infiltration into the ovary or pre-ovulatory follicle could result in the interruption of the ovulatory cascade. Therefore, this chapter describes the effect of acute leptin treatment on the numbers of pre-ovulatory follicles and leukocyte recruitment into the ovary. This study examined the presence of granulocyte neutrophils and monocytes/macrophages by immunohistochemical staining of ovaries pre- and post-ovulation.

4.2 The effect of acute leptin treatment on ovarian morphology

Ovaries were collected from gonadotrophin-primed immature leptin or saline treated rats (4 - 5 rats per group) at pre- and post-ovulation (Chapter 2.2.6). Ovaries were embedded in Jung tissue freezing media, frozen in liquid nitrogen-cooled isopentane and stored at -80°C (Chapter 2.2.6). Fresh frozen tissue was serially sectioned (130 μm) and fixed in 95 % ethanol, stained in haematoxylin and eosin, cleared and mounted (Chapter 2.3).

All ovaries collected comprised of healthy and atretic follicles from primordial to antral stages. Pre-ovulatory follicles were healthy follicles >576 μm in the largest cross section (Figure 4.2A, Figure 4.3A) as determined by video image analysis software (Chapter 2.3.6), while atretic follicles were discounted from the results. The follicles present in the pre- and post-ovulatory ovaries ranged from Class 1 to Class 5 follicles (Chapter 2.3.6) regardless of the treatment given (Figure 4.2B, 4.3B). We anticipated that a higher level of Class 5 follicles would be predominant in the pre-ovulatory ovaries (Figure 4.2B), however, as early corpora lutea in post-ovulatory ovaries (Figure 4.3B) could not be

A



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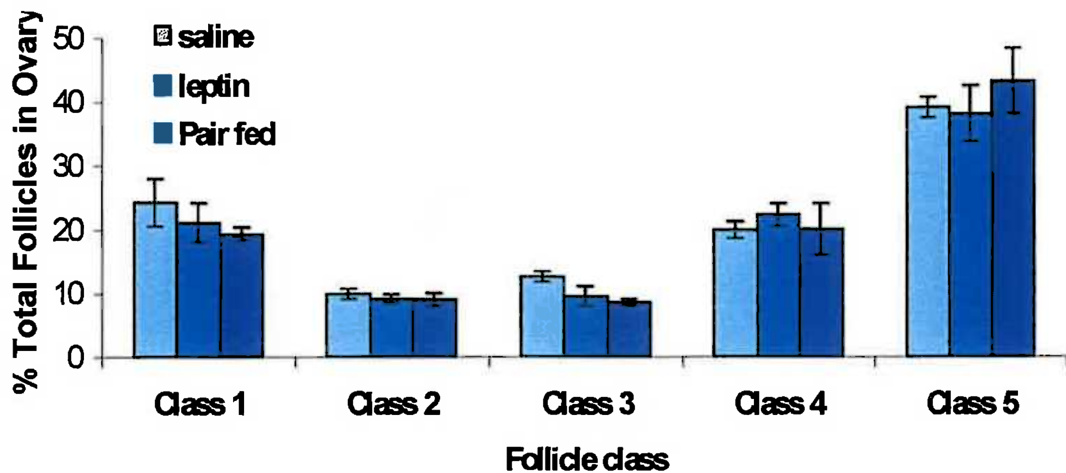


Figure 4.2 Distribution of antral follicles in the pre-ovulatory ovaries of gonadotrophin-primed immature rats treated with leptin, saline or pair feeding. Ovaries were collected from pre-ovulatory rats and stained for morphological analyses and follicle population determination. Antral follicles were classified on the basis of mean diameters and placed into one of the five following classes: class 1 (275-350 μm); class 2 (351-400 μm); class 3 (401-450 μm); class 4 (451-575 μm); or class 5 (≥576 μm), where class 5 follicles are classified as pre-ovulatory follicles. A) A haematoxylin and eosin stained pre-ovulatory ovarian follicle with an oocyte and cumulus cell complex (arrow) and B) the distribution of follicles of various classes in pre-ovulatory ovaries of gonadotrophin primed saline, leptin, or pair fed rats, n = 4.

A

B

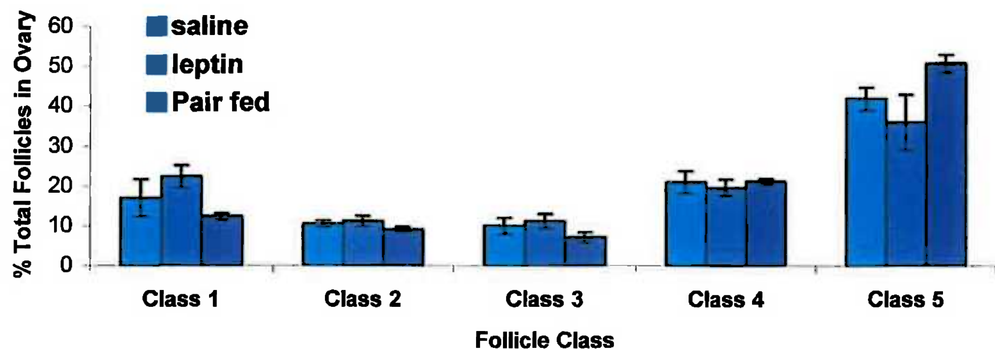


Figure 4.3 Distribution of antral follicles in ovaries of gonadotrophin-primed immature rats treated with leptin, saline or pair feeding post-ovulation. Ovaries were collected from rats post-ovulation and stained for follicle population determination. Antral follicles were classified on the basis of mean diameters and placed into one of the five following classes: class 1 (275-350 μm); class 2 (351-400 μm); class 3 (401-450 μm); class 4 (451-575 μm); or class 5 (≥576 μm), where class 5 follicles are classified as pre-ovulatory follicles. A) A haematoxylin and eosin stained ovulated ovarian follicle and B) the distribution of follicles from various classes in ovaries post-ovulation of gonadotrophin primed rats treated with saline, leptin or pair feeding, n = 4.

differentiated from Class 5 follicles, the levels of Class 5 follicles is similar between the pre- and post-ovulation ovaries.

4.3 Acute leptin treatment affects ovarian leukocyte recruitment

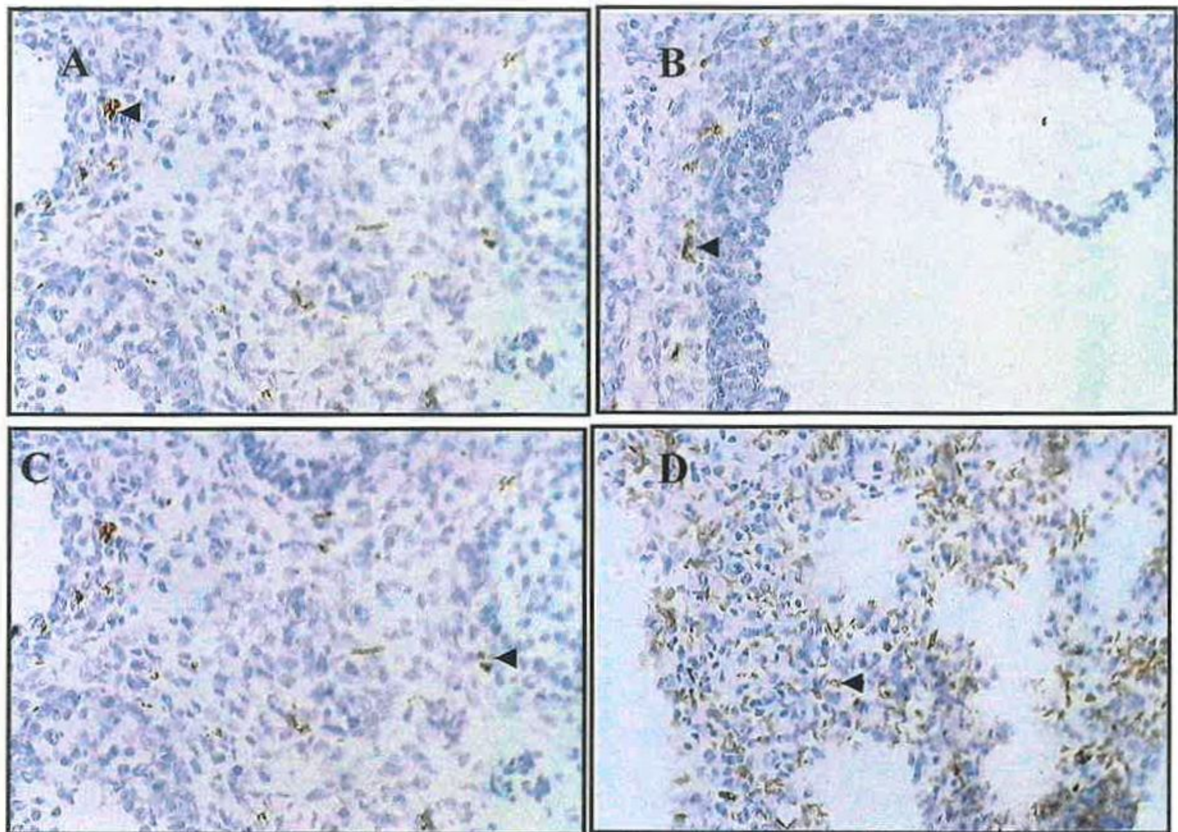
To assess the effect of leptin treatment on the recruitment of leukocytes into the ovary, immunohistochemistry using an indirect immunoperoxidase technique was employed, on sections cut from pre-ovulatory ovaries of immature-gonadotrophin primed rats treated with leptin or saline (Chapter 2.4). Fresh frozen tissue were serially sectioned (every 130 μm), fixed in acetone and leukocyte distribution was assessed using monoclonal antibodies (Chapter 2.3.2). Slides were counter-stained in haematoxylin, cleared and mounted (Chapter 2.3.5) and the distribution of leukocytes determined by quantifying the level of immunoperoxidase stain using a video image analysis system and Video Pro software (Chapter 2.3.6). All slides were stained in the same experiment to avoid inter-assay variation and were analysed on the one system. An independent observer carried out analyses on random samples using a blind protocol labeling system; the data generated between the two observers between these samples never differed more than 15 %. A cell showing the brown DAB signal was defined as a positive cell. The percent positivity was determined by dividing the number of positively stained cells (brown stain - neutrophils or monocytes/macrophages in the theca interna) by the total stain (brown stain and blue haematoxylin counterstain) in the pre-ovulatory follicle. The VIA software uses contrast for analysis and requires limits to be set manually. The mean positivity (3 thecal areas) for each pre-ovulatory follicle in the ovarian sections was tallied and this value was divided by the total number of follicles in all ovarian sections. The mean positivity of the stromal regions for each ovarian section was evaluated by analysing six random stromal regions per

section, then calculating the average for the ovary. Only healthy follicles were analysed. Atretic follicles and corpora lutea were distinguished from the remaining follicles according to the descriptions of Petrovska (1996). Positive control tissue (spleen, liver) and negative controls (where primary antibody incubation was omitted) were included. Both ovaries of four rats from each treatment group were analysed. Statistical analyses were performed using a parametric ANOVA followed by Tukey-Kramer Multiple Comparisons Test and significance was accepted if $P < 0.05$.

Granulocyte neutrophils were found to be located in both the thecal and stromal layer of pre-ovulatory follicles of all ovaries (Figure 4.4). Likewise, monocytes/macrophages were also distributed throughout the thecal and stromal layers of pre-ovulatory leptin, saline and pair fed treated ovaries (Figure 4.5). Leukocytes were not localised to the granulosa layer of pre-ovulatory follicles at any time (Figure 4.6).

4.4 Conclusions and discussion

The results in the previous chapter clearly show that leptin can reduce ovulation rates *in vivo* and *in vitro* when administered acutely. The mechanism of action however, remains uncertain. In this study, we set out to investigate whether leptin treatment of animals impaired pre-ovulatory follicle development. However, our results indicate that the numbers of pre-ovulatory follicles recruited for the ovulatory pool are unaffected by leptin treatment. Therefore, the decrease in ovulation observed in the previous chapter could not be attributed to a decrease in the number of pre-ovulatory follicles as both the number of these follicles and ovarian weights (Chapter 3.5) were unchanged in leptin-treated animals. It is possible that oocytes became trapped inside the follicles but this was not detected with



