Interactions between Leptin, Cytokines and CRF in the Brain

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Abstract

Cytokines are primary mediators of host defence responses to disease and infection such as fever and cachexia. Leptin, the product of the *ob* gene also has marked effects on energy balance, to reduce food intake and increase energy expenditure. Leptin and its receptor possess several 'cytokine-like' properties. Therefore leptin may also be involved in responses to infection. The work described in this thesis investigated the hypothesis that leptin acts as a mediator of food intake, body weight and core body temperature in rodents, and studied the relationships with proinflammatory cytokines and neuropeptides in the brain.

Injection of leptin into the lateral cerebral ventricles (icv) of rats reduced food intake, inhibited body weight gain, and increased core body temperature in a dose and time-dependent manner, similar to the responses to proinflammatory agents such as bacterial lipopolysaccharide (LPS) or the cytokine, interleukin (IL)-1β. The naturally occurring receptor antagonist to IL-1 (IL-1ra) significantly attenuated these effects. Responses to peripheral leptin administration were similar to those induced by central injection of leptin and were also inhibited by IL-1ra. Leptin significantly increased levels of immunoreactive (ir) IL-1β and IL-6 in rat hypothalami. Central injection of leptin failed to influence food intake and body weight in mice lacking the IL-1 type I receptor gene. Conversely, IL-6-deficient mice displayed increased sensitivity to the effects of leptin on food intake and body weight. Immunohistochemistry revealed that icv injection of

leptin stimulated increased expression of irIL-1β in choroid plexus cells, hypothalamic microglia and meningeal macrophages in the brain.

Several actions of IL-1 in the brain are mediated by the release of prostaglandins (PGs, which are products of cyclo-oxygenase activity) and corticotrophin releasing factor (CRF). Peripheral injection of the cyclo-oxygenase inhibitor, flurbiprofen, abolished leptin-induced fever, but failed to influence its effects on food intake or body weight. The CRF receptor antagonist, D-Phe-CRF_{12-41}, significantly and dose-dependently attenuated leptin effects on food intake and body weight, but not body temperature.

The lack of effect of the CRF receptor antagonist on febrile body temperature contrasted with published data. Further investigations revealed that the actions of CRF on body temperature might be more complex than thought previously. Separate studies indicated that injection of CRF induced dose-dependent hypothermia, which was inhibited by a CRF receptor antagonist. The response to CRF varied by altering housing conditions, method of measuring core body temperature and ambient temperature, underlining the importance of experimental conditions when investigating the role of CRF in thermoregulation.

The data presented suggest that IL-1 mediates actions of leptin on body temperature and food intake. The responses to IL-1 appear to be mediated by the activation of two distinct pathways, involving PGs and CRF respectively. Thus leptin may act as a neuroimmune mediator which may be involved in responses to disease and infection.

Declaration

I, the undersigned declare that no portion of this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

Jason Dudley Gardner 13/02/1999

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Preface

I obtained my Bachelor of Science degree in Anatomical Sciences (Upper Second Class with Honours) in the School of Biological Sciences at the University of Manchester in 1995. I continued to work towards my Ph.D. in this department, under the supervision of Professor Nancy J. Rothwell and Dr Giamal N. Luheshi.

Acknowledgement of materials and assistance received

I would like to thank Professor Andrew S. Loudon, who provided me with the first sample of leptin, and without whose critical advice, the work undertaken in this Ph.D. would no doubt have taken a completely different course. Thanks to Dr Steve Poole at the NIBSC for providing seemingly endless quantities of IL-1β, and the S-328 antibody used in IL-1β ELISA and expression studies. Thanks also to Professor Jean Rivier at the SALK Institute for Biological Studies, for generous gifts of CRF and α-helical CRF_{9-41}.

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Finally, I would like to thank the Ministry of Agriculture, Fisheries and Food for their financial support.

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It's amazing how many people I can say have helped me over the last 3 years, whether at work, at home, on the rugby pitch, or just in friendship:

I would like to thank my supervisor-in-chief, Nancy Rothwell, for allowing me the opportunity to work in her laboratory, and for all her invaluable advice, enthusiasm, generosity and well directed death threats. Truly inspirational.

As for the big fella himself, Giamal Luheshi, my supervisor and mentor - what a boy! I am eternally grateful for his support, and constructive comments on practically everything that I have done and written. Cheers mate!

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My Mum and Dad, who have managed to endure 26 years of supporting their number one son; far, far above and beyond the call of duty. Thank you for your unquestioning love, understanding, generosity, and for just being there. And thanks to Rupert - that damn pesky brother of mine.

Finally, a huge sloppy thank you to Joanne - what would I do without you?

Thank you all - doesn't time fly when you're having fun!?

Dedication

This thesis is dedicated to

Mum, Dad, Rupert, Grandma,

and my little pickle.

Cheers!

List of Abbreviations

°C	degrees Celsius
µg	micrograms
µl	microlitres
Ab	antibody
ACTH	adrenocorticotrophic hormone
AGRP	agouti-related protein
ANOVA	analysis of variance
BAT	brown adipose tissue
BDNF	brain-derived neurotrophic factor
BMI	body mass index
BSA	bovine serum albumin
BSU	biological services unit
cm	centimetres
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CRF	corticotrophin releasing hormone
CRF-BP	CRF-binding protein
CRF-R1 or 2	CRF receptor type 1 or 2
DAB	3,3'-diaminobenzidine tetrahydrochloride
dH_2O	distilled water
DNA	deoxyribose nucleic acid
ECGF	endothelial cell growth factor
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FGF	fibroblast growth factor
g	gram(s)
G,M,GM-CSF	granulocyte, macrophage and granulocyte/macrophage colony stimulating factor(s)
GDNF	glial-derived neurotrophic factor
GDP	guanosine diphosphate
h	hours
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HMSO	Her Majesty's Stationary Office
HPA axis	hypothalamo-pituitary adrenal axis
ICE	interleukin-1 converting enzyme (caspase 1)
icv	intracerebroventricular
IFN	interferon
IL	interleukin
IL-1ra	IL-1 receptor antagonist
IL-1R-AcP	IL-1 receptor accessory protein
IL-1RI (-/-)	IL-1 type I receptor knockout mice
IL-1RI or II	IL-1 receptor type 1 or 2
IL-1Rrp	IL-1 receptor-related protein

IL-6 (-/-)	IL-6 knockout mice
ip	intraperitoneal
ir	immunoreactive
IU	international units
iv	intravenous
JAK	janus kinase
kDa	kilodalton(s)
kg	kilogram(s)
l	litre(s)
LAL test	limulus amoebocyte lysate test
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
MANOVA	multiple analysis of variance
MC	melanocortin
MCP	monocyte chemotactic protein
mg	milligram(s)
MGSA	melanoma growth stimulatory activity
min	minute(s)
MIP	macrophage inflammatory protein
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
mRNA	messenger RNA
MSH	melanocyte stimulating hormone
n	number
NaOH	sodium hydroxide
NAP	neutrophil activating protein
NDS	normal donkey serum
ng	nanogram(s)
NGF	nerve growth factor
nm	nanometre(s)
NPY	neuropeptide Y
NT	neurotrophin
O_2	oxygen
OD	optical density
OM	oncostatin M
OPD	0-phenylenediamine dihydrochloride
p	probability
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PFA	paraformaldehyde
pg	picogram(s)
PG	prostaglandin
pmol	picomole(s)
POMC	pro-opiomelanocortin
PVN	paraventricular nucleus
RANTES	regulated upon activation normal expressed and secreted
RNA	ribose nucleic acid

s	second(s)
sc	subcutaneous
SD	Sprague-Dawley
SEM	standard error of the mean
STAT	signal transducer and activator of transcription
TBS	tris-buffered saline
TGF	transforming growth factor
TNF	tumour necrosis factor
UCP	uncoupling protein
UK	United Kingdom
US	United States
VMH	ventromedial hypothalamus
vs	versus
WHO	World Health Organisation

Chapter 1

General Introduction

1.1 OBESITY

The concept of an ideal body weight has altered considerably throughout history (Beller, 1977). It is likely that our human ancestors conferred greater chances of survival on individuals who possessed a large, fatty phenotype, than those that exhibited a leaner figure. The underlying basis of this concept is that fat people carried around their own natural energy stores to utilise in periods of famine. This may explain why primitive societies and cultures may consider obesity an indication of good health, fertility (as obese women were able to provide adequate nourishment for their offspring) and status. In contrast, the modern consensus on obesity is undoubtedly, that the condition that may have been a fundamentally successful survival tactic, is now a serious health problem in modern civilisation.

Figure 1.1.1.1. Mr Daniel Lambert (1770-1809) aged 38, and weighing 52 stones.

1.1.1 Epidemiology of Obesity

The incidence of obesity in the United Kingdom (UK) alone has practically doubled between 1986 and 1995, as reported by Her Majesty's Stationary Office (HMSO) of Population Censuses and Surveys (HMSO, 1993; HMSO, 1997). These reports indicate that obesity in men aged 16-64 has risen from 7% to 15%, and in women from 12% to 16.5%. Although these surveys report more women than men to be obese, the more recent report (HMSO, 1997) states that 44% of men compared to 33% of women are overweight or obese, based on body mass index (BMI >25 kg/m^2 signifies overweight, >30 kg/m^2 signifies obese). These statistics suggest that over 14 million adults in the UK are overweight, and a further 6 million are obese.

These figures are not exclusive to the UK. In Europe, more than half of all adults aged 35-65 are overweight or obese (Seidell & Flegal, 1997). In the United States (US), reports suggest similar statistics (Millar & Stephens, 1987; Kuczmarski et al., 1994), that upward of 34 million adults in the US are obese (Colditz, 1992). It must be noted that comparisons between countries are difficult because of the differing monitoring protocols and statistical techniques. Nevertheless, the World Health Organisation (WHO) has concluded that the prevalence of obesity is higher in the US than in Europe (WHO, 1997).

1.1.2 Consequences of Obesity

Obesity is a major factor in increasing mortality by associated illness, and has significant negative effects on the quality of an individual's life (Pi-Sunyer, 1993). In fact the health implications of obesity are so extensive that the number

of obesity related deaths in the US in 1990 (300 000) were second only to mortality linked with smoking (400 000) (McGinnis & Foege, 1993).

The cost of obesity to an economy for direct medical treatment was $39.3 billion in 1986 in the US (Colditz, 1992). In approximately 10 years that figure increased by 25%, such that in 1995, the same research team (Wolf & Colditz, 1998) reported US expenditure on obesity related health care to be $51.6 billion. Furthermore, the inclusion of indirect costs to the economy such as lost productivity, produced a total figure of $99.2 billion.