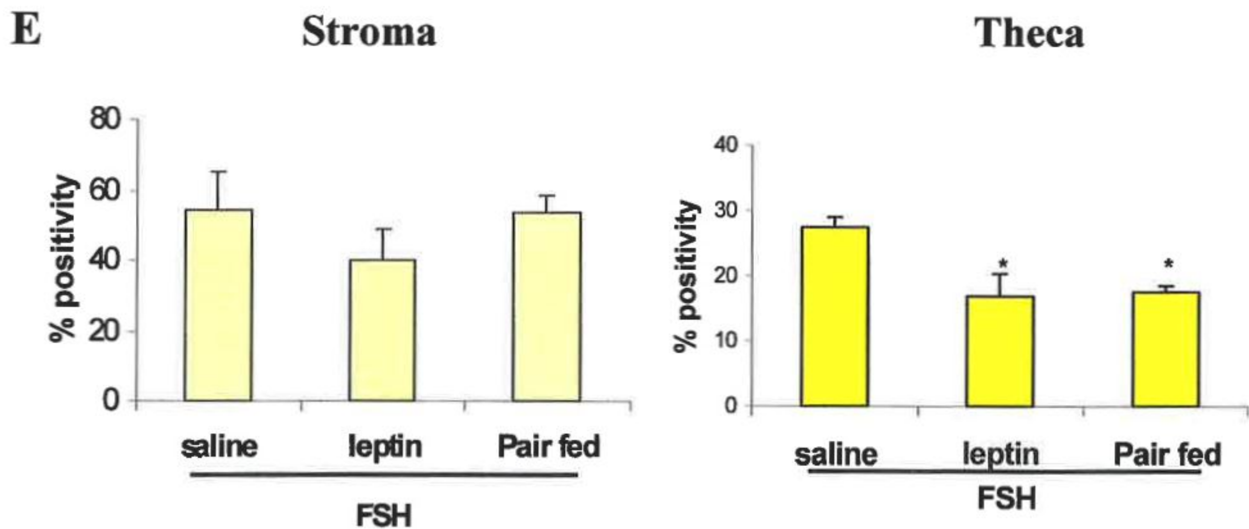
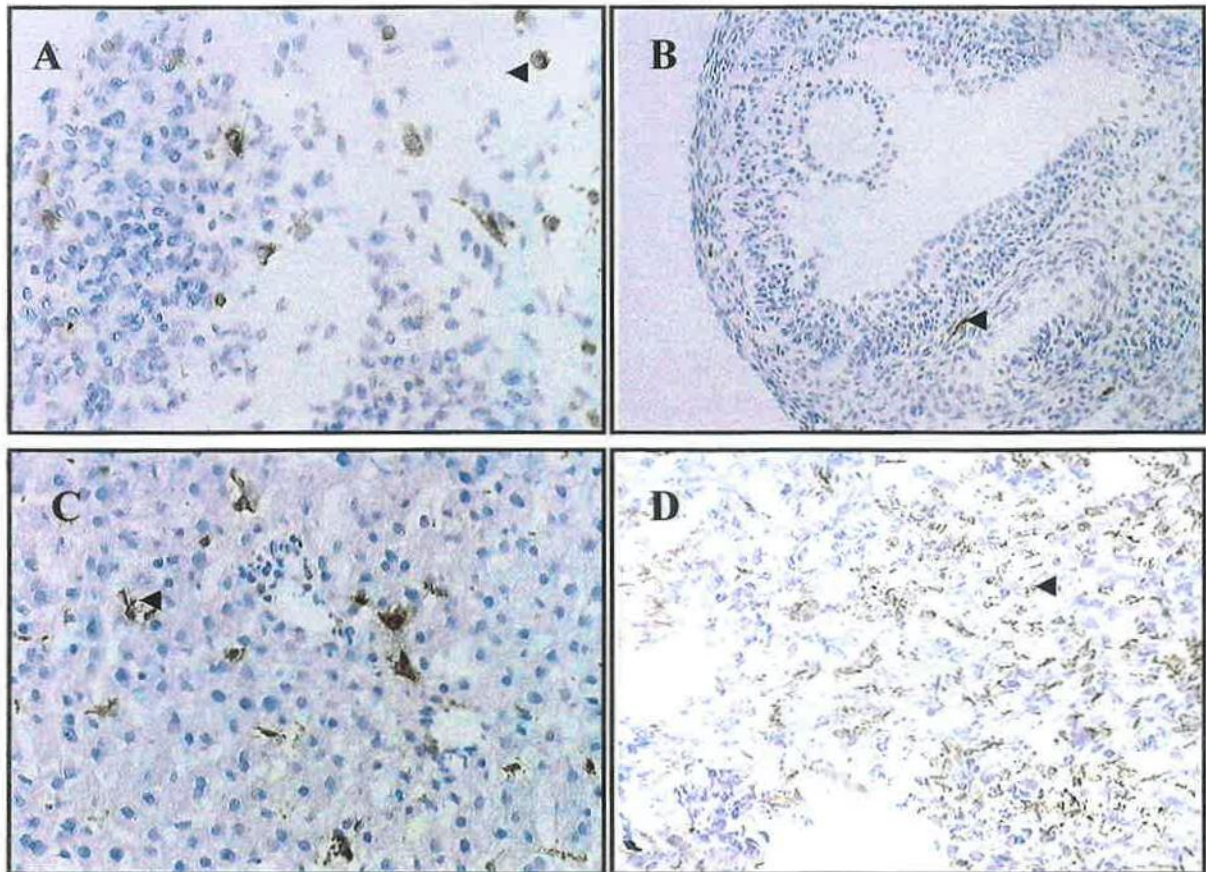


Figure 4.4 Immunohistochemical localisation of granulocyte neutrophils in the pre-ovulatory rat ovary. Ovaries were collected from gonadotrophin primed immature rats treated with leptin, saline or pair feeding. Neutrophils are stained with a monoclonal antibody against antigens on the surface of neutrophilic granulocytes. Neutrophilic granulocyte detection (arrow) in the A) stroma and B) theca of a pre-ovulatory follicle. Control tissues: C) liver and D) lung. E) Percent positive stain as assessed by video image analysis in the stroma and theca of pre-ovulatory follicles. * significantly different from gonadotrophin stimulated saline treated rats, as assessed by ANOVA and the Tukey Kramer Multiple Comparisons Test, $P < 0.05$. Data are presented as mean ± SEM, n = 4.



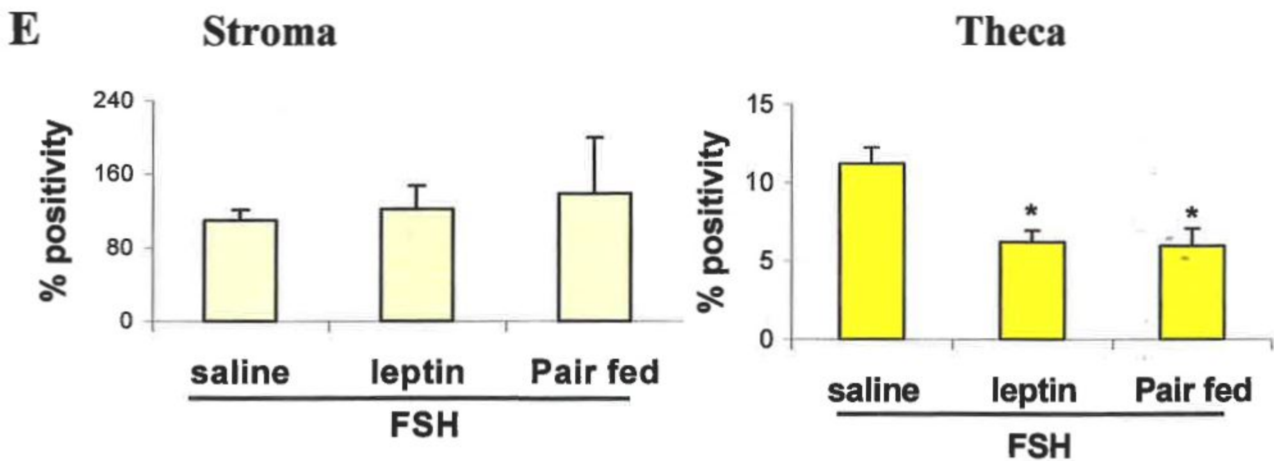


Figure 4.5 Immunohistochemical localisation of monocytes/macrophages in the pre-ovulatory rat ovary. Ovaries were collected from gonadotrophin primed immature rats treated with leptin, saline or pair feeding. Monocytes/macrophages are stained with a monoclonal antibody against the antigens on the surface of monocytes/macrophages. Monocytes/macrophages staining in the A) stroma and B) theca of a pre-ovulatory follicle. Control tissues: liver C) and lung D). E) Percent positive stain as assessed by video image analysis in the stroma and theca of pre-ovulatory follicles. * significantly different from gonadotrophin stimulated saline treated rats, as assessed by ANOVA and the Tukey Kramer Multiple Comparisons Test, $P < 0.05$. Data are presented as mean ± SEM, $n = 4$.

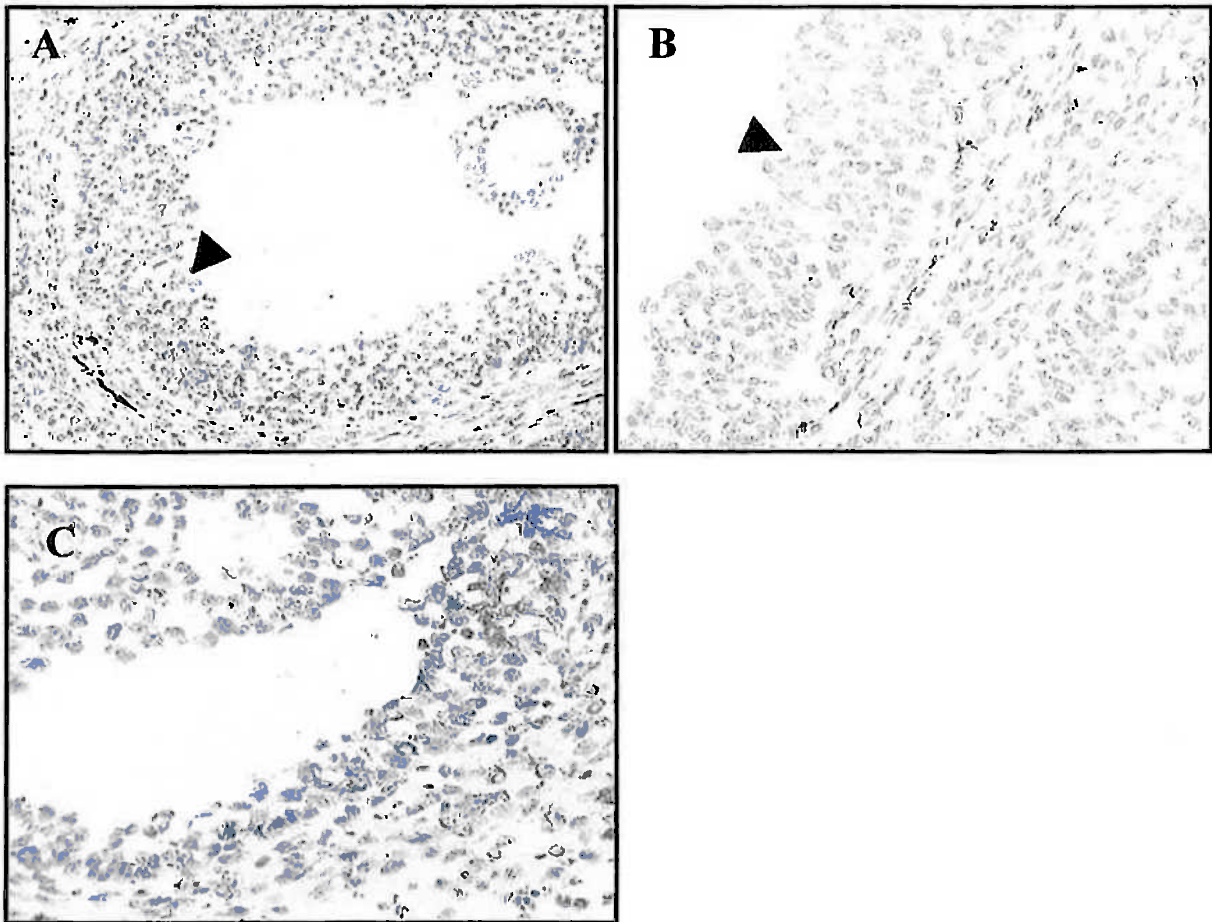


Figure 4.6 Immunohistochemical localisation of neutrophilic granulocytes and monocytes/macrophages in the granulosa layer of pre-ovulatory rat ovaries. Ovaries were collected from gonadotrophin primed immature rats treated with leptin, saline or pair feeding. Representative staining of A) neutrophils and B) monocytes/macrophages in the granulosa layers of pre-ovulatory follicles. C) A representative negative control ovarian follicle (where primary antibody was omitted).

histopathology. If this was the case, then leptin administration may have interfered with LH action, thereby preventing the release of the oocytes from the pre-ovulatory follicle. *In vitro* studies in dispersed ovaries performed using IGF-I suggested that leptin can impair LH action (Agarwal *et al.*, 1999).

Next, we hypothesised that the inhibition in ovulation observed in leptin treated animals may be the result of an inhibition in the infiltration of leukocytes into the ovary at the time of ovulation. The data presented in this chapter show that, as in other studies (Brannstrom *et al.*, 1993b; Brannstrom *et al.*, 1995), both neutrophils and monocytes/macrophages are found to be distributed throughout the thecal layer interna of pre-ovulatory follicles, but are virtually absent in the granulosa layer of these follicles. We found the numbers of thecal neutrophils and monocytes/macrophages to be significantly reduced ($P < 0.05$) in ovaries of rats treated with leptin and rats subjected to feed restriction, when compared to ovaries of rats treated with saline. This demonstrates that the number of neutrophils and monocytes/macrophages recruited at ovulation decrease significantly in response to a decrease in food intake. The current data extends previous observations (Hudson *et al.*, 1999) that a small (5 %) reduction in food intake is able to dramatically alter the numbers of macrophages in the mouse uterus.

In the previous chapter, ovulation rates in pair fed rats were not significantly different from FSH/saline treated controls and hence ovulation was unaffected by reduced food intake. Therefore this study shows that the reduction in thecal neutrophil or monocyte/macrophage numbers observed here is not likely to be the direct cause of the ovulation inhibition seen in leptin-treated rats as we hypothesised. The numbers of neutrophils and monocytes/macrophages found in the stroma were not affected by any of the treatments.

Therefore, the decline in infiltrating neutrophils and monocytes/macrophages in leptin-treated and feed restricted animals was a specific event isolated to the theca interna of pre-ovulatory follicles in response to decreased food intake.