1.1.3 Energy Balance and Obesity

Body energy content is determined by the balance between energy intake (food) and energy expenditure (basal metabolic rate, physical activity, and thermogenesis). There is considerable evidence to suggest that body energy content is regulated (e.g. Rothwell & Stock, 1981), and like other homeostatic factors such as body temperature, is under the control of the hypothalamus. However, unlike body temperature, the limits for the regulation of body weight and energy content as a physiological parameter are wide, as determined by the limits for survival. Nevertheless, body weight often remains remarkably constant over many years, although with increasing time and age, fat may be deposited at a constant rate, resulting in the development of obesity.

Acute deviations in body energy content may be corrected by eliciting changes in the systems controlling energy intake (satiety) or expenditure (metabolism). Therefore, there is still debate as to whether obesity is a result of a dysfunction

in energy balance, or the increased energy intake and decreased expenditure, associated with a sedentary lifestyle.

1.1.4 Genetics of Obesity

Undoubtedly, environmental factors exert a significant effect on the prevalence of obesity, and resulting obesity related disorders (Hill & Peters, 1998). Nevertheless, other studies have consistently suggested that between 40% and 70% of obese characteristics such as BMI, skin fold thickness, fat mass, and hormone levels are heritable (Comuzzie et al., 1994; Allison et al., 1996; Comuzzie et al., 1996). This argument is supported further by studies showing that monozygotic and dizygotic twins exhibit a significantly similar BMI, fat mass, and fat-free mass (Bouchard et al., 1988; Bouchard et al., 1990; Selby et al., 1991). Moreover, studies of adoptees show a clear correlation between the BMI of the adult adoptee and those of their biological, rather than their adoptive parents (Stunkard et al., 1986a; Stunkard et al., 1986b; Sorensen et al., 1992a; Sorensen et al., 1992b). These studies suggest that obesity, or at least a predisposition to obesity may be genetically linked.

There are now a considerable number of genes that have been implicated in energy balance regulation and the development of obesity (Bouchard & Perusse, 1996; Bouchard, 1997; Leibel, 1997a; Leibel et al., 1997b; Chagnon et al., 1998). The most likely candidates are listed in the following table (**Table 1.1.4.1**), and are selected on the basis of animal models, physiology, and human studies.

Gene	Product	Indication	Reference
adrb3	Adrenoreceptor β3	Adipocyte differentiation	(Mitchell et al., 1998)
asip	Agouti signalling protein	Obesity	(Michaud et al., 1997)
cckar	Cholecystokinin A receptor	Satiety	(Huppi et al., 1995)
crf	Corticotrophin releasing factor	Energy balance	(Vale et al., 1981)
mcr3 & 4	Melanocortin receptors 3 & 4	Feeding behaviour	(Magenis et al., 1994; Huszar et al., 1997)
npyr5	Neuropeptide Y receptor 5	Appetite regulation	(Nakamura et al., 1997)
ob	Leptin	Obesity	(Zhang et al., 1994)
obr	Leptin receptor	Obesity	(Tartaglia et al., 1995)
pomc	Pro-opiomelanocortin	Obesity	(Boston et al., 1997; Mountjoy & Wong, 1997)
tnfa	Tumour necrosis factor α receptor	Obesity	(Norman et al., 1995)
tub	Tub protein	Obesity	(Noben-Trauth et al., 1996)
ucp1-3	Uncoupling proteins 1-3	Thermogenesis	(Cassard et al., 1990; Fleury et al., 1997; Solanes et al., 1997)

Table 1.1.4.1. Candidate genes implicated in obesity

The potential targets for the pharmaceutical and genetic therapy of obesity that have created the most interest over the last four years have been the leptin (*ob*) and leptin receptor (*obr*) genes. Indeed, trials studying their efficacy are already ongoing (Campfield et al., 1998). These are based on the assumption that genetic and experimental models of obesity in rodents are directly relevant to human obesity. This relationship and the background to leptin and its receptor are described in the next section.

1.2 LEPTIN

Leptin - derived from the Greek word *leptos*, meaning thin.

1.2.1 History of leptin

The hypothalamus is a primary centre in the brain for homeostatic mechanisms including the regulation of energy balance, and is implicated in the pathophysiology of obesity (Wilding et al., 1997; Flier & Maratos-Flier, 1998; Plata-Salaman, 1998b). This involvement of the hypothalamus in energy balance was first proposed following a study where bilateral hypothalamic lesioning induced an obese phenotype even though food intake (after an initial increase) had returned to basal levels (Hetherington & Ranson, 1942). Further studies involving parabiosed rats suggested an interaction of the hypothalamus with a blood-borne hormone (Hervey, 1958). This study reported that destruction of the ventromedial hypothalamus (VMH) in one rat caused death by starvation of its unlesioned parabiosed partner. This effect was hypothesised to be a result of high levels of a circulating satiety hormone produced by the lesioned rat.

Further parabiosis studies involving obese *ob/ob* and *db/db* mice (**Figure 1.2.1.1**) elucidated that the *ob/ob* mouse was sensitive to the actions of this hormone, but produced a defective form of the circulating factor; whereas the *db/db* mouse produced an active form of the hormone, but was insensitive to its action (Coleman, 1973; Coleman, 1978). These gene products have now been identified as leptin (*ob*) and its receptor (*obr*) (**Section 1.2.2**).

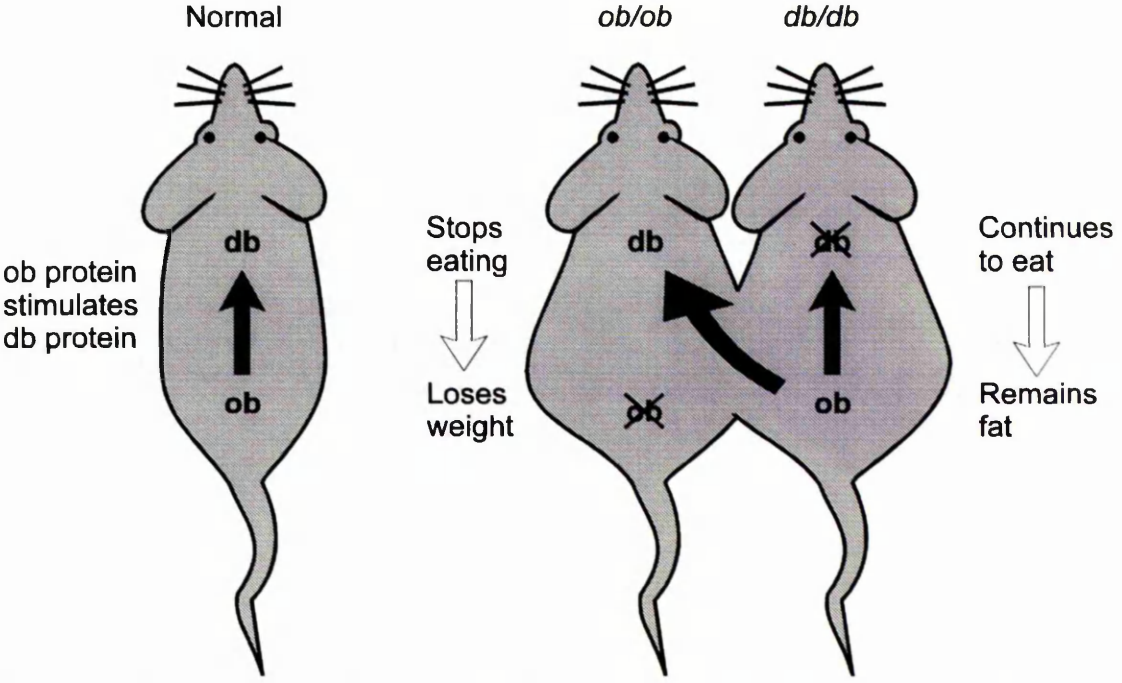

Figure 1.2.1.1. Schematic representation of *ob/ob* and *db/db* mice parabiosis experiments (Coleman, 1973) adapted from Campfield *et al.*, 1996

1.2.2 Leptin Molecule

The *ob* gene was localised to chromosome 6 in the mouse, as early as 1978 from studies by Coleman in the *ob/ob* mouse (Coleman, 1978), which expresses an inactive leptin molecule. However, the mouse and human forms of the leptin molecule were not identified until 1994 (Zhang et al., 1994). Rat leptin was identified a year later (Murakami & Shima, 1995). Zhang and colleagues described the leptin molecule as a 167 amino acid protein that is synthesised by adipose tissue (Zhang et al., 1994). The N-terminal of the protein consists of a 21 amino acid secretary signal sequence that is cleaved from the molecule before secretion and release into circulation as a 146 amino

acid active form with a molecular weight of 16 kDa. Leptin comprises four α-helix domains and two β-sheet regions joined by a C-terminal disulphide bond. Molecular modelling suggests that the tertiary structure of the leptin molecule is globular, and similar to cytokines such as the interleukin family and granulocyte-macrophage colony stimulating factor (Madej et al., 1995; Rock et al., 1996; Zhang et al., 1997; Kline et al., 1997). Leptin has a high degree of homology between species (**Table 1.2.2.1**), which suggests that the gene is phylogenetically old and has been highly conserved. Therefore the function of leptin is likely to be of considerable physiological importance.

Species	Genetic Homology	Reference
Mouse vs Rat	96%	(Murakami & Shima, 1995)
Mouse vs Human	84%	(Zhang et al., 1994)
Human vs Rat	83%	(Ogawa et al., 1995)

Table 1.2.2.1. Leptin cDNA homology between species

1.2.3 Leptin Receptor

The leptin receptor was first identified and cloned by Tartaglia and colleagues in 1995 (Tartaglia et al., 1995) and as proposed by Coleman (Coleman, 1973; Coleman, 1978), is encoded by the *db* (*obr*) locus and is defective in the *db/db* mouse (Chen et al., 1996; Chua et al., 1996). The leptin receptor is also defective in the *fa/fa* Zucker rat, which as a result exhibits an obese phenotype (Takaya et al., 1996; Iida et al., 1996; Phillips et al., 1996; Chua et al., 1996;

Yamashita et al., 1997). The amino acid sequence of the human leptin receptor extracellular domain has 78% homology, and the intracellular domain has 71% homology to the mouse leptin receptor (Tartaglia et al., 1995; Chen et al., 1996). Furthermore, the rat leptin receptor has 91% and 76% homology to the mouse and human leptin receptors, respectively. The *obr* gene has been reported to encode for at least six alternatively spliced forms - five short forms (OB-Ra,c,d,e, and f) and one long form (OB-Rb) (Tartaglia et al., 1995; Lee et al., 1996; Wang et al., 1996; Tartaglia, 1997). OB-Rb is a single transmembrane-spanning protein with a cytosolic C-terminal tail (Tartaglia et al., 1995). The short a,c,d and f forms of the receptor possess small C-terminal tails which are likely to anchor these receptors to the cell membrane. However, the OB-Re terminates prior to the transmembrane domains, and so is suggested to function as a soluble leptin receptor (Takaya et al., 1996; Liu et al., 1997; Tartaglia, 1997). It is therefore hypothesised that the different leptin receptors have separate roles in leptin physiology (**Table 1.2.3.1**).