Leukocytes are a major cellular component in the pre-ovulatory follicle prior to ovulation (Brannstrom *et al.*, 1993b; Brannstrom *et al.*, 1995; Norman *et al.*, 1997). This study demonstrates for the first time that even a small decrease in food intake, in otherwise normal animals (approximately 3.1 g), results in a decrease in leukocyte infiltration of pre-ovulatory follicles. The reduction in leukocyte infiltration into the pre-ovulatory follicle in response to decreased food intake may be an evolutionary response to prevent ovulation and hence pregnancy in times of low food availability. The cause of the specific leptin-induced inhibition in ovulation, independent of caloric restriction, in our previous study is not clear. The decline in neutrophils and monocytes/macrophages with acute leptin treatment prior to ovulation suggests that excessive leptin in circulation may play an inhibitory role in the behaviour of pre-ovulatory follicles at the time of ovulation.

In summary, our results indicate that leptin is able to affect factors involved in the ovulation cascade. This study shows that leptin's feed restrictive effects cause a decline in neutrophil and monocytes/macrophage infiltration into the theca layer of pre-ovulatory follicles. This suggests that although pair fed animals consume the same amounts of food as the leptin treated animals, the pair fed animals must have a compensatory mechanism to cope with inhibitory effects on ovulation. It appears that neutrophils and monocytes/macrophages are highly sensitive to a decline in feed restriction at the time of ovulation as reflected by decreased infiltration of these leukocytes following the feed

restriction, however, leptin was unable to affect the numbers of pre-ovulatory follicles being recruited into the ovulatory pool.

Chapter 5

Leptin treatment on meiotic maturation, granulosa cell proliferation, steroid hormone and prostaglandin E_2 synthesis in rat ovarian follicle culture.

5.1 Introduction

Leptin has been shown to have significant negative effects on cellular components of the ovary (Spicer and Francisco, 1997; Zachow and Magoffin, 1997; Spicer and Francisco, 1998; Agarwal *et al.*, 1999). Several lines of evidence indicate that high leptin concentrations can inhibit the stimulatory actions of many hormones important in the selection of dominant follicles (IGF-I, TGF-β, FSH and insulin). 1) High leptin concentrations can block the stimulatory effects of IGF-I on FSH-stimulated rat granulosa cell oestradiol but not progesterone production (Zachow and Magoffin, 1997; Agarwal *et al.*, 1999). 2) Leptin can suppress the positive effect of TGF-β on FSH-dependent oestrone and oestradiol levels in cultured rat granulosa cells, aromatase mRNA expression and aromatase activity (Zachow *et al.*, 1999). 3) Leptin inhibits insulin-induced granulosa cell oestradiol production and decreases the insulin-induced increases in bovine granulosa cell progesterone production (Spicer and Francisco, 1997). 4) Leptin can suppress FSH and dexamethasone induced pregnenolone, progesterone and 20α-hydroxy-4-pregnen-3-one production in rat granulosa cells, a result partly caused by a reduction of an enzyme in the P450scc system (adrenodoxin) (Barkan *et al.*, 1999). 5) Leptin inhibits insulin stimulated progesterone production from human luteinised granulosa cells (Brannian *et al.*, 1999). The presence of leptin receptors on rat theca cells has not been demonstrated and the finding that hypophysectomised rat thecal cells lack a functional leptin receptor (Zachow *et al.*, 1999) means that leptin research in the rat ovary has mainly focussed on the effects of leptin on granulosa cells. However, several studies have demonstrated that leptin has inhibitory effects on human and bovine thecal cells (Spicer and Francisco, 1998; Agarwal *et al.*, 1999).

We have reported that acute leptin administration to immature gonadotrophin-primed rats inhibits ovulation independent of caloric restriction in earlier chapters. The previous chapter also shows that an inhibition of leukocyte infiltration is not the direct cause of the inhibition in ovulation. To investigate the cause of the inhibition in ovulation in leptin treated immature gonadotrophin primed rats, a series of experiments were designed. We investigated whether key factors in the ovulatory process, namely meiotic maturation, granulosa cell DNA synthesis, steroid and prostaglandin synthesis and interleukin-1 secretion, were disturbed by high concentrations of leptin, *in vitro*.

5.2 Meiotic maturation

Meiotic maturation of cultured follicles following 4 and 12 hours of incubation was assessed (Chapter 2.6). The rate of meiotic maturation was not affected by leptin treatment, regardless of the amount of incubation (Figure 5.1). Analysis of follicles revealed that incubations with IGF-I, control or leptin did not induce meiotic maturation, while incubations with the gonadotropins FSH and LH induced meiotic maturation of follicle-enclosed oocytes. Addition of leptin to cultures did not inhibit meiotic maturation in any instance.

5.3 Steroid hormone, prostaglandin and interleukin-1β synthesis

Oestradiol and progesterone production from both 4h and 12h follicle cultures was assessed (Chapter 2.8). Control, leptin or IGF-I treatments stimulated little oestradiol production with 4 or 12 hours of incubation (Table 5.1). The addition of the gonadotrophins, FSH or LH, stimulated oestradiol production at 4h and 12h, regardless of whether IGF-I or leptin were

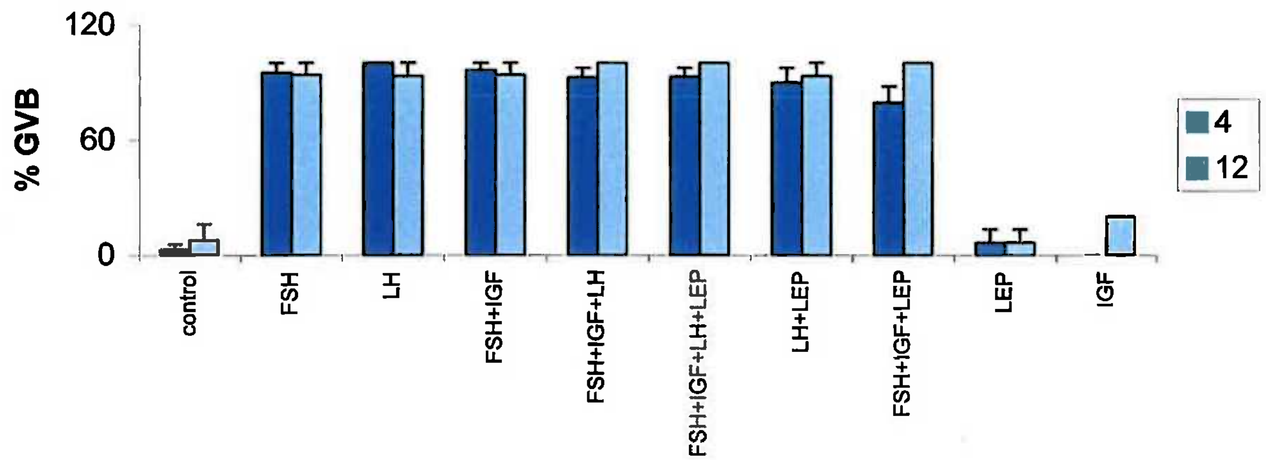


Figure 5.1 Percentage germinal vesicle breakdown (% GVB) following 4 or 12h follicle culture. Follicles were cultured with FSH (500mIU), LH (100ng/ml), IGF-I (50ng/ml) or leptin (300ng/ml) for a period of 4 or 12 h, and the meiotic maturation of follicle enclosed oocytes assessed.

Table 5.1 Oestradiol, progesterone and prostaglandin E_2 production from 4 and 12 h follicle culture. Letters indicate significant difference to the control, as analysed by the Dunnett's multiple comparisons test ($p < 0.001$).

FSH	-	-	+	-	+	+	-	-	+	+
LH	-	-	-	+	-	+	-	+	-	+
IGF-I	-	+	-	-	+	+	-	-	+	+
Leptin	-	-	-	-	-	-	+	+	+	+
4h E_2/ follicle nmol/L	1.5 ± 0.2	1.48 ± 0.5	8.6 ± 1.3 [a]	6.5 ± 2.0 [a]	5.5 ± 0.3 [a]	6.8 ± 1.2 [a]	1.0 ± 0.7 [a]	6.1 ± 1.5 [a]	6.2 ± 0.3 [a]	6.4 ± 1.1 [a]
4h Prog/ follicle nmol/L	0.2 ± 0.01	0.3	6.1 ± 0.13 [a]	29.9 ± 1.9 [a]	6.7 ± 0.22 [a]	34.1 ± 3.6 [a]	0.2	38.2 [a]	7.8 ± 0.54 [a]	36 ± 2 [a]
12h E_2/ follicle nmol/L	1.86 ± 0.3		7.1 ± 0.6 [a]	8.0 ± 2.1 [a]	9.1 ± 2.5 [a]	6.8 ± 0.6 [a]	3.7 ± 0.4 [a]	7.8 ± 2.0 [a]	7.5 ± 1.2 [a]	7.8 ± 1.2 [a]
12h Prog nmol/L	0.48 ± 0.2		8.5 ± 0.2 [a]	24.9 ± 4.8 [a]	12.2 ± 1.3 [a]	23 ± 4 [a]	1.25 ± 0.12	24.5 ± 3.9 [a]	10.3 ± 0.4 [a]	27.3 ± 4.3 [a]
12h PGE_2/ follicle ng/ml	0.09 ± 0.02		1.2 ± 0.3	3.4 ± 0.5	1.6 ± 0.2	3.9 ± 0.6	0.1 ± 0.02	2.3 ± 0.6	1.0 ± 0.3	3.6 ± 0.5
IL-1β pg/ml	65.8 ± 5.8		61.6 ± 2.7	68.7 ± 3.4	61.3 ± 2.3	64.7 ± 2.3	67.2 ± 2.5	71.8 ± 1.9	70.1 ± 2.1	70.3 ± 2.1

added. Progesterone production was stimulated above control values when FSH was added, however, the stimulation was far greater when LH was added. Addition of IGF-I in combination with FSH, enhanced progesterone production above the levels observed with FSH alone. Leptin addition to the cultures did not influence progesterone production at either time point. Prostaglandin E_2 levels were measured after 12h of culture. The addition of LH, FSH or FSH and IGF-I to the cultures enhanced prostaglandin E_2 production above basal levels. The addition of leptin to cultures incubated with LH, FSH and IGF-I, or FSH, IGF-I and LH resulted in an insignificant trend of decreased prostaglandin E_2 production ($p=0.056$). Interleukin-1β levels in the culture media of treated follicles were similar regardless of the culture treatment.

5.4 Granulosa cell proliferation assay

Incorporation of ^3H into the DNA of cells challenged with FSH and IGF-I in the presence or absence of leptin was used to assess the effect of leptin on granulosa cell proliferation (Chapter 2.6.3). FSH addition to cells enhanced the level of proliferation above the basal level (Figure 5.2). Leptin treatment in the presence of FSH and IGF-I or IGF-I alone did not alter the level of cell proliferation.

5.5 Granulosa cell culture

Granulosa cells were challenged with androstenedione (0.1 μM) in the presence of FSH, IGF-I and leptin (Chapter 2.6.4) in order to confirm previous studies, which have shown that leptin inhibits the IGF-I augmentation of FSH-dependent oestradiol production (Zachow and

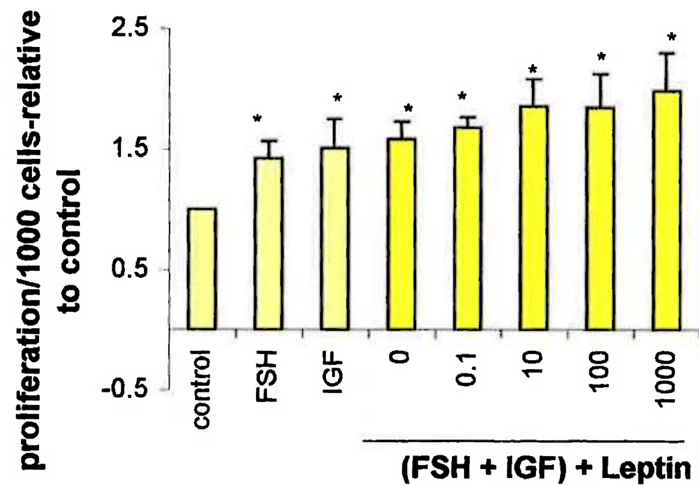


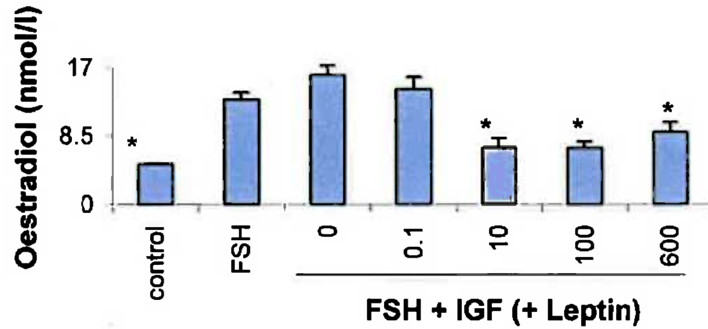
Figure 5.2 The level of granulosa cell proliferation, as assessed by measuring the level of ^{3}H incorporation into granulosa cell DNA. DNA synthesis (relative to control) following treatment with FSH, IGF-I, or FSH and IGF-I in the presence of varied concentrations of leptin is shown. * significantly different to control, as assessed by ANOVA and the Tukey Kramer Multiple Comparisons Test, $P < 0.05$. Data are presented as mean ± SEM.

Magoffin, 1997). FSH induced both oestradiol and progesterone production above control levels (Figure 5.3). The addition of IGF-I with FSH induced oestradiol and progesterone production above FSH levels. There was no significant difference between FSH with IGF-I treatment and FSH and IGF-I in the presence of 0.1 ng/ml leptin. Leptin concentrations above 10 ng/ml, in the presence of FSH and IGF-I, resulted in an inhibitory effect on oestradiol production while progesterone production was unaffected.