Leptin Receptor Isoform	Membrane Bound	Proposed Function
OB-Ra,c,d,f	✔	Transmembrane transport Inactivate free leptin
OB-Rb	✔	Full signal transduction
OB-Re	✖	Soluble inactivator of circulating leptin

Table 1.2.3.1. Proposed physiology of leptin receptor isoforms

The structure of the leptin molecule has been compared to cytokines (Madej et al., 1995; Rock et al., 1996; Zhang et al., 1997; Kline et al., 1997). Interestingly, the leptin receptor has a similar relationship, in that the OB-R molecule has been categorised as a class I cytokine receptor (Tartaglia et al., 1995; White & Tartaglia, 1996; Tartaglia, 1997) and shares a high degree of homology with gp130, the signal transducing component of the IL-6 receptor (Nakashima et al., 1997b). The leptin receptor (OB-Rb) activates signal transducers and activators of transcription, STATs 3,5 and 6 (Baumann et al., 1996; Ghilardi et al., 1996; Vaisse et al., 1996), also known as 'fat-stats' (Darnell, 1996), by janus kinase (JAK)-mediated tyrosine phosphorylation (Ghilardi & Skoda, 1997) and shares overlapping (but distinct) signal transduction mechanisms with gp130 (Nakashima et al., 1997a; Nakashima et al., 1997b).

Leptin receptors are distributed widely throughout the central nervous system (CNS), indicating diverse actions of leptin. Studies have reported OB-Rb mRNA, immunoreactive OB-R protein, and high affinity leptin binding sites in the thalamus, choroid plexus, meninges and hypothalamus and surrounding blood vessels (Couce et al., 1997; Golden et al., 1997; Yarnell et al., 1998; Corp et al., 1998; Elmquist et al., 1998; Bjorbaek et al., 1998). The fact that leptin receptors are highly expressed in the hypothalamus (Tartaglia et al., 1995; Schwartz et al., 1996b; Mercer et al., 1996b; Hakansson et al., 1998; Yarnell et al., 1998; Elmquist et al., 1998) and that expression is increased in response to fasting (Baskin et al., 1998), further links actions of leptin to energy balance regulation.

1.2.4 Biology of Leptin

Leptin exists in the circulation freely, or bound to serum proteins (Houseknecht et al., 1996; Birkenmeier et al., 1998) or soluble leptin receptors (Liu et al., 1997). These binding properties are likely to increase the half-life of leptin in circulation, and regulate the bioavailability of the molecule. Leptin has an estimated half-life in circulation of 24.9±4.4 min, which is independent of adiposity (Klein et al., 1996). Serum levels of leptin in normal, healthy, lean humans have been reported to be 7.5±9.3 ng/ml, whereas obese subjects exhibited levels of 31.3±24.1 ng/ml (Considine et al., 1996). In this study, levels of leptin were highly correlated to percentage body fat. Other studies have also described similar serum levels of leptin (lean, 5.6±1.3 ng/ml; obese, 43.0±9.4 ng/ml) (Hosoda et al., 1996) and shown a correlation with fat mass and BMI (Maffei et al., 1995; Rosenbaum et al., 1996). Similar relationships have also been established in lean and obese rodents (Maffei et al., 1995). Furthermore, subjects suffering from anorexia nervosa exhibit significantly lower leptin concentrations than normal individuals (Eckert et al., 1998), although these levels are correlated to the BMI rather than the specific disorder (Ferron et al., 1997). Serum leptin levels are thought to be related to gender, independently of fat mass or BMI, such that females exhibit higher concentrations of leptin per mass of fat than males in both rodents (Frederich et al., 1995) and humans (Hickey et al., 1996; Rosenbaum et al., 1996).

Differences in leptin levels may also be seen when measured at different times during the circadian cycle. Rats exhibit diurnal variation in *ob* gene expression, increasing during the dark phase, after rats start eating (Saladin et al., 1995).

Diurnal rhythms are also seen in humans, such that leptin levels peak during night-time sleep, and then decrease to reach a nadir in the late afternoon (Simon et al., 1998). These variations are associated with changes in body temperature, plasma glucose and insulin (Simon et al., 1998), and are inversely related to levels of adrenocorticotrophic hormone (ACTH) and cortisol (Licinio et al., 1997). Furthermore, leptin levels are pulsatile, with 32.0±1.5 pulses per day, and each pulse having a duration of 32.8±1.6 min (Licinio et al., 1997). Serum concentrations of leptin in humans are also directly controlled by energy intake as shown by studies in which leptin levels were progressively increased in response to feeding, and decreased by fasting (Dallongeville et al., 1998). Furthermore, leptin concentrations are altered during lactation such that the hyperphagia exhibited does not elicit increases in leptin levels (Pickavance et al., 1998), and so hyperphagia persists.

Serum leptin can enter the brain by crossing the blood brain barrier via a saturable transport system (Banks et al., 1996; Caro et al., 1996; Girard, 1997; Golden et al., 1997; Bjorbaek et al., 1998). Autoradiography has detected radiolabelled leptin uptake in the choroid plexus, arcuate nucleus of the hypothalamus, and median eminence (Banks et al., 1996). The transport of leptin into the brain is believed to be mediated via short forms of the leptin receptor that are expressed in brain microvessels of the choroid plexus, meninges, hypothalamus and cerebellum (Bjorbaek et al., 1998). This transport mechanism acts by endocytosis of the leptin molecule, and is a saturable, specific, and temperature-dependent system (Golden et al., 1997). In obese individuals, who exhibit high leptin levels in proportion to body fat (Considine et

al., 1996; Hosoda et al., 1996; Rosenbaum et al., 1996), the capacity for leptin transport into the brain appears to be lower than in lean individuals (Caro et al., 1996). This down regulation of leptin transport into the brain may provide a mechanism for leptin resistance in obesity. Alternatively, the reduced transport efficiency may result from a deficiency in the leptin receptor as exhibited by *fa/fa* rats and *db/db* mice, and may also be linked to the pathology of obesity.

1.2.5 Actions of Leptin

There is extensive evidence to suggest that leptin is a hormonal link between peripheral fat mass and the CNS (Campfield et al., 1996; Campfield et al., 1996; Misra & Garg, 1996; Campfield, 1997; Considine & Caro, 1997; Auwerx & Staels, 1998; Friedman, 1998). These studies propose that leptin enters the brain from circulation and acts primarily in the hypothalamus to regulate energy balance. The evidence supporting this hypothesis is convincing. Leptin is synthesised and released into circulation by adipose tissue in proportion to body fat mass (Frederich et al., 1995; Considine et al., 1996; Hosoda et al., 1996; Rosenbaum et al., 1996). Leptin does appear to act peripherally, since leptin receptors are expressed in peripheral tissues (Tartaglia et al., 1995; Chen et al., 1996; Lee et al., 1996), and leptin has been shown to induce significant biological responses in tissue cultures of adipocytes (Bai et al., 1996; Muller et al., 1997), haemopoetic cells (Gainsford et al., 1996; Ghilardi & Skoda, 1997), pancreatic cells (Shimabukuro et al., 1997), and hepatocytes (Cohen et al., 1996). However, leptin is also suggested to enter the brain (Banks et al., 1996), which is probably its primary site of action on food intake and energy expenditure (Jacob *et al.*, 1997; Campfield *et al.*, 1995; Seeley *et al.*, 1996;

Halaas *et al.*, 1997) . Lesions of the hypothalamus induce obesity (Hetherington & Ranson, 1942; Hervey, 1958; Bray et al., 1982) and increase adipocyte *ob* gene expression (Funahashi et al., 1995). Furthermore the leptin receptor is expressed primarily in the hypothalamus (Tartaglia et al., 1995; Schwartz et al., 1996b; Couce et al., 1997; Elmquist et al., 1998) – an area that is activated in *ob/ob* mice in response to peripheral injection of leptin (Woods & Stock, 1996).

Most of the physiological effects of leptin have been identified from observed responses to injection of leptin in rodents. Studies published by different research groups shortly after the identification of leptin described how food intake and body weight are reduced in normal rodents or *ob/ob* mice, in response to administration of leptin intravenously (iv), intraperitoneally (ip), or into the cerebral ventricles (icv) (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995; Campfield et al., 1996). Similar responses are also reported in response to injection of a recombinant adeno-associated virus vector encoding mouse leptin in *ob/ob* mice (Murphy et al., 1997). Furthermore, effects of endogenous leptin are inhibited by injection of antibodies raised against leptin (Brunner et al., 1997) or inactive leptin mutant forms (antagonists) (Verploegen et al., 1997). The specificity of these leptin-induced effects on food intake and body weight is verified by their absence in *db/db* mice and *fa/fa* rats (Campfield et al., 1995; Halaas et al., 1995; Seeley et al., 1996) that possess defective leptin receptors and so are insensitive to leptin. The weight loss induced by leptin is specific to the depletion of adipose tissue (Halaas et al., 1995), which appears to be mediated by apoptotic mechanisms (Qian et al., 1998). This is qualitatively distinct from the responses induced by food

restriction, which include loss of both fat and lean body mass (Halaas et al., 1995).

Leptin also increases resting energy expenditure. Injection of leptin normalises core body temperature (Pelleymounter et al., 1995; Harris et al., 1997; Harris et al., 1998) and increases oxygen consumption (Hwa et al., 1996; Hwa et al., 1997) in *ob/ob* mice, which exhibit reduced basal temperatures. However, there is no convincing evidence that leptin increases core body temperature above normal values in normal animals.