5.6 Conclusions and discussion

The inhibitory effects of high leptin concentrations on the ovary have been described in many studies. We have previously demonstrated that chronic leptin treatment *in vivo* inhibits ovulation. The current study was designed to investigate the cause of the ovulation inhibition reported previously. In our previous studies, we established that the leptin initiated decrease in food intake resulted in a decrease in leukocyte infiltration. However, this was unlikely to be the sole factor responsible for the inhibition in ovulation observed in leptin treated animals, as feed restricted animals also experienced lower ovarian leukocyte infiltration, while their ovulations were similar to ad-libitum fed rats. The current study investigated the direct effects of high concentrations of leptin on gonadotrophin-primed follicles, cultured *in vitro*. This data shows that leptin treatment does not hamper meiotic maturation, steroid hormone secretion, interleukin-1β secretion or granulosa cell DNA synthesis. However, a slight effect may occur on prostaglandin production. Furthermore, this study confirms previous findings (Zachow and Magoffin, 1997) and shows the effect of leptin treatment on oestradiol production of FSH and IGF-I cultured granulosa cells in the presence of androstenedione.

A



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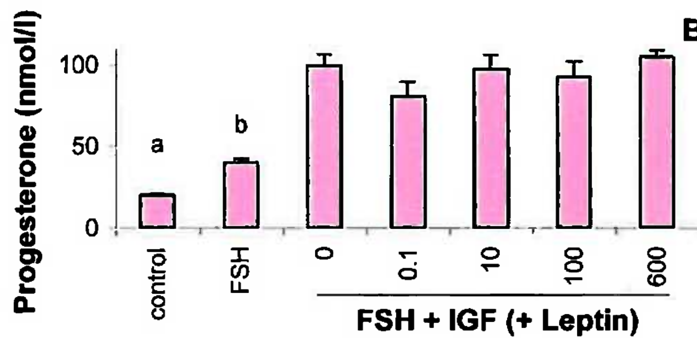


Figure 5.3 The level of steroid hormones, A) oestradiol and (B) progesterone, produced by granulosa cells challenged with androstenedione. Granulosa cells were cultured in the presence or absence of FSH, IGF-I, or FSH and IGF-I in the presence of varied concentrations of leptin (ng/ml) and challenged with androstenedione. * or letters indicate significant difference to remaining groups, as assessed by ANOVA and the Tukey Kramer Multiple Comparisons Test, $P < 0.05$. Data are presented as mean ± SEM, $n = 4$.

In vitro studies in dispersed ovaries performed with IGF-I suggested that leptin can impair LH action (Agarwal *et al.*, 1999). Our previous results also indicated that leptin may inhibit LH action in the ovary. We therefore set out to investigate the effects of leptin on LH action in isolated rat follicles. Gonadotrophin stimulated follicle culture produced similar levels of oestradiol and progesterone as previously reported (Tornell *et al.*, 1995). Leptin did not influence steroid synthesis when cultured in the presence of FSH, LH or IGF-I. These results contradict previous results of isolated components of the ovary, as granulosa cell cultures have clearly demonstrated that leptin has an adverse effect on the action of sensitising agents, such as IGF-I. However, we believe our culture system provides a new dimension to current leptin research in the ovary. Our culture system minimises follicle-follicle interaction. Previous research has shown that co-culture of more than 1 follicle with direct contact between follicles results in the growth and development of one follicle while the other is depressed (Nayudu and Osborn, 1992; Spears *et al.*, 1996). In order to confirm previous findings and validate the activity of the hormones used in our study, we cultured granulosa cells in the presence of androstenedione and leptin and measured oestradiol and progesterone production. Our data confirms previous findings (Zachow and Magoffin, 1997) and shows that isolated granulosa cells cultured with leptin, inhibit oestradiol production, giving a different result to that found with whole cultured follicles.

Like other studies (Gutierrez *et al.*, 1997), we found that physiological concentrations of FSH led to proliferation of granulosa cells, as indicated by DNA synthesis, and the inclusion of IGF-I also enhanced proliferation. However, granulosa cell DNA synthesis was unaffected by leptin treatment, a finding that has also been demonstrated in bovine granulosa cells (Spicer and Francisco, 1997). Our results indicate that high leptin concentrations inhibit ovulation using a pathway other than the path of granulosa cell DNA synthesis. Systemic changes, in response to

food availability and hence the concentration of leptin, are reflected in parallel to changes that occur in follicular fluid essentially as the result of leptin diffusion from blood. This follows as leptin concentrations in blood and follicular fluid correlate in both humans (Agarwal *et al.*, 1999) and pigs (Galeati *et al.*, 2000). Leptin treatment (10 ng/ml) of oocytes retrieved from prepubertal gilts reduced the number of oocytes in GV from 60 to 33 % (Galeati *et al.*, 2000). Preliminary data from *in vitro* mouse oocyte culture from our laboratory also indicates that leptin inhibits germinal vesicle breakdown. However, our data shows that the meiotic competence of follicle-enclosed oocytes is not compromised with high concentrations of leptin in rats, indicating that follicle enclosed oocytes will respond to gonadotrophin stimulus regardless of the concentration of leptin. Our results indicate that a species difference in the way leptin interacts with oocytes is important.

The importance of prostaglandins in the ovulation process has been established (Espey and Lipner, 1994). Prostaglandins E and F begin to increase in ovarian follicles during the first several hours of the ovulatory process. These prostaglandins reach a peak at the time of follicular rupture and levels decline and return to normal toward pre-ovulatory values following ovulation. Prostaglandins have roles in pro- and anti-inflammation. We therefore hypothesised that chronic leptin treatment may diminish the prostaglandin levels at the time of ovulation. Leptin appeared to have an insignificant effect on prostaglandin production, however, future studies may ascertain the mechanism by which ovulation is inhibited as the mechanism of action remains uncertain. Our data indicates that elevated leptin concentrations are able to exert a direct inhibitory effect on ovarian function by inhibiting prostaglandin E_2. However, leptin treatment of neonatal rat hypothalami with leptin appears to stimulate the production of prostaglandin E_2 (Brunetti *et al.*, 1999).

The processes within the ovary that follow the LH surge and lead to ovulation have been well characterised (Brannstrom and Norman, 1993c; Norman *et al.*, 1996; Norman *et al.*, 1997; Norman *et al.*, 1999). LH initiates an increase in interleukin-I production as well as the induction of a variety of other cytokines, collagenases, plasminogen activators and enzymes (Norman *et al.*, 1999). Follicular fluid contains detectable concentrations of immunoactive cytokines including interleukins, with granulosal-luteal cells capable of producing detectable levels of these (Norman *et al.*, 1996). Leptin appears to be able to affect pro-inflammatory cytokines. Leptin action on food intake is believed to be mediated by IL-1 (Luheshi *et al.*, 1999) and therefore, we hypothesised that interleukin-1 may be influenced by leptin treatment. The level of interleukin-1β in the follicle culture media was similar within treatments, possibly indicating that interleukin-1β was not secreted from the follicles into the media. However, levels within the follicle may have been affected by the various treatments. Future experiments are required to investigate the effects of leptin on interleukin-1β production inside ovarian follicles.

In summary, this study demonstrates that the effects of high leptin levels on ovarian follicles *in vitro*. In our studies we found high concentrations of leptin were unable to inhibit germinal vesicle breakdown in oocytes of gonadotrophin stimulated enclosed follicles. Steroid synthesis was unaffected by leptin, while interleukin-1β levels were similar regardless of the treatments. We found that prostaglandin synthesis was marginally inhibited by leptin treatment, while granulosa cell DNA synthesis was unaffected. Leptin treatment of isolated granulosa cells cultured in the presence of androstenedione, FSH and IGF-I showed inhibitory effects on oestradiol production.

Chapter 6

Leptin receptor (*OB-RB* & *OB-RA*) expression throughout the oestrous cycle in the mature rat ovary.

6.1 Introduction

Two main sites of leptin action relating to the reproductive system are the brain and ovary (Chapter 1.1.5, Chapter 1.3). The importance of the leptin protein in the brain has been well documented (Campfield *et al.*, 1996; McCowen *et al.*, 1998). Recent studies have demonstrated the significance of leptin in the regulation of reproductive processes in the human and rat ovary (Cioffi *et al.*, 1997; Agarwal *et al.*, 1999; Barkan *et al.*, 1999; Cunningham *et al.*, 1999; Keisler *et al.*, 1999; Kitawaki *et al.*, 1999; Zachow *et al.*, 1999) and the previous chapters have discussed the inhibitory effect of acute leptin treatment on the ovulation process in the rat.

Leptin and the secretion of steroid hormones appear to be directly linked (Chapter 1.3.2). Leptin levels in peripheral blood samples vary throughout the human menstrual cycle (Licinio *et al.*, 1998; Lukaszuk *et al.*, 1998; Messinis *et al.*, 1998) with leptin peaking in the luteal phase in a similar manner to 17β-oestradiol and progesterone (Slieker *et al.*, 1996; Lukaszuk *et al.*, 1998). Leptin gene expression in fat decreases with ovariectomy (Yoneda *et al.*, 1998), while leptin stimulates the production of oestrogen in periovarian fat and increases the production of progesterone and testosterone in the ovary (Cannady *et al.*, 2000). Therefore, the variations in leptin levels across the menstrual cycle may alter the responsiveness of the ovary to leptin.

Leptin has a short regulatory loop controlling leptin receptor mRNA synthesis in the brain. Six isoforms of the leptin receptor have been identified (Chapter 1.1.4). The long isoform of the leptin receptor (*OB-RB*) is believed to be the main form responsible for leptin signal transduction. *OB-RB* gene expression (Zachow *et al.*, 1999) and the *OB-RB* receptor have

been localised in the rat ovary (Kim *et al.*, 1999). Several lines of evidence indicate that the leptin receptors are regulated and respond to changes in circulating steroid hormones and leptin levels. Firstly, leptin receptors are differentially expressed in the hypothalamus at different stages of the oestrous cycle. The lowest level of total leptin receptor transcripts occurs in the choroid plexus at pro-oestrus, corresponding inversely to the highest level of oestradiol in the 4-day cycle of the rat (Bennett *et al.*, 1999). Secondly, leptin levels vary with the length of the day and food deprivation (Saladin *et al.*, 1995; Caro *et al.*, 1996) and studies in Siberian hamsters and mice have shown that leptin receptor (*OB-RB* and others) mRNA expression varies in response to the changes in leptin (Baskin *et al.*, 1998; Mercer *et al.*, 2000). Thirdly, leptin infusion into the brain results in an increase in leptin receptor transcripts (Dickson and Bennett, 1999). Fourth, leptin deficient mice (Mercer *et al.*, 1997) and fasting rats (Bennett *et al.*, 1998) have increased leptin receptor gene expression in the brain and leptin treatment in these animals reduces leptin receptor transcripts (Mercer *et al.*, 1997). Finally, leptin administration into New Zealand Obese mice does not result in weight loss, however, *OB-RB* mRNA is reduced by 30 % in the arcuate nucleus, a main site of *OB-RB* localisation (Baskin *et al.*, 1998). This feedback loop also appears to work in the opposite direction in adipose tissue, as reduced copy number for the functional leptin receptor results in a diminished feedback signal to the leptin gene (Zhang *et al.*, 1997). Therefore, leptin appears to regulate leptin receptor gene expression.

A species difference between rodents and humans exists in the circulation of leptin levels across the reproductive cycle, with leptin levels remaining constant across the oestrous cycle of the mouse (Chehab *et al.*, 1997) and rat (Bennett *et al.*, 1999). This led to the hypothesis that leptin receptor levels vary across the rat oestrous cycle in order to alter the ovarian responsiveness to leptin. This chapter addresses whether ovarian leptin receptor

expression varies throughout the oestrous cycle in response to the changing hormonal environment of the ovary, thus modulating the sensitivity of the leptin protein. The purpose of this study therefore, was to determine the relationship between plasma leptin, steroid hormone secretion and leptin receptor (*OB-RB* and *OB-RA*) expression at the various stages of the oestrous cycle using the highly sensitive technique, real time RT-PCR.

6.2 Determination of oestrous cycle stage

The technique of vaginal smears (Figure 6.1) was used to identify the stage of the oestrous cycle (Chapter 2.2.8). Several qualitative criteria were enforced in order to minimise variation and ensure accurate assessment of the oestrous cycle (Chapter 2.7.1). The vaginal population of cells varies with the stage of oestrous cycle (Table 2.1), with different cell populations being predominant at the different stages (Figure 6.2).