Brown adipose tissue (BAT) is involved in the regulation of non-shivering thermogenesis and core body temperature in small mammals (Himms-Hagen, 1984). BAT is also important in the regulation of energy balance as a mediator of diet-induced thermogenesis in rodents (Rothwell & Stock, 1979; Rothwell & Stock, 1997). Ablation of BAT in mice results in weight-specific reduction in metabolic rate such that core body temperature in these mice is approximately 0.9°C below control temperatures (Klaus *et al.*, 1998). The very high rates of heat production in BAT have been ascribed to uncoupling of oxidative phosphorylation via a proton conductance pathway (Rial & Nicholls, 1987). Exposure of rodents to cold, over feeding or administration of leptin all stimulate activity of this pathway, and induce synthesis of uncoupling proteins (UCPs) in BAT via the sympathetic nervous system (Cusin et al., 1998; Kotz et al., 1998).

Obesity is linked with infertility and delayed sexual development (Norman & Clark, 1998). Leptin is also involved in reproductive mechanisms, as demonstrated by studies that have reported how the sterility exhibited by *ob/ob*

mice is reversed by leptin administration (Mounzih et al., 1997), and that leptin accelerates the onset of puberty in normal mice (Ahima et al., 1997).

1.2.6 Mediators of Leptin Actions

The mechanisms by which leptin induces physiological responses are largely unknown, although several candidate mediators involved in the regulation of energy balance have been suggested (Flier & Maratos-Flier, 1998; Sahu, 1998).

Among other effects, neuropeptide Y (NPY) is potent in stimulating appetite, reducing energy expenditure, and has been implicated in the pathology of obesity (Wettstein et al., 1995; Tomaszuk et al., 1996). There is also evidence, linking NPY with actions of leptin. NPY mRNA expression is reduced by 42% in the arcuate nucleus of the hypothalamus of *ob/ob* mice (that exhibit high baseline levels), and by 24% in normal rats in response to injection of leptin (Schwartz *et al.*, 1996a; Schwartz *et al.*, 1996b). Furthermore hypothalamic NPY secretion is reduced by leptin (Lee & Morris, 1998). Within the arcuate nucleus, NPY-containing neurones co-express leptin receptors in normal (Hakansson et al., 1996; Mercer et al., 1996a; Hakansson et al., 1998) and *ob/ob* mice (Mercer et al., 1997). Moreover, the obese phenotype exhibited by *ob/ob* mice is reduced by deletion of the NPY gene (Erickson et al., 1996b). However, NPY is not the sole mediator of energy balance or of leptin actions, since NPY-knockout mice display normal food intake and body weight, and respond normally by reducing food intake in response to leptin administration (Erickson et al., 1996a). NPY is closely and inversely linked with the neuropeptide corticotrophin releasing factor (CRF) (Heinrichs et al., 1992;

Heinrichs et al., 1993; Mercer et al., 1996c; Wilding et al., 1997), which has also been implicated in mediating actions of leptin.

CRF is most noted for its involvement in stress responses and activation of the pituitary-adrenal axis (Dunn & Berridge, 1990; Lehnert et al., 1998). However, CRF is also involved in regulating food intake and energy expenditure (Krahn et al., 1986; Arase et al., 1988; Rothwell, 1990b), and is linked to actions of leptin. Leptin has been reported to increase CRF mRNA expression in the paraventricular nucleus (PVN) of the rat hypothalamus (Schwartz et al., 1996b). Interaction in the brain between leptin and CRF is further supported by findings that leptin receptors are localised to CRF-containing neurones in the parvocellular region of the PVN (Hakansson et al., 1998). Furthermore, leptin elicits stimulation and release of CRF from superfused brain slice preparations containing hypothalamus or amygdala (Raber et al., 1997; Costa et al., 1997).

However, the link between leptin and CRF appears to be more complicated in obese conditions, since in *ob/ob* mice, the hypothalamo-pituitary-adrenal (HPA) axis is activated and levels of adrenal steroids are elevated (Heiman et al., 1997). Paradoxically, this study reports that in *ob/ob* mice, leptin infusion reduces CRF release. Furthermore, leptin prevents the induction of CRF synthesis in the PVN of food-deprived *ob/ob* mice, and hinders the elevation of arcuate nucleus NPY synthesis (Huang et al., 1998). Together these results suggest an inverted role for leptin in the excessive response of the CRF system of the *ob/ob* mouse.

Additional proteins that have been suggested to mediate leptin effects are pro-opiomelanocortin (POMC) and its cleavage product α-melanocyte stimulating

hormone (α-MSH). Leptin receptors are expressed on POMC-containing neurones in the hypothalamus (Cheung et al., 1997). The reduced levels of POMC mRNA exhibited in the hypothalamus of leptin-deficient *ob/ob* mice are normalised by leptin administration (Schwartz et al., 1997; Thornton et al., 1997). However, this effect is not induced in leptin receptor deficient *db/db* mice (Schwartz et al., 1997). These findings suggest that leptin stimulates hypothalamic POMC gene expression via a pathway involving leptin receptors. The lethal yellow, agouti (*AY/a*) mouse has a defect in POMC-α-MSH signalling in the brain that leads to leptin insensitivity and obesity (Michaud et al., 1997). These mice express agouti-related protein (AGRP) in the brain, a peptide which antagonises the effects of α-MSH at the melanocortin (MC) 1,3 and 4 receptors (Ollmann et al., 1997; Ollmann et al., 1998), the latter of which appears to be particularly important in the pathology of obesity (Huszar et al., 1997; Fan et al., 1997). However, *AY/a-ob/ob* mice that express AGRP and are leptin-deficient exhibit independent and additive obese phenotype (Boston et al., 1997), suggesting that in POMC and leptin pathways may be separate. This proposal is strengthened by the observation that starvation results in increased levels of POMC in both *ob/ob* and *db/db* mice (Boston et al., 1997; Mizuno et al., 1998).

Leptin may of course stimulate food intake and energy expenditure directly, or may act via as yet undetermined mediators such as the product of the *TUB* gene (Kleyn et al., 1996). Alternatively, other molecules known to be involved in regulating energy balance such as cytokines, may mediate actions of leptin. Potential pathways mediating leptin actions are depicted below (**Figure 1.2.6.1**).

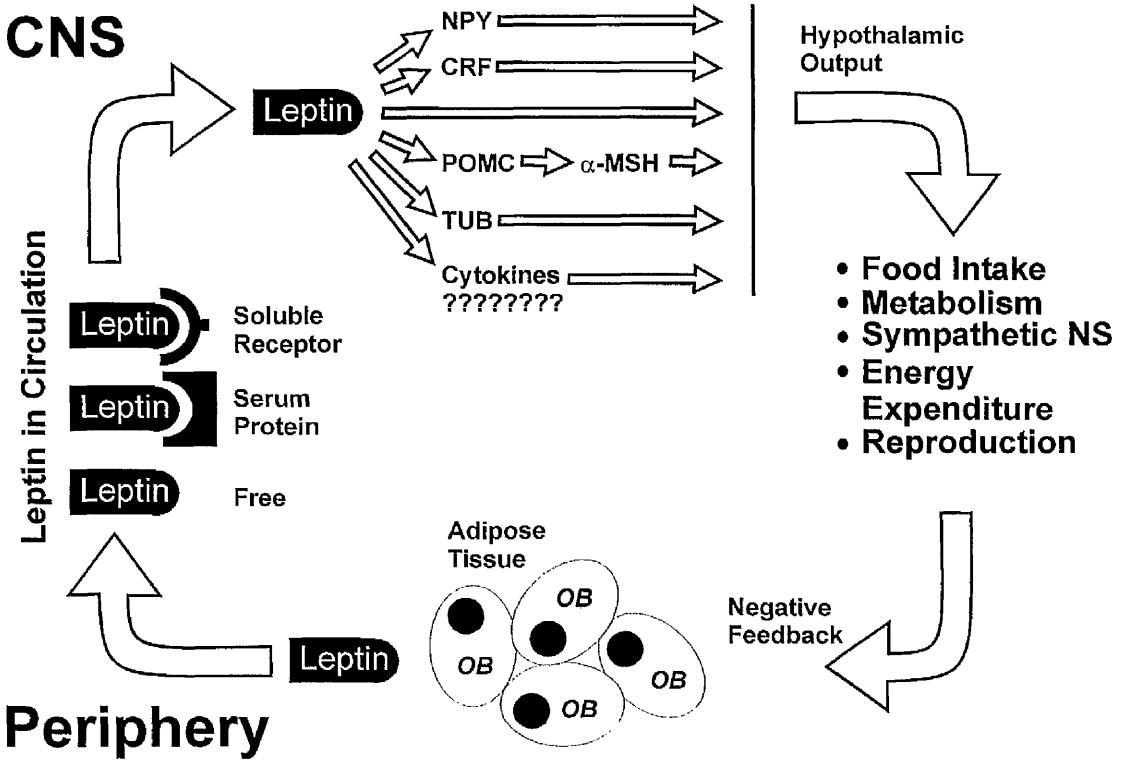

Figure 1.2.6.1. Expression and actions of leptin

1.2.7 Is Leptin a Cytokine?

Leptin and its receptor have similar structural properties to cytokines (Tartaglia et al., 1995; Madej et al., 1995; Rock et al., 1996; Zhang et al., 1997; Kline et al., 1997) as described earlier (**Section 1.2.2-1.2.3**), to the extent that they are now being categorised in this family of molecules (White & Tartaglia, 1996; Tartaglia, 1997). Furthermore, effects of leptin on energy balance by reducing food intake and body weight, and increasing energy expenditure, are similar actions to the neuroimmune responses reported to be mediated by cytokines (Rothwell & Hopkins, 1995; Rothwell et al., 1996). Therefore leptin and

cytokines may be intimately related in mediating cachectic responses to disease and infection.

1.3 CYTOKINES

Cytokines are a heterogeneous family of endogenous, hydrophilic 8-40 kDa proteins that are produced in response to a variety of physiological and pathophysiological stimuli (Hopkins & Rothwell, 1995; Liles & Van Voorhis, 1995). They have many diverse effects, and generally are considered to influence target cell growth, differentiation, survival and also death (Sachs & Lotem, 1994). Cytokines could be classed as hormones if it were not for the distinction that hormones are by convention released from specific endocrine glands, whereas cytokines are produced by practically every cell type within the body and are particularly associated with the motile cells of the immune system (Hopkins & Rothwell, 1995). The pluripotency of the cytokines has resulted in their classification into a number of different subgroups, although further categorisation has been suggested on the basis of structure, expression, receptors, and nomenclatures applied during the disjointed discovery of the different molecules (Rothwell & Hopkins, 1995; Hopkins & Rothwell, 1995). The major cytokine families are listed in the following table (**Table 1.2.7.1**).