6.3 Generation of standard *OB-RA* and *OB-RB* DNA

Ovaries were collected from rats at different stages of the cycle and frozen until RNA extraction (Chapter 2.7.3). RNA was extracted and quantified prior to being subjected to a reverse transcription reaction. Fragments of the long and short isoforms of the leptin receptor were generated by PCR (Figure 6.3) using the appropriate primers and cloned into the TOPO TA cloning vectors (Chapter 2.7.5). The identity of the inserts was confirmed by direct sequencing and enzymatic digestion (Figure 6.4A). The vector-inserts were linearised for the preparation of standards for *OB-RB* and *OB-RA* (Figure 6.4B). Standards were diluted to provide a range of concentrations in order to prepare a standard curve (Figure 6.5) and real time PCR performed. Standard curve data is expressed as the amount (fg) of

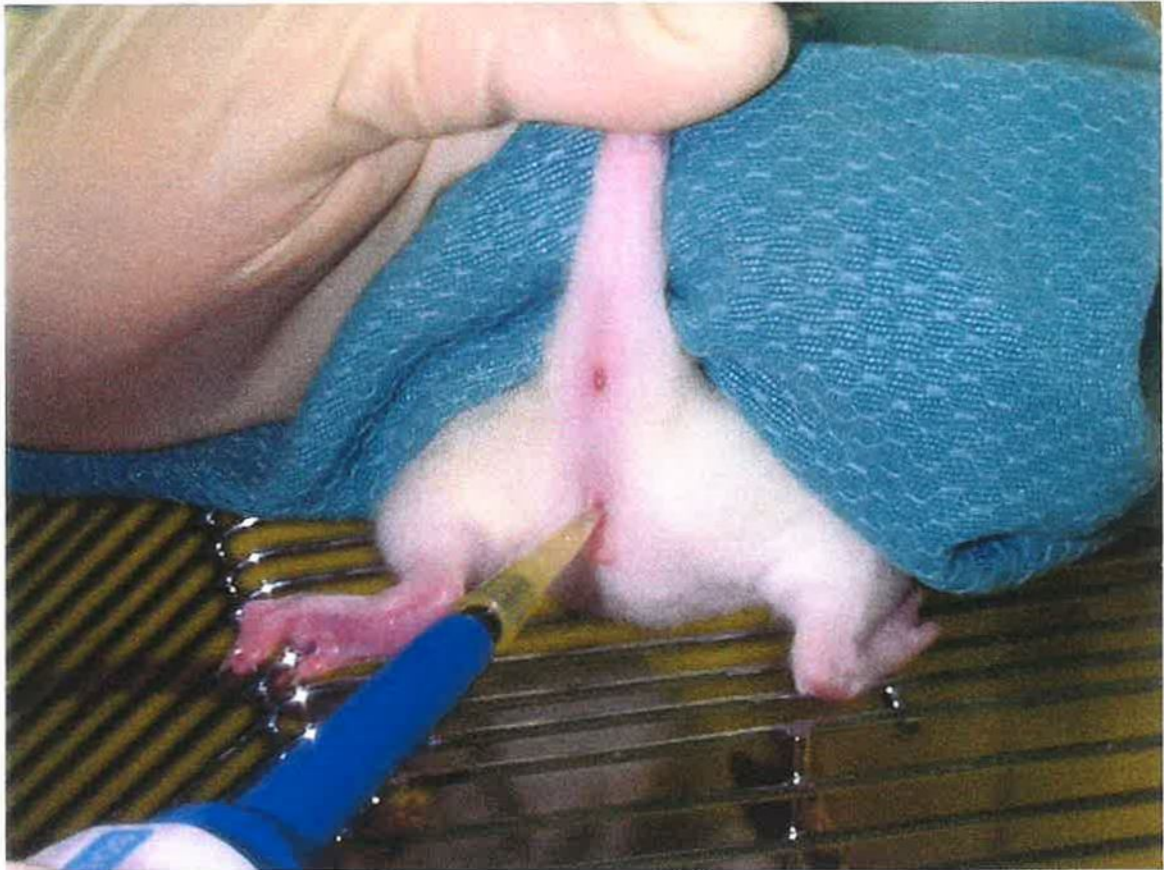


Figure 6.1 The technique of vaginal smearing. Approximately 30 µl of sterile saline is flushed into the vagina, twice and the sample viewed as an unstained wet preparation on a microscope slide with a coverslip under an inverted microscope. The stage of oestrus is determined according to Table 2.1.

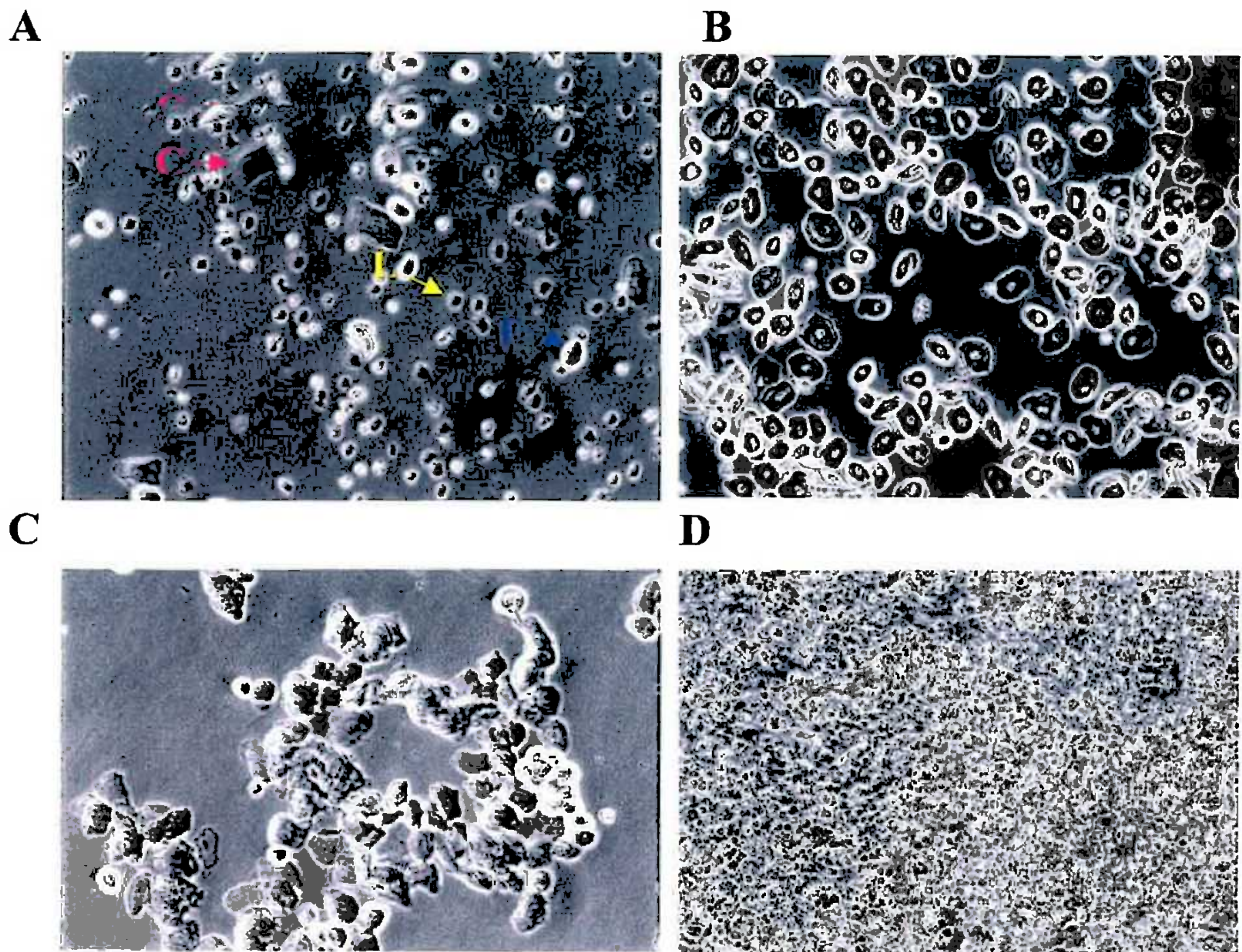


Figure 6.2 Oestrous cycle can be identified by the presence/absence of cell populations (epithelial, cornified and leukocyte) in the vagina. A) Pro-oestrus: a majority of epithelial (E), with occasional populations of cornified (C) and leukocyte (L) cells. B) Met-oestrus I: cornified cells only, C) Met-oestrus II: a majority of cornified and leukocyte populations, with occasional epithelial cells appearing. D) Mostly epithelial and leukocyte populations with mucous also apparent.

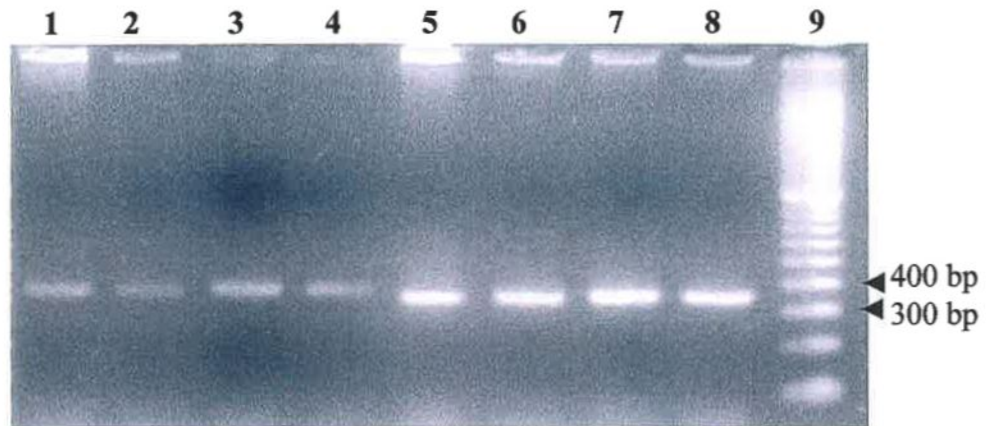


Figure 6.3 Amplification of *OB-RA* and *OB-RB* PCR products. RNA extracted from whole rat ovaries was reverse transcribed and PCR products generated. Total products from the PCR reactions were electrophoresed on a 2 % agarose gel and visualised using ultraviolet light and ethidium bromide. Lanes 1-4 - *OB-RB* PCR products (375 bp), lanes 5-8 - *OB-RA* PCR products (346 bp) and Lane 9 - 100 bp ladder.

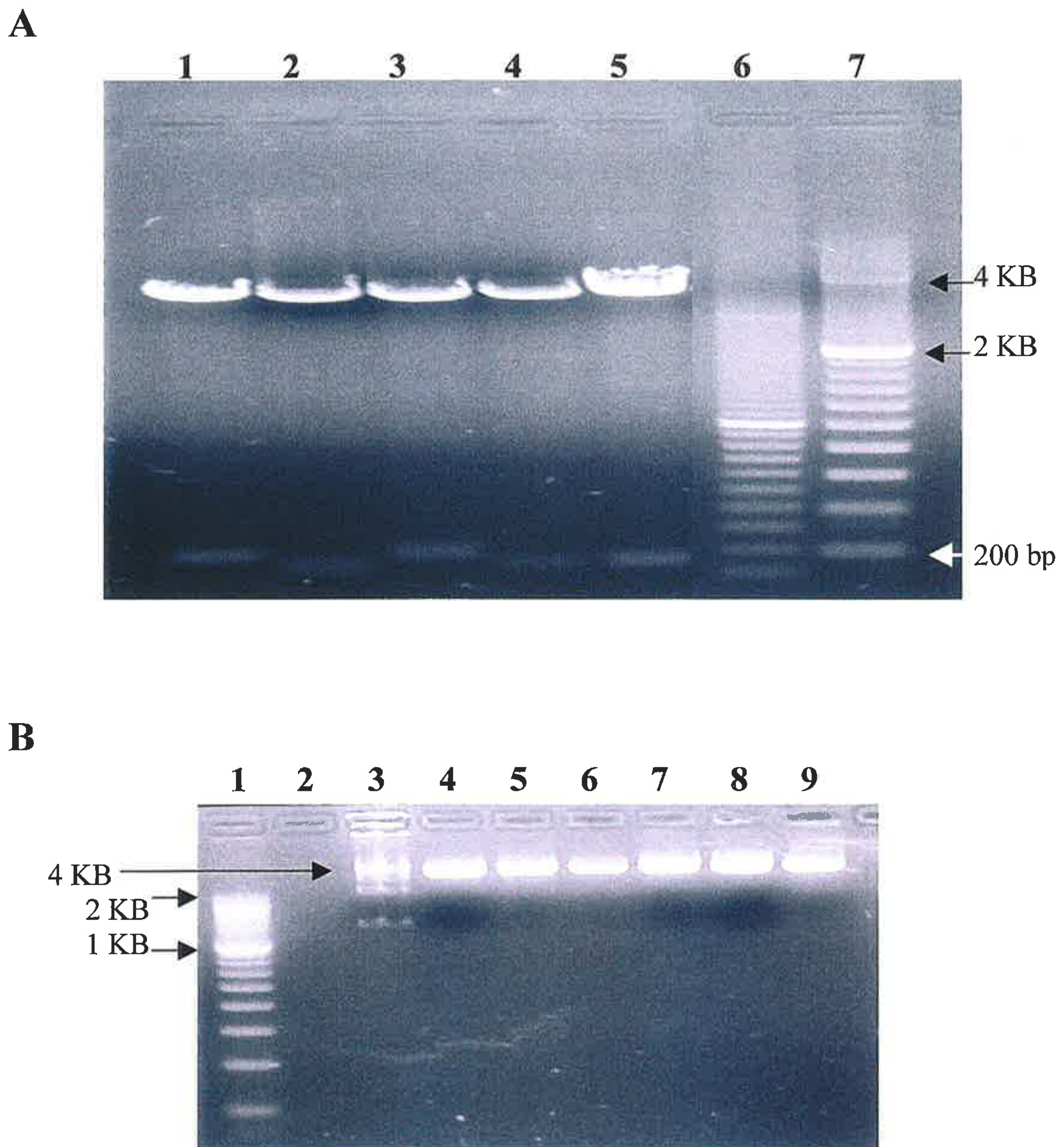


Figure 6.4 Confirmation of PCR product insert insertion into TOPO TA cloning vectors. Clones containing TOPO TA with insert (*OB-RA* or *OB-RB*) were miniprepped and the DNA purified for enzymatic digestion. The presence of the insert was assessed with EcoRV enzymatic digestion and positive clones, releasing 217 bp & 4029 bp for *OB-RA* (A) were linearised using HindIII for *OB-RA* (and KpnI for *OB-RB* – data not shown). Lane contents: (A) Lanes 1-5 - digested TOPO vector, releasing *OB-RA*, Lane 6 - 100 bp ladder and Lane 7 - 200 bp DNA ladder. (B) Lane 1 - 100 bp ladder, lane 2 - negative control, lane 3 - 1 KB DNA ladder, lanes 4-9 - linearised TOPO-*OB-RA* complex.