Family	Members	Activities	References
Chemokines	IL-8/NAP-1 and 2, MIP-1α and β, MCP-1, MGSA, RANTES	Leukocyte chemotaxis & cellular activation	(Miller & Krangel, 1992)
Colony Stimulating Factors	IL-3, G,M and GM-CSF, other ILs	Colony cell formation in the bone marrow and leukocyte activation	(Cannistra & Griffin, 1988)
Growth Factors	EGF, FGF, PDGF, TGF-α and β, ECGF	Cell growth and differentiation	(Bennett & Schultz, 1993; Letterio & Roberts, 1998)
Interferons	IFN-α,β and γ	Inhibition of intracellular viral replication and regulation of cell growth	(Baron & Dianzani, 1994)
Interleukins	IL-1α,β and ra, IL-2-18	Multiple immunoregulatory activities	(Mizel, 1989; Kelso, 1998)
Neuropoietins	LIF, CNTF, OM, IL-6	Act in CNS via receptor-related complex	(Murphy et al., 1997)
Neurotrophins	BDNF, NGF, NT-3-6, GDNF	Neuronal growth and differentiation	(Ebadi et al., 1997)
Tumour Necrosis Factors	TNF-α and β	Similar to IL-1 plus tumour cytotoxicity	(Gruss & Dower, 1995)

Abbreviations: *brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), endothelial cell growth factor (ECGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), glial-derived neurotrophic factor (GDNF), granulocyte, macrophage and granulocyte/macrophage colony stimulating factor(s) (G,M and GM-CSF), interferon (IFN), interleukin (IL), leukaemia inhibitory factor (LIF), monocyte chemotactic protein (MCP), melanoma growth stimulatory activity (MGSA), macrophage inflammatory protein (MIP), neutrophil activating protein (NAP), nerve growth factor (NGF), neurotrophin (NT), oncostatin M (OM), platelet-derived growth factor (PDGF), regulated upon activation normal expressed and secreted (RANTES), transforming growth factor (TGF), tumour necrosis factor (TNF).*

Table 1.2.7.1. Cytokine families (adapted from Hopkins & Rothwell, 1995)

Many cytokines share biological actions and are also capable of affecting the release and receptor expression of other cytokines, a scenario known as the cytokine network (Kelso, 1998). This cytokine network is essential when normal homeostasis is threatened by tissue damage or infection (Rothwell & Hopkins, 1995; Hopkins & Rothwell, 1995; Dinarello, 1996; Kelso, 1998). Cytokines were

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initially considered to be associated specifically with the peripheral immune system. Further studies have now demonstrated diverse sources and actions of cytokines as mediators of host defence responses in a variety of organs including the brain (Rothwell, 1991a; Rothwell, 1991b; Luheshi & Rothwell, 1996b). It is in the brain that cytokines have a primary effect as neuroimmune mediators of host defence responses, and elicit fever, sickness behaviour, reduced food intake, increased energy expenditure and cachexia (Rothwell, 1990a; Rothwell, 1990c; Rothwell & Hopkins, 1995; Hopkins & Rothwell, 1995; Rothwell, 1997).

1.3.1 Cytokines in Cachexia

Cachexia, or wasting (literally - 'bad condition') is the most visible response to chronic diseases such as cancer, and parasitic or viral infections. It is characterised by loss of appetite (anorexia), weight loss, muscle wasting, haematological abnormalities (e.g. anaemia) and dysfunction in protein, lipid and carbohydrate metabolism (Balducci & Hardy, 1987; Langstein & Norton, 1991; Tisdale, 1997b). Cachexia arises because energy expenditure exceeds energy intake, and where body protein (lean tissue) as well as fat stores are utilised. However, such is the complexity of the condition that cachexia cannot be treated by increasing energy intake alone (Moldawer & Copeland, 1997; Tisdale, 1997a). Treatment therefore has focused on several candidate mediators of cachexia including cytokines such as IL-1, IL-6, TNF and IFN-γ (Moldawer et al., 1992; Matthys & Billiau, 1997; Tisdale, 1997a; Tisdale, 1997b). These cytokines have been reported extensively to induce cachectic responses in experimental animals (Hellerstein et al., 1989; Darling et al., 1990;

Plata-Salaman et al., 1996; Plata-Salaman, 1998a). Cytokine expression (particularly circulating IL-6) is upregulated in disease and infection (Gelin et al., 1988; Murray et al., 1997; Arsenijevic et al., 1997). Furthermore, studies have reported inhibition of tumour-induced cachexia by blocking actions of a number of cytokines (Rambaldi et al., 1991; Strassmann et al., 1993).

In addition to its role in cachexia, and as suggested earlier (**Table 1.1.4.1**), TNFα has been implicated in the pathology of obesity (Argiles et al., 1997). However, probably the most extensively investigated cytokine (with nearly 23 500 Medline hits) that is highly involved in anorexia (Plata-Salaman et al., 1996; Plata-Salaman, 1998a) and cachexia (Laviano et al., 1995) is IL-1 (Dinarello, 1997a).

1.4 IL-1

IL-1 was originally described as a heat-labile protein found in acute granulocytic exudate that induces fever when injected in animals or humans (Atkins, 1960). It was initially called endogenous pyrogen, until it was redefined as interleukin-1 in 1979 (Aarden et al., 1979). IL-1 was first cloned in 1984 from human blood monocytes (Auron et al., 1984) and a P388D mouse macrophage cell line (Lomedico et al., 1984). The proteins identified in each of these studies were structurally different and were termed IL-1α and IL-1β. A third isoform that possessed antagonist properties was later identified from the urine of patients with monocytic leukaemia, and was termed IL-1 receptor antagonist (IL-1ra) (Seckinger et al., 1987; Mazzei et al., 1990). These three different isoforms are

expressed from separate genes, apparently as a result of evolutionary gene duplication and modification (Dinarello, 1994; Alheim & Bartfai, 1998). All isoforms recognise the same receptors on target cells where IL-1α and IL-1β act as agonists, but IL-1ra fails to elicit signal transduction (Dripps et al., 1991; O'Neill, 1997) and functions as the only known naturally occurring pure antagonist (Dinarello, 1991).

Although IL-1α and β only share a 25-27% homology, they possess similar biological activities (Dinarello, 1997a). However, these actions may not represent the true nature and functions of the endogenous molecules, since IL-1β is generally regarded as the primary secreted form, whereas IL-1α is proposed to regulate intracellular mechanisms (Dinarello, 1996). Both forms of IL-1 are synthesised as 31 kDa precursor molecules (pro-IL-1) before cleavage into the 17 kDa mature forms. Pro-IL-1α is fully active (Mosley et al., 1987), although pro-IL-1β requires enzymatic cleavage by interleukin-1β-converting enzyme (ICE) to achieve its active form (Black et al., 1988).

There are two known immunoglobulin-like interleukin-1 receptors, the interleukin-1 type 1 receptor (IL-1RI) and the interleukin-1 type 2 receptor (IL-1RII) (Sims et al., 1988). An IL-1 receptor accessory protein (IL-1R-AcP) (Greenfeder et al., 1995), and two IL-1 receptor-related proteins (Lovenberg et al., 1996; Parnet et al., 1996) have been identified, which facilitate ligand binding and signal transduction. IL-1RI is an 80 kDa membrane-bound glycoprotein that is widely distributed in the brain and periphery (Shirakawa et al., 1987; Deyerle et al., 1992; Yabuuchi et al., 1994). It is this form of the IL-1 receptor that is considered to be the primary receptor involved in signal

transduction (Sims et al., 1993; Cremona et al., 1998a). However, the mechanisms by which IL-1 receptors amplify and transduce a signal to activate cytosolic effectors remains unclear, although several pathways have been proposed (O'Neill, 1997; O'Neill & Greene, 1998). The IL-1RII form appears to act as a 'decoy' molecule, since although it binds IL-1 with high affinity, it does not transduce a signal (Colotta et al., 1993; Sims et al., 1993). IL-RII blockade potentiates IL-1-induced anorexia (Cremona et al., 1998b), indicating that free IL-1 levels are regulated by this receptor.

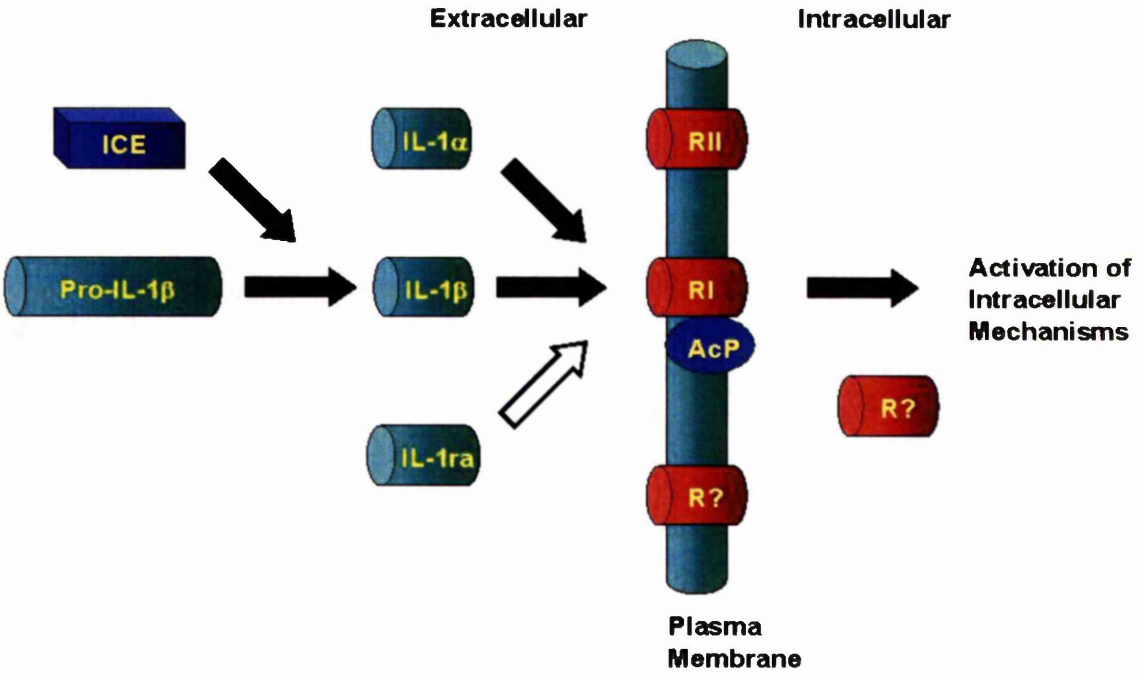

Figure 1.3.1.1. Schematic diagram of the IL-1 pathway

1.4.1 IL-1 and Food Intake

IL-1 mediates various host defence responses to disease and infection (Dinarello, 1996) and has a marked inhibitory influence on food intake and body weight. IL-1, IL-1ra and IL-1RI mRNAs are upregulated in the brain in response to systemic administration of the proinflammatory agent bacterial lipopolysaccharide (LPS) (Ilyin et al., 1998). This upregulation is most apparent in the hypothalamus - a region of the brain highly involved in regulating food intake and energy balance (Rothwell, 1992; Plata-Salaman, 1998b). IL-1 induces anorexia and weight loss when injected peripherally or centrally in experimental animals (McCarthy et al., 1986; Hellerstein et al., 1989; Kent et al., 1994; McCarthy et al., 1995; Sonti et al., 1996b) at pathophysiological doses (Plata-Salaman et al., 1996; Sonti et al., 1996a; Finck & Johnson, 1997), by reducing meal size, frequency and duration (Plata-Salaman, 1994). These studies demonstrate that central doses of IL-1 required to elicit such responses are considerably less than peripheral doses, indicating that IL-1 is likely to act primarily in the CNS in affecting appetite. Furthermore, inhibition of these responses by IL-1ra indicates that the effects are specific to IL-1 receptor activation (Plata-Salaman & Ffrench-Mullen, 1992; Plata-Salaman, 1994; Sonti et al., 1996a). The involvement of IL-1 in anorexic responses to disease appears to be acute, since animals become tolerant to chronic IL-1 infusion (Mrosovsky et al., 1989), probably as a result of receptor down-regulation.