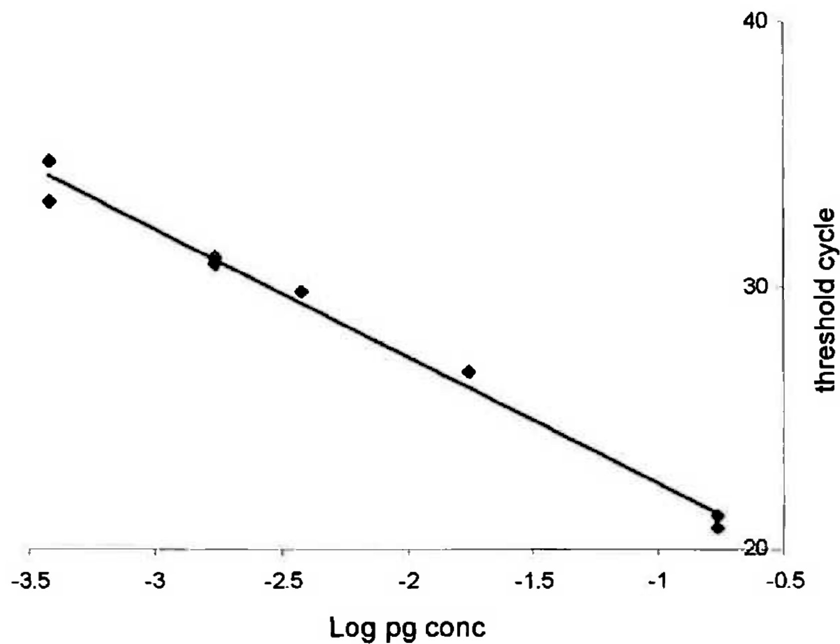


Figure 6.5. A typical standard curve showing log standard concentration (pg) of the leptin receptor (*OB-RB*) against PCR cycle number. Leptin receptor insert-TOPO vector was diluted to give various concentrations of vector + insert (standard) DNA. The concentration of *insert alone* was established to quantitate the amount of full-length cDNA generated from mRNA samples. Real time PCR analysis revealed the cycle number at which the curve was in an exponential phase. This cycle number was plotted against the known standards concentration to provide a standard curve for unknown samples.

insert DNA alone (from the insert-vector), therefore, the results reflect the amount of full length cDNA generated from mRNA samples. The threshold cycle, the cycle at which point the reporter dye emission intensity is above background noise, was used to determine the cycle number at which PCR amplification was in an exponential phase (Figure 6.6).

6.4 Real time RT-PCR of *OB-RA* and *OB-RB*

Real time RT-PCR for *OB-RA* and *OB-RB* isoforms was performed to establish the expression pattern of the leptin receptor genes throughout the oestrous cycle (Chapter 2.7.6). *OB-RA* real time RT-PCR analysis revealed that leptin receptor expression was at its maximum in the met-oestrus phases with the highest level of expression noted in the met-oestrus II stage (Figure 6.7A). Real time RT-PCR of *OB-RB* revealed a similar expression pattern with the highest levels of *OB-RB* gene expression recorded at met-oestrus, while the lowest levels were recorded at pro-oestrus and di-oestrus (Figure 6.7B). PCR products were also visualised using gel electrophoresis to confirm the production of a single, correctly sized product (Figure 6.8A). After the 40 cycles, gel electrophoresis revealed that the end quantity of the PCR products of each tube, regardless of starting concentrations, were identical (Figure 6.8A). Therefore, by using real time RT-PCR we were able to quantify the small differences among samples, accurately, by analysing data in the exponential phase. Genomic DNA was quantified in ovaries in order to control for variation between tissues. Data is expressed as fg RNA/μg total DNA.

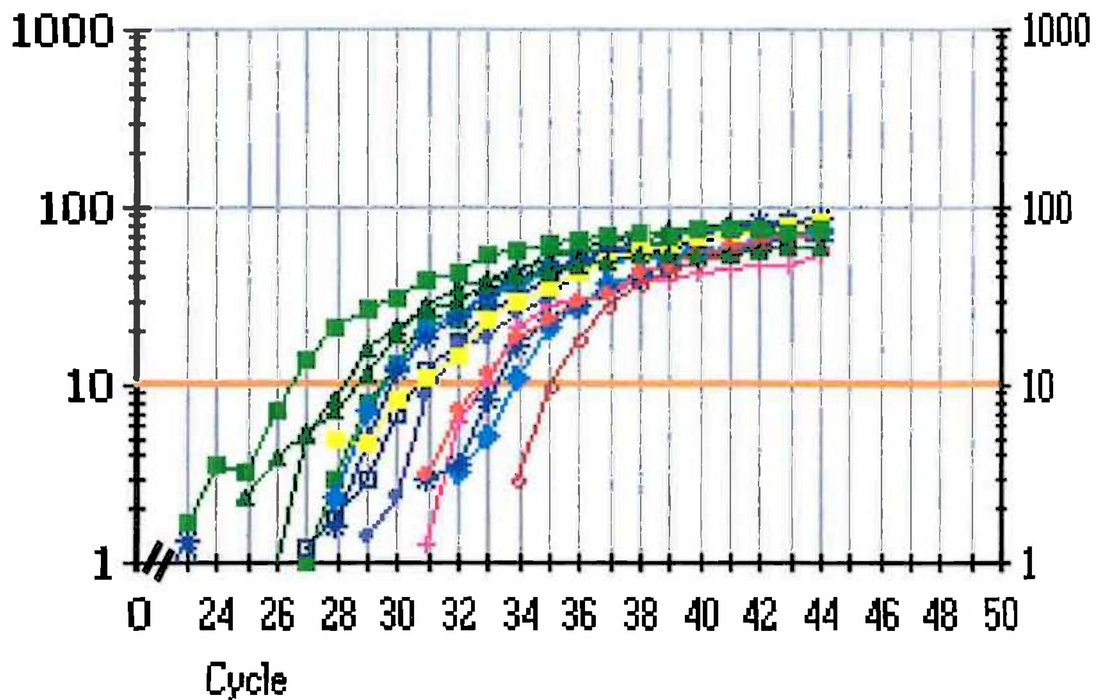
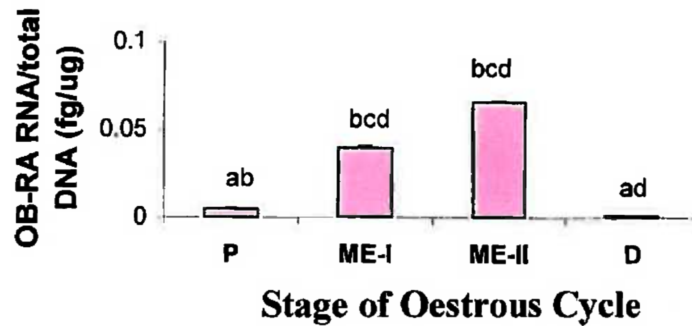


Figure 6.6 A sample graph of real time RT-PCR. The amplification of the PCR products (log scale) is shown against PCR cycle number. Cycles prior to the 25th cycle are considered as baseline cycles, with random fluorescence and 'noise' fluorescence being recorded by the computer. The threshold cycle is indicated by a bold horizontal line and is the point at which reporter dye emission intensities rise above the background noise and when the collected data curve is in an exponential phase.

A



B

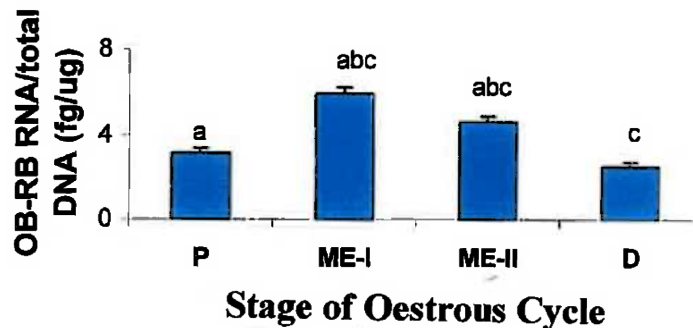


Figure 6.7 Standard concentration of ovary *OB-RA* (A) and *OB-RB* (B) RNA (fg or 10^{-15} g) throughout the oestrous cycle of the rat. The oestrous cycle of mature Sprague-Dawley rats was determined by vaginal cytology (P = pro-oestrus, ME-I = met-oestrus-I, ME-II = met-oestrus II, D = di-oestrus). Ovaries were collected for RNA extraction and processed for real time RT-PCR analyses. Data are expressed as the mean standard concentration of RNA from both ovaries of each receptor ± SEM. Same letters indicate a significant difference between groups. A) a indicates $p < 0.05$, while b, c and d indicate $p < 0.001$. B) a and c indicate $p < 0.001$, while b indicates $p < 0.01$.

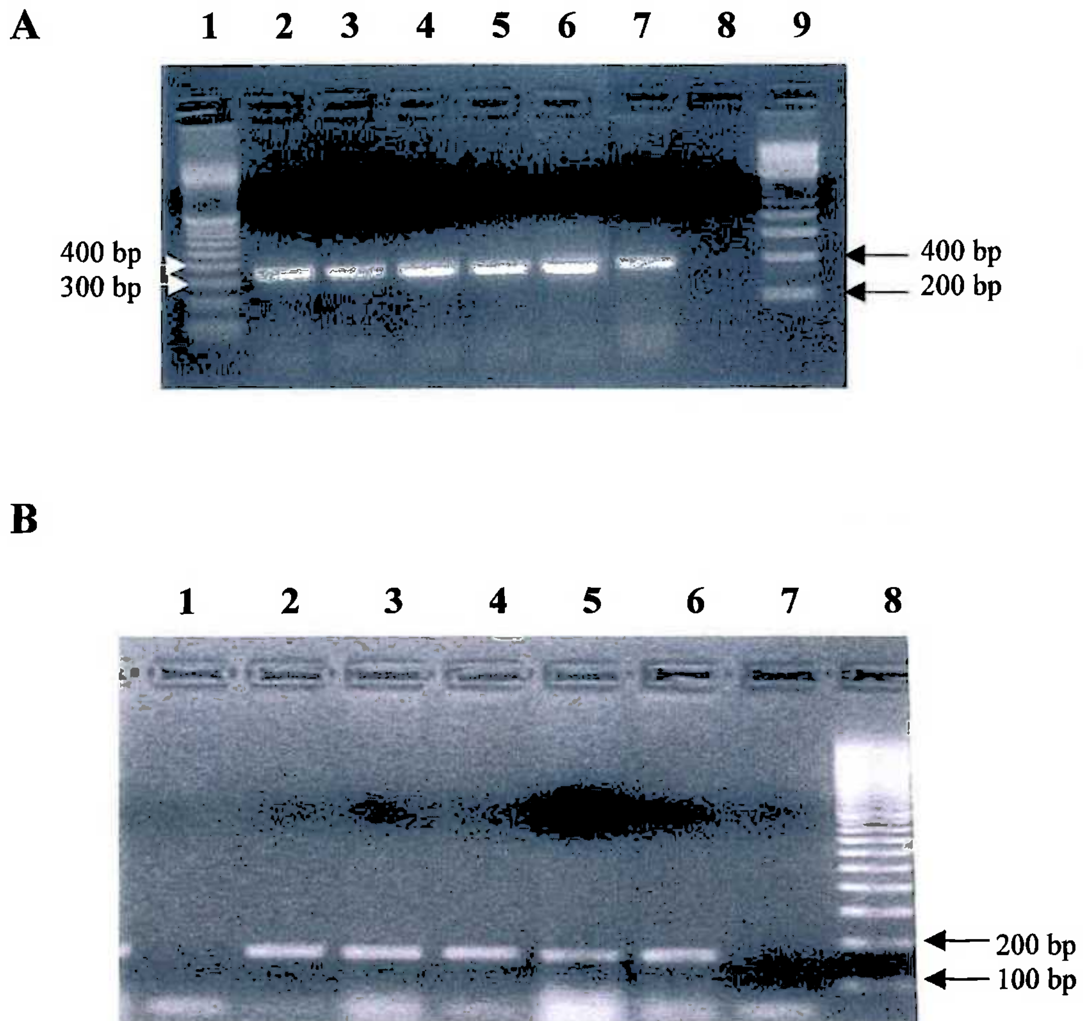


Figure 6.8 Visualisation of PCR products amplified in the real time RT-PCR reaction. A) The production of a correctly sized PCR product from the real time amplification reaction for *OB-RB*. Lane 1 - 100 bp DNA ladder, lanes 2-4 - increasing concentrations of TOPO TA-*OB-RB* were seeded into the PCR reactions and amplified. Lane 5-7 - different concentrations of cDNA were seeded and amplified. Lane 8 - negative control (cDNA omitted) and lane 9 - 200 bp DNA ladder. The resultant products appeared identical in intensity, regardless of the starting concentration. B) RT-check® RNA control was amplified by PCR to check RNA integrity, variation across tissues for RNA transcription in the RT step and to check the robustness of the PCR. Lanes 1 and 7 - negative controls, lanes 2-6 - RT-check PCR products, lane 8 - 100 bp ladder.