Effects of IL-1 on food intake are proposed to be mediated largely by inhibition of NPY, which is a potent appetite stimulator (Wettstein et al., 1995; Tomaszuk et al., 1996). Central administration of IL-1 in rats upregulates brain expression

of the IL-1 system components and reduces hypothalamic NPY expression (Gayle et al., 1997). IL-1 antagonises NPY-induced feeding in rats, and conversely, NPY inhibits IL-1β-induced anorexia (Sonti et al., 1996b). NPY is closely and inversely related to brain CRF (Wilding et al., 1997), which is also proposed to mediate IL-1 actions (De Souza, 1993).

CRF induces anorexia in experimental animals (Gosnell et al., 1983; Levine et al., 1983; Negri et al., 1985; Krahn et al., 1986; Arase et al., 1988) and is positively related to IL-1. Injection of IL-1 induces activation (by increased c-fos expression) of CRF-containing neurones and CRF release in the hypothalamus (Barbanel et al., 1990; Veening et al., 1993). Hypothalamic CRF mRNA expression is increased in response to LPS administration is blocked by IL-1ra (Kakucska et al., 1993). IL-1-induced hypophagia is blocked by CRF immunoneutralisation (Uehara et al., 1989b). These studies suggest a link between IL-1 and CRF in hypophagia.

IL-1 hypophagia also appears to be mediated by prostaglandins (PGs), which are activated by cyclo-oxygenase enzymes (Kaufmann et al., 1997). PGs suppress food intake in experimental animals (Doggett & Jawaharlal, 1977), and inhibition of cyclo-oxygenase activity blocks hypophagia induced by IL-1 (Hellerstein et al., 1989; Uehara et al., 1989a; McCarthy et al., 1995). This supports the proposal that PGs are involved in IL-1 suppression of appetite. However, in addition to effects on food intake, these molecules (IL-1, CRF and PGs) are associated with thermogenic and febrile responses to disease and infection (Rothwell & Cooper, 1992; Kluger et al., 1995; Luheshi & Rothwell, 1996b; Dinarello & Bunn, 1997b; Blatteis & Sehic, 1998; Milton, 1998).

1.4.2 IL-1 as an Endogenous Pyrogen

IL-1 was initially thought to be the major circulating endogenous pyrogen and was named such (**Section 1.4**), although subsequent studies have shown that it is a much more potent pyrogen when injected centrally (Dascombe et al., 1989; Rothwell, 1990c). Furthermore, injection of IL-1ra or neutralising antibodies to IL-1β into the brain of rodents attenuate endotoxin-induced fever (Rothwell, 1990c; Hill et al., 1997; Fleshner et al., 1998), indicating a primarily central role in fever (Dinarello & Bunn, 1997b). This suggestion is supported by the finding that IL-1 is difficult to detect in the plasma (Bristow et al., 1991), but is detected in the hypothalamus (Hagan et al., 1993) after peripheral endotoxin administration in rodents.

The main circulating endogenous pyrogen is now considered to be IL-6, whose plasma bioactivity correlates strongly with fever induced by LPS in rats (LeMay et al., 1990b), and may induce fever by stimulating IL-1 in the brain (LeMay et al., 1990a; Blatteis & Sehic, 1998). Endogenous pyrogens are proposed to signal the brain, either by directly entering the CNS where the blood-brain barrier is compromised (circumventricular organs) (Saper & Breder, 1992), by active transport into the brain, via release of secondary mediators (e.g. PGs), or by direct neuronal afferent signals from the periphery (Fleshner et al., 1998). In a generalised host defence response the brain is probably signalled by several mechanisms (Watkins et al., 1995).

There is extensive evidence that IL-1 acts as an endogenous pyrogen within the brain. Central injection elicits fever at nanogram doses (Busbridge et al., 1989; Dascombe et al., 1989), IL-1β knockout mice exhibit attenuated fever in

response to LPS (Kozak et al., 1995) and central administration of IL-1β antiserum or IL-1ra attenuates fever induced by peripheral administration of LPS or turpentine (Long et al., 1990; Smith & Kluger, 1992; Luheshi et al., 1996a). However, LPS fever is not attenuated by IL-1α antiserum, even at a dose known to inhibit IL-1α fever (Long et al., 1988), suggesting that IL-1β is the primary mediator of fever in response to a generalised systemic response to infection.

It is generally accepted that IL-1 fever is mediated in the brain by PG-dependent mechanisms, since cyclo-oxygenase inhibitors such as indomethacin attenuate the febrile response induced by IL-1 (Coceani et al., 1988; Murakami et al., 1990; Milton, 1998). However, IL-1α and IL-1β appear to exert their pyrogenic effects in the brain via different mechanisms, since febrile effects of IL-1β, but not IL-1α, are inhibited by pre-treatment with either the CRF receptor antagonist α-helical CRF_{9-41}, or with a CRF-neutralising antibody (Busbridge et al., 1989; Rothwell, 1989). Furthermore, CRF and POMC mRNA is increased in response to injection of IL-1β, but not IL-1α (Harbuz et al., 1992). This suggests that IL-1α and IL-1β activate different IL-1 receptors, which in turn produce a thermogenic response via separate pathways – one dependent on CRF and the other independent of CRF. Pre-treatment with a cyclo-oxygenase inhibitor (ibuprofen) or CRF antagonist (α-helical CRF_{9-41}) (Rothwell, 1990c), indicate that these distinct thermogenic pathways are not exclusive to IL-1 (**Table 1.4.2.1**).

Endogenous Pyrogen	Febrile Mechanisms
IL-1 α, IL-1β, TNF-α	PG-dependent
IL-1β, IL-6, IL-8	CRF-dependent

Table 1.4.2.1. Mechanisms of febrile effects of cytokines in the brain

It has already been suggested that IL-1 stimulates the synthesis and release of CRF in the brain (Berkenbosch et al., 1987; Barbanel et al., 1990; Kakucska et al., 1993; Veening et al., 1993), and that CRF mediates IL-1-induced anorexia (**Section 1.4.1**). Evidence suggesting that CRF also mediates the febrile response to IL-1β is derived by studies demonstrating that CRF receptor antagonists or neutralising antibodies block the febrile response to IL-1β (Busbridge et al., 1989; Rothwell, 1989; Nakamori et al., 1993). However, there appears to be a cascade involving PGs and CRF in IL-1-induced fever. Although PGE_2 can mediate fever (Dascombe, 1985; Rothwell, 1989; Morimoto et al., 1989), its effects are independent of CRF (Morimoto et al., 1988; Rothwell, 1989). $PGF_{2\alpha}$ also induces fever (Morimoto et al., 1988) and stimulates CRF release (Bernardini et al., 1989). Moreover, $PGE_{2\alpha}$ fever is blocked by a CRF receptor antagonist, and effects of CRF and $PGE_{2\alpha}$ (but not PGE_2) on body temperature are additive (Rothwell, 1990a). Therefore, the thermogenic effects of IL-1β are considered to act via $PGF_{2\alpha}$ and then CRF, rather than PGE_2.

1.5 CRF

CRF is a 41 amino acid peptide that has been implicated in a variety of CNS disorders (Behan et al., 1996). It mediates several endocrine, metabolic, behavioural and autonomic responses to stress and infection (Dunn & Berridge, 1990; Owens & Nemeroff, 1991), and mediates many actions of cytokines in the brain (Dunn & Berridge, 1990). CRF was first identified in 1981, in the sheep hypothalamus (Vale et al., 1981), and has now been identified in the cow, goat, horse, human, pig, rat, suckerfish, and xenopus toad (Turnbull & Rivier, 1997; Vale et al., 1997). The human and rat forms are identical, and differ by seven amino acids from the ovine sequence (Vale et al., 1983). CRF also shares significant homology with related peptides from lower species such as sauvagine and urotensin (Vale et al., 1983). Moreover, a related mammalian peptide called urocortin has been identified recently (Spina et al., 1996; Donaldson et al., 1996), thereby further extending the CRF peptide family.

CRF is synthesised in precursor form as prepro-CRF (Jingami et al., 1985b) and is suggested to be cleaved to its mature form by intracellular pro-hormone converting enzymes (Brar et al., 1997). CRF is localised particularly to the dorsomedial parvocellular neurosecretory cells of the PVN (Merchenthaler et al., 1984b), but also has a diverse distribution throughout the brain including the cortex, and regions of the limbic system such as the locus coruleus, amygdala and hippocampus (Swanson et al., 1983; Merchenthaler, 1984a). CRF is also present in various peripheral tissues (Petrusz et al., 1985).

CRF receptors exist in several isoforms - a proposal that was suggested when a discrepancy was found between brain and peripheral CRF receptor molecular

weights (Grigoriadis & De Souza, 1988). Further research resulted in the cloning and characterisation of two distinct CRF receptors - CRF receptor type 1 (CRF-R1) (Perrin et al., 1993; Chen et al., 1993; Chang et al., 1993) and CRF receptor type 2 (CRF-R2) (Ross et al., 1994; Lovenberg et al., 1995). CRF-R2 has since been found to exist as three splice variants: CRF-R2α, CRF-R2β (Lovenberg et al., 1995) and recently CRF-R2γ (Kostich *et al.*, 1998), thereby creating a family of four distinct CRF receptor subtypes (Chalmers et al., 1996).