6.5 Negative and positive controls

Negative controls included omitting RNA in the RT step or omitting cDNA in the real time RT-PCR step. Negative controls resulted in background level noise on the I-cycler PCR machine and no PCR product was visualised by gel electrophoresis (Figure 6.8A). The RT-check™ kit was used as an endogenous control to test the integrity of the RT step and the amplification efficiency of the PCR (Figure 6.8B). The PCR products produced in the RT-check™ step were labeled with [^{32}P]-dCTP, visualised by gel electrophoresis and the bands excised and counted in a scintillation counter. The radioactivity counts from the various PCR products validated that the RT step and amplification efficiency was similar from tissue to tissue (less than 8 per cent variation across all tissues). Due to the low tube to tube variation, data generated from the real time experiments required no correction.

6.6 Body weight, ovarian weight, plasma steroid hormone and leptin levels

Body weights of animals at the time of tissue removal were similar between groups (Table 6.1). Left and right ovaries of animals, regardless of the stage of the oestrous cycle, were of similar weight (Table 6.1). Plasma oestradiol levels (pmol/L) were highest at the time of pro-oestrus (71.7 ± 4.8), and similar in the remaining three stages measured (met-oestrus I: 50.8 ± 6.9, met-oestrus II: 46.0 ± 3.6, di-oestrus: 38.1 ± 3.2) ($p < 0.05$). Plasma progesterone levels (nmol/L) were higher in the luteal phases of the cycle (met-oestrus II: 59.2 ± 7.2, di-oestrus: 46.6 ± 8.1) than the pro-oestrus (16.3 ± 2) and met-oestrus I (19.0 ± 2.7) phases ($p < 0.05$). Plasma leptin levels (Chapter 2.8) were only detectable in the pro-

Table 6.1 Body weights, ovarian weights, levels of serum leptin, oestradiol and progesterone in the rat at different stages of the oestrous cycle.

	Pro-oestrus	Met-oestrus I	Met-oestrus II	Di-oestrus
Body weight (g)	184 ± 13	163 ± 9	152 ± 5	168 ± 6
Ovary weight (mg)	310 ± 11	262 ± 27	295 ± 3	292 ± 2
Serum Leptin (ng/ml)	0.35 ± 0.05	ND	ND	ND
Oestradiol (pmol/L)	71.7 ± 4.8^{abd}	50.8 ± 6.9^{d}	46.0 ± 3.6^{b}	38.1 ± 3.2^{a}
Progesterone (nmol/L)	16.3 ± 2^{ab}	19.0 ± 2.7^{cd}	59.2 ± 7.2^{ac}	46.6 ± 8.1^{bd}

All measurements reported are at the time of tissue collection. ND signifies not detected. Results are reported as mean ± SEM per animal (n = 6-7). Data was analysed using a one-way ANOVA (Tukey-Kramer Multiple comparisons test), with the same letters indicating significant difference between groups, with a and c indicating $p < 0.001$ and b and d indicating $p < 0.01$ and $p < 0.05$, respectively.

oestrus phase (0.35 ± 0.05 ng/ml) of the oestrous cycle and were below the detection limit of the assay at other stages of the oestrous cycle.

6.7 Conclusions and discussion

Gene expression of the long form (*OB-RB*) of the leptin receptor has been detected in human granulosa, theca, interstitial and cumulus oophorus cells of the ovary (Billig *et al.*, 1997; Cioffi *et al.*, 1997; Karlsson *et al.*, 1997; Agarwal *et al.*, 1999). The short form (*OB-RA*) of the leptin receptor has been detected in human granulosa, theca and cumulus oophorus cells of the ovary (Cioffi *et al.*, 1997; Karlsson *et al.*, 1997; Agarwal *et al.*, 1999). Research in the rat has shown *OB-RA* and *OB-RB* expression in granulosa cells, with only *OB-RA* expression notable in theca cells of hypophysectomised rats (Zachow *et al.*, 1999). However, the expression of *OB-RA* and *OB-RB* in the ovary at different times of the oestrous cycle has not been quantified in any species. The current study was designed to investigate the direct effect of oestrous cycle stage on leptin receptor (*OB-R*) expression in the rat ovary using the specific and quantitative technique of real time RT-PCR.

This study clearly demonstrates that *OB-R* expression varies in response to the changing environment of the ovary. The onset of the met-oestrus phases correlate with an increase in the expression of *OB-RA* and *OB-RB*, with the lowest expression levels observed at pro-oestrus and di-oestrus. It is interesting to note the expression patterns of both leptin receptors (*OB-RA* and *OB-RB*) increased and decreased in a similar manner. Our experimental design allowed us to specifically identify the natural expression patterns of ovarian *OB-R* in response to normal fluctuations of steroid hormones and the leptin protein, throughout the oestrous cycle. This design allowed a focus on the ovary, while it remained

an intact unit with the hypothalamic-pituitary-ovarian axes and suggests that the variation in *OB-R* expression throughout the oestrous cycle complements the cyclic variation in ovarian steroids.

Several studies have reported the interaction between leptin and steroid hormones in the human menstrual cycle (Hardie *et al.*, 1997; Licinio *et al.*, 1998; Lukaszuk *et al.*, 1998; Mannucci *et al.*, 1998; Messinis *et al.*, 1998; Messinis *et al.*, 1999). This prompted us to measure leptin and steroid hormone levels in the rat across the oestrous cycle. As seen in our study, steroid hormone levels vary across the oestrous cycle, with maximum oestradiol levels recorded at pro-oestrus (Butcher *et al.*, 1974; Kalra and Kalra, 1974; Bennett *et al.*, 1999). It has previously been proposed that leptin can regulate oestradiol oscillations in the human (Licinio *et al.*, 1998) and that leptin gene expression is regulated by ovarian steroid hormones, with ovariectomised rats having lower levels of *ob* gene expression in fat (Yoneda *et al.*, 1998; Machinal *et al.*, 1999). In agreement, our findings show that leptin levels are at a maximal level at pro-oestrus, a time when oestradiol is at its peak. Research indicates that following an increase in oestradiol in pro-oestrus, *OB-R* transcript levels fall in the rat choroid plexus (Bennett *et al.*, 1999). Our results show that this pattern is also present in the ovary. *OB-R* mRNA concentrations increased in met-oestrus I and II following the oestradiol surge, and declined again in di-oestrus, immediately prior to the oestradiol surge at pro-oestrus. Our results indicate that oestradiol may be an important factor in fluctuations of leptin and *OB-R* at the time of pro-oestrus. Research in porcine ovaries has shown that *OB-R* expression increases with maximal progesterone levels (Ruiz-Cortes *et al.*, 2000). Leptin has a stimulatory effect on luteal function in the pig, with leptin mRNA and protein expression increasing with progesterone accumulation, *in vitro* (Murphy and Dobias, 1999; Pescador *et al.*, 1999). We therefore hypothesised that leptin

levels may vary with progesterone levels in the oestrous cycle. In our study, *OB-R* levels were maximal in stages anticipatory of maximal progesterone concentrations (met-oestrus I and II) and declined dramatically following high progesterone concentrations.

This study analysed *OB-R* expression in whole ovarian tissue, therefore our findings relate to the change in *OB-R* expression in the ovary as a whole. It is possible that luteal cells, endothelial cells and leukocytes may contribute to the differences in *OB-R* expression observed in the met-oestrus I and II stages. The results presented for met-oestrus I and II are at a time following ovulation and development of corpora lutea. Due to the changing physiology of the ovary, with granulosa cells becoming luteal cells, the increase in *OB-R* transcripts probably leads to increases in intact *OB-R* protein. Porcine granulosa cell *OB-R* expression increases in granulosa cells as they luteinise (Ruiz-Cortes *et al.*, 2000), therefore, our findings comply with previous research and support the hypothesis that luteinisation in the ovary promotes an increase in *OB-R*. In addition, dense staining for the *OB-R* in the theca-luteal layer has been located in mouse corpora lutea (Ryan *et al.*, 2001). The role of leptin in angiogenesis (Bouloumie *et al.*, 1998) may also provide an explanation for the increase in *OB-R* at met-oestrus I and II, as luteinisation results in neovascularisation in the ovary due to ovulation at the oestrus stage. Leukocytes and cytokines are important components of the ovulatory cascade and infiltrate the ovary at ovulation (Brannstrom and Norman, 1993c). The presence of *OB-R* on leukocytes has been identified (Gainsford *et al.*, 1996; Lord *et al.*, 1998; Santos-Alvarez *et al.*, 1999), and the contribution of these cells to the total ovarian *OB-R* expression can not be dismissed. Whether the increase in *OB-R* arises due to the influx of leukocytes at this time, remains to be defined.

In our study, we found that leptin levels were only detectable in the pro-oestrus phase and undetectable in all other stages. Other researchers have reported unchanged circulating leptin concentrations across the oestrous cycle of the mouse (Chehab *et al.*, 1997) and rat (Bennett *et al.*, 1999). One of these studies (Bennett *et al.*, 1999) reports pro-oestrus leptin levels approximately nine times above those in the current report. The reason for the discrepancy between the results may be attributed to the strain of the animal or the assay technique used (RIA versus ELISA). In our analysis, we used a larger volume of serum in the leptin assay, in an attempt to detect low levels of leptin. Assuming that the changes in *OB-R* transcripts are reflected in translated receptor levels, the decrease in *OB-R* expression observed at di-oestrus and pro-oestrus could be linked to the increase in serum leptin at pro-oestrus. The decrease in the *OB-R* expression could be used to reduce the amount of available functional receptors (*OB-RB*) for receptor-ligand interaction and/or to reduce the amount of *OB-RA* receptor-leptin binding in circulation or tissue. *OB-RA* has been hypothesised to be a transporter molecule for leptin (Stephens and Caro, 1998) which promotes leptin stability, therefore a decrease in *OB-RA* may promote the metabolism of leptin.

Although results from the previous chapters have demonstrated that excessive leptin is inhibitory to ovulation, it has also been implied that insufficient leptin-*OB-RB* interaction, due to leptin resistance, results in anovulation (Schwartz *et al.*, 1996; Cunningham *et al.*, 1999). We speculate that acute leptin administration in our previous study may have 'programmed' the ovary to be in a post-ovulatory stage of the oestrous cycle. Physiologically, post-ovulation is a time of increased leptin action, due to the increases in *OB-R*. Indeed, if this was the case, the ovary may respond by inhibiting ovulation, as observed in our study. We believe that the varying nature of the *OB-R* throughout the

oestrous cycle provides a mechanism for the regulation of normal physiological processes, in order to allow a different response to leptin across the oestrous cycle where leptin levels may otherwise only vary slightly. This would not be surprising given the variability in receptor number of other key regulators such as the FSH receptor and LH receptor in granulosa cells at different stages of the oestrous cycle.

In summary, we have investigated the effect of the stage of oestrous cycle on *OB-R* expression in the mature rat ovary. In this study we found significant decreases in the level of *OB-R* expression at the time of pro-oestrus and di-oestrus, stages which are preparatory for ovulation. The stages following ovulation appeared to have an increase in the level of *OB-R* expression, stages at which leptin in circulation was undetectable. The increase in *OB-RB* expression at these stages may be a mechanism to provide less leptin action prior to ovulation while increasing leptin action for the corpora lutea. A simultaneous increase in *OB-RA* may promote the stability of leptin at times of high *OB-R* action, as *OB-RA* has been postulated to be a transporter molecule for leptin. The unique relationship that leptin appears to have with the ovarian axis at physiological levels is in contrast to studies which have demonstrated an inhibitory action of leptin on steroidogenesis (Zachow and Magoffin, 1997; Spicer and Francisco, 1998) and ovulation.

Chapter 7

Summary and future work

7.1 Summary

At the onset of this study, the direct effects of leptin treatment on the rat ovary were unclear. The major aims of this study were therefore to investigate the effects of acute leptin administration on the ovulation process, *in vivo* and *in vitro*. In particular, we wished to investigate the effects of exogenous leptin on ovarian follicles and ovarian leukocyte biology at the time of ovulation, using histopathology and immunohistochemistry. In addition, we wished to investigate the effects of acute leptin administration on proliferation and development of ovarian compartments *in vitro* and the effects of physiological levels of leptin throughout the oestrous cycle on the expression of the functional leptin receptor. The knowledge from these studies enable a better understanding of leptin biology in the ovary and may be extrapolated in order to understand the effects of acute leptin concentrations, as in obese individuals, on peripheral tissues such as the ovary.

A large amount of leptin research has been carried out in humans and the genetically obese Lep^{ob}/Lep^{ob} mouse. With the rat as an ideal experimental animal for studying the effects of exogenous hormone treatment with short oestrous cycles, we have used Sprague Dawley rats to investigate the effects of leptin on the ovary.

Initially, the hypothesis that acute leptin treatment inhibits ovulation in gonadotrophin primed animals was investigated. The data from the first study supported the hypothesis and showed a marked decline (300 %) in ovulation in leptin treated rats, while feed restricted (pair fed) animals ovulated similarly to control animals. However, steroid synthesis in these animals was unaffected by leptin treatment. The use of exogenous gonadotrophins indicates that the effects on ovulation occurred by a mechanism within the

induction of the hypothalmic/pituitary axes. The *in vitro* perfusion studies further indicate that the inhibition in ovulation was specifically a result of inhibition at the ovarian level. In obese and infertile women, peripheral tissues are exposed to high leptin levels. This study may therefore have implications on the treatment of infertility observed in obese women.

Having established the effects of acute leptin treatment on the ovulation process, it was important to determine the possible contributors to this inhibition. With the clearly established interactions between immune cells and the ovulatory process and the effects of leptin on the immune axis, we investigated the effects of exogenous leptin treatment on ovarian morphology and leukocyte distribution into pre-ovulatory follicles. Leptin treatment did not affect the recruitment of pre-ovulatory follicles into the ovulating follicle pool. However, leptin treatment resulted in a decrease in food intake, a factor that significantly reduced the infiltration of neutrophils and macrophages/monocytes selectively into the theca layer of pre-ovulatory follicles. The inhibition in ovulation observed in leptin treated animals was not seen in feed restricted animals, indicating that food restricted animals had a compensatory mechanism which allowed ovulation to occur.