Each receptor has distinct localisation in the brain and therefore, like the IL-1 receptors, may also mediate different actions of the ligand. The CRF-R1 is localised mainly in the neocortical, cerebellar, and sensory relay structures (Chalmers et al., 1995). CRF-R2α is expressed in the subcortical structures including the PVN, VMH, choroid plexus and amygdala, whereas CRF-R2β is found in cerebral arterioles throughout the brain amygdala and hypothalamic nuclei, and in a variety of peripheral tissues (Lovenberg et al., 1995). CRF-R2γ is predominantly expressed in the septum and hippocampus (Kostich *et al.*, 1998). As a result of receptor distributions, the CRF-R1 is generally regarded as the primary neuroendocrine pituitary CRF receptor and important in cortical, cerebellar and sensory roles of CRF. The anatomical distribution of the CRF-R2 indicates a role for this novel receptor in hypothalamic neuroendocrine, autonomic and general behavioural actions of central CRF (Chalmers et al., 1995). Further functional categorisation of the receptors may be derived by comparing the localisation and affinity for CRF and urocortin. The coincidence of urotensin-like immunoreactivity with CRF-R2 in brain, and the observation that urocortin is more potent than CRF at binding and activating CRF-R2, as

well as at inducing c-fos in regions enriched in CRF-R2 receptors, indicate that this new peptide could be an endogenous ligand for CRF-R2 (Vaughan *et al.*, 1995).

In addition to these receptors, a CRF-binding protein (CRF-BP) was initially isolated, cloned and expressed from human plasma (Potter et al., 1991), and subsequently found in the brain in neuronal and astrocytic cells (Behan et al., 1995) co-localised with CRF-expressing cells (Potter et al., 1992). Some of this protein may therefore be partially membrane-associated, although no obvious transmembrane domains are present in its amino acid sequence. Hence, the function of this protein could be as a decoy receptor for CRF, in a regulatory role similar to that of IL-1RII and IL-1 (Behan et al., 1995). Alternatively, Lowry discusses the possibility that CRF-BP acts as a receptor to an as yet undiscovered CRF-like ligand (Lowry, 1995).

CRF is perhaps most noted for its role in the HPA axis as the major stimulus in the release of POMC products such as ACTH and β-endorphin from the anterior pituitary which, in the case of the former induces glucocorticoid release from the adrenal glands (Vale et al., 1981; Rivier & Plotsky, 1986). Glucocorticoids impose a rapid inhibitory effect on the release of CRF and ACTH (Plotsky & Vale, 1984; Jingami et al., 1985a), thereby creating a negative feedback loop, important in regulating stress responses (Turnbull & Rivier, 1997). There has been much interest in the potential for cytokines to activate the HPA axis in neuroimmune-endocrine responses to disease and infection (Rivier & Rivest, 1993; Tilders et al., 1994; Rivier, 1995; Turnbull et al., 1998). This has led to the

understanding that many cytokine-HPA axis interactions are mediated via CRF. One of these interactions is stimulation of thermogenesis.

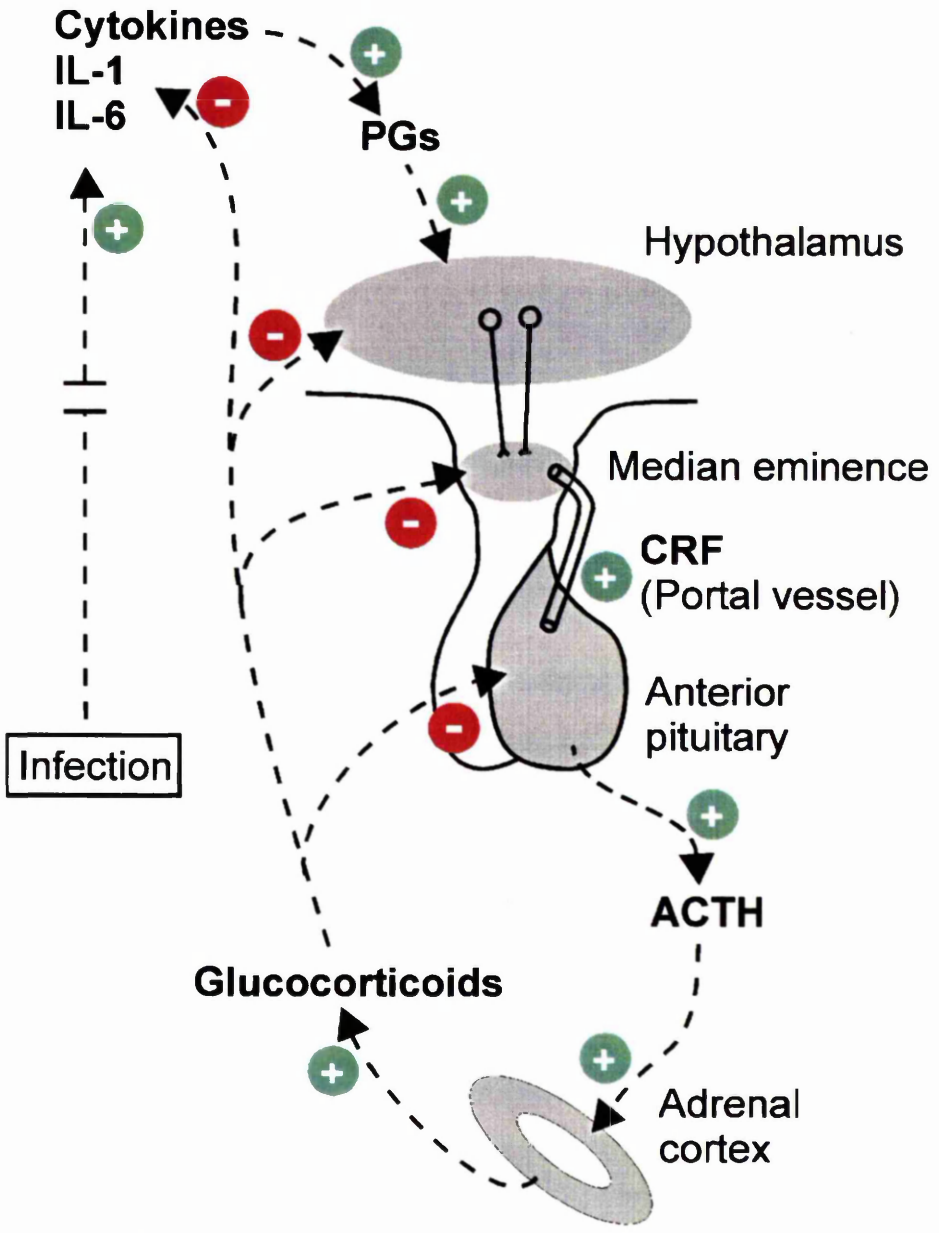

Figure 1.4.2.1. Schematic diagram of the interactions between the HPA axis and cytokines and prostaglandins in infection

1.5.1 CRF and Body Temperature

CRF in the brain, has been implicated in the regulation of energy balance since it reduces food intake and increases energy expenditure by increased thermogenesis and activity, resulting in elevated body temperatures (Rothwell, 1990b). The involvement of CRF in thermogenesis was identified initially because of its known activation of the sympathetic nervous system and increased energy expenditure (Brown et al., 1982; Brown et al., 1985). Increased sympathetic nervous system activity in response to central injection of CRF results in activation of BAT (Holt & York, 1989), eliciting increased GDP binding (a marker of thermogenic activity) and temperature in this tissue (LeFeuvre et al., 1987; Arase et al., 1988). Interestingly, chronic infusion of CRF elicits greater effects on BAT activity in obese Zucker rats than in lean animals (Arase et al., 1989a; Arase et al., 1989b). CRF also increases core body temperature as measured by the rectal probe method (LeFeuvre et al., 1987; Rothwell, 1990a; Rothwell et al., 1991; Strijbos et al., 1992), and similar thermogenic responses are induced by injection of the CRF-related peptides sauvagine and urotensin (LeFeuvre et al., 1989).

Thermogenic effects of CRF are considered to be independent of ACTH, since injection of ACTH or vasopressin do not mimic CRF actions on temperature (LeFeuvre et al., 1987). However, other POMC-derived products may be involved in CRF-induced thermogenesis. Rothwell *et al.* described how CRF thermogenesis is inhibited in the rat by monoclonal antibodies to γ-MSH, and by the antagonist to β-endorphin actions, naloxone (Rothwell et al., 1991).

Moreover, γ-MSH and β-endorphin themselves induce CRF-like thermogenesis, whereas ACTH and α-MSH do not (Rothwell et al., 1991).

1.6 SUMMARY

It is clear from the literature that the mechanisms regulating energy balance are complex, and that in addition to the influence of concious influences imposed by the environment, these factors are under the control of a plethora of neurochemical mediators. Leptin is highly involved in regulating energy balance in normal physiology, and there is growing evidence to suggest that leptin also influences metabolic responses to disease and infection by its action in the brain. Leptin may exert such effects via molecules such as cytokines (that share certain characteristics with leptin) and neuropeptides (e.g. CRF), which are already known to be important in mediating host defence responses.

1.7 AIMS

The overall aim of this study was to investigate the mechanisms of action of leptin in the CNS, and in particular, study its interactions with cytokines (focusing on IL-1) and other proinflammatory agents using food intake, body weight and body temperature responses as end points.

Chapter 2

General Methods

2.1 ESTABLISHMENT

Surgical procedures and experiments were performed in the laboratories of the School of Biological Sciences and the Biological Services Unit (BSU), Stopford Building, University of Manchester, UK, (Home Office Licence 50/01036 and 40/01819) in accordance with the 1986 Animals Act.

2.2 ANIMALS

A variety of male rodents were used, depending upon the specific investigation:

i. Sprague-Dawley rats (Charles River, UK) weighed 250-300 g and were approximately 50 days old.

ii. Lean (Fa/?; 300-350 g) and genetically obese (fa/fa; 500-550 g) Zucker rats (Harlan-Olac, UK) were approximately 120 days old.

iii. IL-1 RI (-/-) mice (Jackson Laboratories, USA) derived from a C57/129J hybrid. C57BL/6 mice (Charles River, UK) were used as controls. Mice were approximately 70 days old and 30 g in weight.

iv. IL-6 (-/-) mice (BSU) derived from a C57/129J hybrid. C57BL/6 mice (Charles River, UK) were used as controls. Mice were approximately 70 days old and 30 g in weight.