Investigating *in vitro* follicle culture using gonadotrophins and leptin further expanded the effects of acute leptin treatment on ovulation. *In vitro* follicle culture indicated that leptin treatment does not influence the meiotic competence of follicle-enclosed oocytes, steroid synthesis, secretion of interleukin-1β, while an effect may occur on prostaglandin E_2 synthesis. Granulosa cell proliferation is unaffected by leptin treatment, however, there is a clear effect of leptin on IGF-I augmented FSH-dependent oestradiol production in the presence of androstenedione. The results indicate that although the inhibition of ovulation in the *in vivo* study can not be attributed directly to the factors tested, it is likely that leptin

may interact with all of the above. This follows as leptin action has been noted with regard to all of the above factors.

To understand the relative relationship between leptin and leptin receptors and the method of modulating leptin sensitivity across the oestrous cycle, a sensitive and quantitative assay for the investigation of leptin receptor expression was developed. The real-time RT-PCR assay used to assess the normal expression patterns of the leptin receptors throughout the oestrous cycle demonstrated that in rat ovaries, leptin sensitivity is modulated across the oestrous cycle by fluctuations in the amount of leptin receptor available for leptin interaction. The expression patterns of the leptin receptor indicate that higher leptin-leptin receptor interaction is required in the luteal stages, while there is low leptin-leptin receptor activity in the follicular stages. This may relate to the role leptin plays in angiogenesis, and may link to the increased neovascularisation required during the luteal stages.

7.2 Applications of leptin research

The importance of leptin in reproductive processes is a new field. The complicated relationships that leptin has with the adrenal, immune, hypothalmic and pituitary axes needs to be identified. Only then can treatments for obesity and infertility be devised. Generally, a better understanding of how leptin interacts with the ovary will expand our knowledge of leptin biology in reproductive tissues and allow application in many areas of infertility. An appreciation of leptin interactions with the ovary may further benefit obese and anovulatory patients in drug design to treat infertility.

Obesity is not a major concern to animal producers, however quality of carcass, per cent body fat, amount of food intake and reproductive potential are all genetic traits of interest in livestock production. Recently, leptin has been implicated as a useful marker to assist in genetic selection for body composition and reproduction in animals (Comuzzie *et al.*, 1997). This implicates leptin as a useful tool for genetic selection for improved livestock characteristics. Several potentials exist for leptin use in domestic animal reproduction. Furthermore, earlier leptin-induced puberty could prove to be useful in animal livestock breeding and leptin treatment may also be useful in shortening the interval from parturition to oestrus. In addition, the roles of leptin in fetal growth and development indicate that leptin may be useful in enhancing the health of livestock offspring.

7.3 Suggestions for future work

The work described in this thesis provides a basis for future research into the effects of acute leptin on ovarian biology. However due to time constraints, some studies, essential in understanding the involvement of leptin in ovulation, were not completed in this thesis. Future research is required to specifically determine the relationship between leptin and the ovary in normal physiological animals. An enhanced knowledge of how leptin interacts with the ovary and steroid hormones will assist in future application of leptin antagonists in the aim of promoting fertility in obese individuals.

Further work to be considered concerning leptin in the ovary includes:

- The role of leptin and the leptin receptors in corpora lutea formation and development and the importance of high leptin-leptin receptor interaction in the luteal stages of the oestrous cycle.

- The effects of leptin treatment on ovarian leukocytes, cytokine secretion and production, and prostaglandin production by the ovary at the time of ovulation, in order to understand the role of leptin in normal ovulatory physiology.
- The relationship between leptin and blood flow to the ovary. The potential for acute leptin concentrations hampering the effects of normal blood flow to the ovary at the time of ovulation.
- Administration of antibodies against the leptin protein or antisense oligonucleotides against the leptin gene directly into the ovary to investigate the effect of a block against the local action of leptin in the ovary in normal animals.
- A clearer understanding of the relationship between leptin, oestrogen and progesterone in normal physiological animals and human ovarian tissue, for potential use in *in vitro* fertilisation.
- Acute and prolonged leptin treatment of mature rats in order to determine the expression profiles of the leptin receptor in response to leptin treatment. Identifying the response of the leptin receptor to the increased leptin concentrations may assist in understanding the leptin resistance experienced by obese and infertile women.
- Treatment of mature rats with steroids may also help to identify the relationship between circulating steroids and the ovarian leptin receptor in normal homeostasis. This would allow a further appreciation of how steroidogenic hormones may contribute to the expression profiles of the leptin receptor.
- Investigations of the involvement leptin has in apoptosis in ovarian follicles. In particular, whether the leptin induced decrease in ovulation results in an increase in atresia of follicles.
- The interaction that leptin has with other important ovulatory mediators, such as bradykinin and angiotensin.

- The effects of leptin on other cytokines, such as TNF/Fas, would assist in the identification of the mechanism by which leptin interferes with the ovulatory process.
- Translated leptin protein levels throughout the oestrous cycle, by way of western blots, would also assist in establishing the role of leptin in the ovulatory process.

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DAB peroxidase substrate

DAB tablet	1
Urea hydrogen tablet	1

Allow tablets to equilibriate to room temperature. Dissolve tablets in 5 ml Milli Q water, filter through a 0.22 µm filter. Solution to be used within 30 min of preparation.

DEPC water

Diethyl pyrocarbonate (DEPC)	1 ml
Milli Q water	1 L

Shake vigorously, stand overnight at room temperature and autoclave 121°C for 22 min. Store at room temperature.

Dry rat food (pellets)

Caloric content (100g)

MJ/Kg 14.38

Kcal 3437.1

Protein 20.88

Fat 6.89

Fibre 5.44

Calcium 0.76

Phosphate .59

Lysine 1.24

Methionine 0.36

Threonine 0.77

Isoleucine 0.88

Tryptophan 0.24

Leucine 1.49

Arginine 1.19

Phenylalanine 0.96

Valine 1.04

Histidine 0.49

Glycine 1.16

Cholline 1675.37

Salt 0.82

Linoleic Acid 2.25

Ash 5.22

Moisture 10.08

Density 0.68

Dulbecco's PBS (Ca^{2+}, Mg^{2+} free, pH 7.4)

Dulbecco's PBS powder sachet	1
Milli U water	1 L

Dissolve sachet in Milli U water, stir until all dissolved. Cool to 4°C before use.

Ethanol (95 %)

Ethanol (100 %)	95 ml
Milli U water	5 ml

Store at room temperature.

Ethanol (70 %)

Ethanol (100 %)	70 ml
Milli U water	30 ml

Store at room temperature.

Oligonucleotide (primer) dilution

Primers were diluted in 1 x TE buffer, to a final concentration of 1 μg/μl, and further calculated in nmol, based on the scale of the preparation.

rh FSH preparation and minipump filling and insertion

FSH supplied at 50 IU and dissolved in 50 μl saline (supplied). Pump holds 100 μl and releases 12 μl/day, required for 3 days giving 4 IU/day. Minipumps are best prepared the day prior to use, using aseptic technique (as described in manufacturers instructions), and stored in saline at 4°C overnight. The minimpumps must be allowed to equilibrate (4 hours) to room temperature prior to insertion. Minipumps are inserted by anaesthetising rats using gas anesthesia (Chapter 2.2.4), and maintained using an individual nozzle per rat. A small slit (1-2 cm) is made on the back (approximately half way down the back) for minipump insertion. Following minipump insertion, the wound is closed with metal clamp(s).

eCG and hCG

eCG was used directly without any addition (supplied in a liquid form). HCG was prepared from a powder using sterile saline with 0.1 % BSA.

Recombinant Leptin production and solubilisation

Recombinant human leptin used in *in vivo* studies was provided by Dr. Denis Magoffin and was produced using a technique similar to that quoted by Chehab, 1996. Directly quoted from Dr. Denis Magoffin : "A specimen of abdominal fat was obtained from a woman and flash frozen in a dry ice/ethanol bath and stored at -80°C. 235 mg of fat were thawed into 2.0 ml of tri-reagent and homogenized by hand in an all glass homogeniser. The aqueous phase was chloroform extracted and centrifuged. The lipid phase was discarded. The supernatant was ethanol precipitated, washed in 75 per cent ethanol, air dried and resuspended in 20 μl of DEPC water. This yielded 1.88 ng of RNA/μl. An RT reaction was performed on 9.4 ng of RNA followed by PCR for 30 cycles using leptin specific primers that

amplify the mature sequence containing BamH I and Kpn I sites. The amplified product (single band) was purified on a 2% agarose gel. The cDNA was extracted from the gel slice with a QIAQUICK kit (QIAGEN) into 30 μl of water. The purified PCR product was digested with BamH I and Kpn I and ligated in frame into the pQE30 expression vector. The ligated vector was transformed and 10 ml overnight cultures were grown using 100 μg/ml ampicillin and 25 μg/ml kanamycin selection in LB broth. The next morning 500 ml of pre-warmed medium was inoculated with the overnight culture and grown at 37°C until the OD_{600} >0.7. IPTG was added to a final concentration of 2 mM and the cultures were incubated for an additional 5 hours. The bacteria were pelleted and frozen at -80°C. The pellet was thawed and lysed in 7.5 ml of 6M guanidine-HCl, 0.1 M Na phosphate, 0.01 M Tris-HCl, pH 8.0. After centrifugation at 10,000 x g for 15 min at 4°C the supernatant was incubated with 8 ml of a 50% slurry of Ni-NTA resin for 45 min at room temperature with gentle stirring. The resin was packed into a glass column and washed with 40 ml of lysis buffer, then 20 ml of 8M urea, 0.1 M Na_2HPO_4, 0.01 M Tris-HCl, pH 8.0. The column was washed with 8M urea, 0.077 M Na_2HPO_4, 0.077M Tris-HCl, pH 6.3 until the OD_{280} was ~0.01. The leptin was eluted from the column with 4.6 M urea, 0.077 M Na_2HPO_4, 0.077M Tris-HCl, 250 mM imidazole, pH 8.0. The eluate was dialysed against 3M, 2M, 1M, and 0.5 M urea in PBS for 24 hours each, then 3 changes of PBS. This procedure yielded 3.6 mg of leptin that runs as a single band on SDS-PAGE. The leptin was aliquotted and lyophilised." The protein was lyophilised to maximise stability of the protein (for travel). The lyophilised powder was mixed with sterile milli-Q water for injections. Leptin used in *in vitro* studies was prepared using sterile saline with 0.1 % supplementation of BSA.

Equipment sterilisation and RNase Free Protocol

Equipment (scissors, tweezers) used for surgery was sterilised using steam sterilisation (121°C for 21 minutes) and stored in chlorhexidine cetrimide alcohol between manipulations. Equipment used for RNA extraction procedures were handled with gloves, washed with RNase away (company), washed twice with

DEPC treated water, and sterilised using steam sterilisation. To maintain an RNase free environment, gloves were worn and changed regularly throughout the protocol.

Ajax Chemicals, NSW, Australia

Alza corporation, Palo Alto, CA, USA

Ambion Inc, Austin, TX, USA

Amersham Pharmacia Biotech, NJ, USA

Baxter Healthcare Corporation, Deerfield, IL, USA

Bayer AG, Leverkusen, Germany

BDH Laboratory Supplies, Poole, UK

Becton-Dickinson, Maryland, USA

Bio-Rad, Hercules, CA, USA

BOC Gases Australia Ltd, NSW, Australia

Boehringer Mannheim, Mannheim, Germany

CIG, Australia

CSL Biosciences, Adelaide, Australia

Dako Corporation, Carpinteria, CA, USA

Diagnostic System Laboratories Scientific (DSL Scienctific) Webster, TX, USA

Eastman Kodak Co. Rochester, NY, USA

Eppendorf, Hamburg, Germany

Forma Scientific, Marietta, Ohio, USA

Gibco BRL, Rockville, MD

Gibco, Grand Island, NY, USA

Grant Instruments (Cambridge) Ltd, Cambridge, England

Graphpad Software Inc, San Diego, CA, USA

Gro*Pep* Pty Ltd, Adelaide, Australia

ICN, Costa Mesa, CA, USA

Intervet International B.V., Boxmeer, Holland

Invitrogen, Carlsbad, CA, USA

Leading Edge, Adelaide, Australia

Leica Instruments, Nussloch, Germany

Menzel, Braunschweig, Germany

Millipore Corporation

Molecular Probes, Eugene, OR, USA

Molecular Research Center Inc, Cincinnati, OH, USA

Neubauer, Germany

NIADDK, Bethesda, MD, USA

Nova, Copenhagen, Denmark

Olympus Optical Co Ltd, Tokyo, Japan

Operon, Alameda, CA, USA

Organon, Oss, The Netherlands

Orthoclinical Diagnostics, Amersham, UK

Perkin Elmer, Boston, MA, USA

Promega, Madison, WI, USA

QIAgen, Valencia, CA, USA

Ridley Agriproducts, SA, Australia

Rhone-Poulenc Lab Products, Victoria, Australia

Serotec, UK

Shandon Scientific,

Sigma Chemical Co./Diagnostics/Aldrich, St. Louis, MO, USA

Surgipath Medical Industries, IL, USA

Terumo Corporation

Troy Laboratories Pty Ltd, NSW, Australia

Turner Designs, Sunnyvale, CA

Vacuette Greiner Labortechnik, Kremsmunster, Bad Hollestr, Austria

Wild Heerbrugg, Switzerland

Zeneca, Macclesfield, UK