All animals were exposed to a 12 h light-dark (08.00 h - 20.00 h) cycle, at an ambient temperature of 22±1°C and 45±10% humidity, and had free access to food (rat chow, Beekay International, UK) and water.

2.3 DRUGS

All drugs were dissolved using sterile techniques, and when necessary, frozen and stored at -70°C. Where possible, drugs were tested for endotoxin contamination by observing drug effects following heat treatment (30 min at 95°C), or by limulus amoebocyte lysate (LAL) test (**Section 2.6**). Drugs were administered into the right lateral cerebral ventricle (icv), intraperitoneally (ip), subcutaneously (sc), or intravenously (iv) in rodents (**Section 2.2**).

2.3.1 CRF

Recombinant human/rat CRF_{1-41} was obtained from three different sources:

i. Prof. Jean Rivier at the Salk Institute for Biological Studies (USA) kindly donated CRF (95% purity) that was dissolved in sterile water / 0.1% low endotoxin BSA (Sigma, UK).

ii. CRF (95% purity) purchased from Sigma (UK) was dissolved in sterile water / 0.1% low endotoxin BSA.

iii. CRF (76.5% purity) kindly donated by Peninsula Laboratories Inc. (USA) was dissolved in 0.9% saline / 0.05% ascorbic acid (Sigma, USA) as recommended.

CRF was administered to rats in the following doses (**Table 2.3.1.1**).

Injection Route	Dose of CRF per Rat
Icv	0.3, 3, 30 μg
Ip	3 μg
Sc	3 μg
Iv	3 μg

Table 2.3.1.1. Doses of CRF

2.3.2 CRF Receptor Antagonists

α-helical-CRF(9-41) was purchased from Novabiochem (UK), dissolved in sterile water / 0.1% low endotoxin BSA, and injected icv in rats (30 μg/rat).

D-Phe-CRF ([D-Phe^{12},Nle^{21,38},α-Me-Leu^{37}]-CRF(12-41) human/rat) was purchased from Bachem (UK), and dissolved in sterile water for icv injection in rats (1, 2.5 and 5 μg/rat).

2.3.3 Flurbiprofen

The sodium salt of flurbiprofen was a gift from Knoll Pharmaceuticals Ltd (UK), and dissolved (1 mg/ml) in sterile saline plus 1% sodium bicarbonate for injection in rats (1 mg/kg, ip).

2.3.4 IL-1 and IL-1ra

Recombinant rat IL-1α, IL-1β and IL-1ra kindly donated by Dr Steve Poole (NIBSC, UK) were dissolved in phosphate-buffered saline (PBS), and administered in doses (derived from previous studies (Busbridge *et al.*, 1989;

Dascombe *et al.*, 1989; Luheshi *et al.*, 1996)) described in the following table (**Table 2.3.1.1**).

Interleukin	Dose per rat (icv)
IL-1β	5 ng
IL-1α	50 ng
IL-1ra	2 x 100, 200 μg

Table 2.3.4.1. Doses of IL-1 and IL-1ra

2.3.5 Leptin

Leptin (rat or murine) was administered icv to both rats and mice at doses of 0.4, 1 or 4 μg. Rats received ip injections of 1 mg/rat (3.5-4 mg/kg).

Recombinant murine leptin was obtained from two sources. Leptin from Insight Biotechnology Ltd. (UK) was dissolved in sterile water. Leptin kindly donated by Dr Brian Holloway (Zeneca Pharmaceuticals, UK) was already dissolved (0.5 mg/ml) in PBS.

Recombinant rat leptin was purchased from R&D Systems (UK). The protein (1 mg) was dissolved in 1.5 ml sterile HCl (15 mM) / 0.3 ml sterile NaOH (7.5 mM) to bring the pH to approx. 6.

2.3.6 LPS

The gram negative, bacterial glycoprotein, lipopolysaccharide (LPS) was purchased from Sigma (UK; serotype 0128:B12; lot 90H4012), dissolved in sterile saline, and injected icv (0.3 μg/rat) or ip (100 μg/kg rat).

2.3.7 Prostaglandin

PGE_2 was purchased from Sigma (UK), dissolved in sterile saline (0.9%) and administered to rats (500 ng, icv).

2.3.8 Water and Saline

Sterile water for injections and sterile saline (0.9%) were purchased from B. Braun Medical Ltd. (UK).

2.4 Surgical Procedures

Animals were anaesthetised (verified by absence of tail-pinch and corneal reflex) prior to surgery using a gaseous mixture of 3% halothane (Fluothane, Zeneca, UK) in oxygen at 2 l/min using a flow-chamber. Animals were then transferred to a stereotaxic frame (Stoelting, USA) equipped with a specially constructed mouthpiece delivering the same anaesthetic mixture, to enable surgery (**Figure 2.3.8.1**).

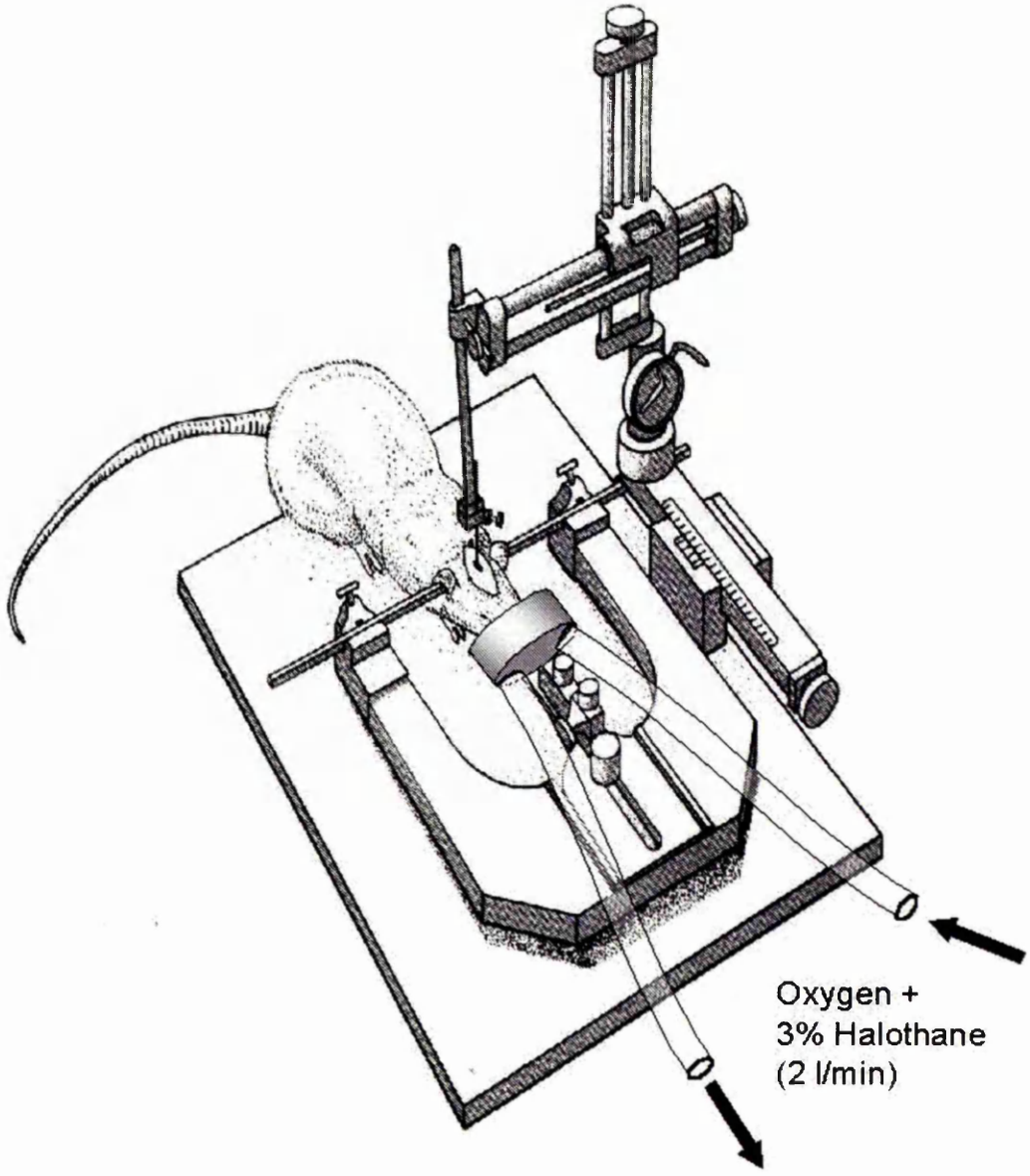

Figure 2.3.8.1. Schematic diagram depicting stereotaxic surgery (adapted from Carlson, 1991)

2.4.1 Implantation of Lateral Ventricle Guide Cannulae in Rats

Anaesthetised animals (**Section 2.4**) were secured in a stereotaxic frame by ear bars (positioned in the external auditory meatae) and a jaw bar holding the superior incisors at a level of 3.3 mm below the intra-aural line.

Once animals were secured in the frame, the dorsal aspect of the head (from eyes to occipital notch) was shaved and swabbed with iodine solution. A 2-3 cm incision was then made along the median sagital plane of this area and the skin clamped back to reveal the underlying tissue. This loose connective tissue and periosteum was displaced by the use of a cotton bud to expose the surface of the skull.

Holes were drilled in the left caudal and right rostral quadrants of the skull with a dental drill (Radio Spares Ltd., Corby, UK; burr size 1.5 mm). Screws (4 mm long x 2 mm diameter) were inserted into these holes taking care not to pierce the meninges on the under-surface of the cranium. The guide cannula (constructed from sterile 0.8x40 mm hypodermic needles (Microlance 3, BD Ltd., UK) cut to 3 mm length) was secured to the vertical arm of the stereotaxic manipulator, and positioned so that the tip rested on bregma which was taken as zero for stereotaxic reference. From there the cannula was moved 0.8 mm caudal, 1.5 mm lateral (right) and 3 mm ventral (length of guide cannula).

Another hole was drilled at these co-ordinates to allow the guide cannula to be lowered to penetrate the brain. This resulted in the tip of the guide cannula being situated 2 mm superior to the right lateral ventricle (**Figure 2.4.1.1**). The guide cannula was secured in position by moulding acrylic dental cement (Simplex Rapid, Associated Dental Products Ltd, UK) around the screws and cannula, taking care that no sharp or abrasive edges were sculpted.

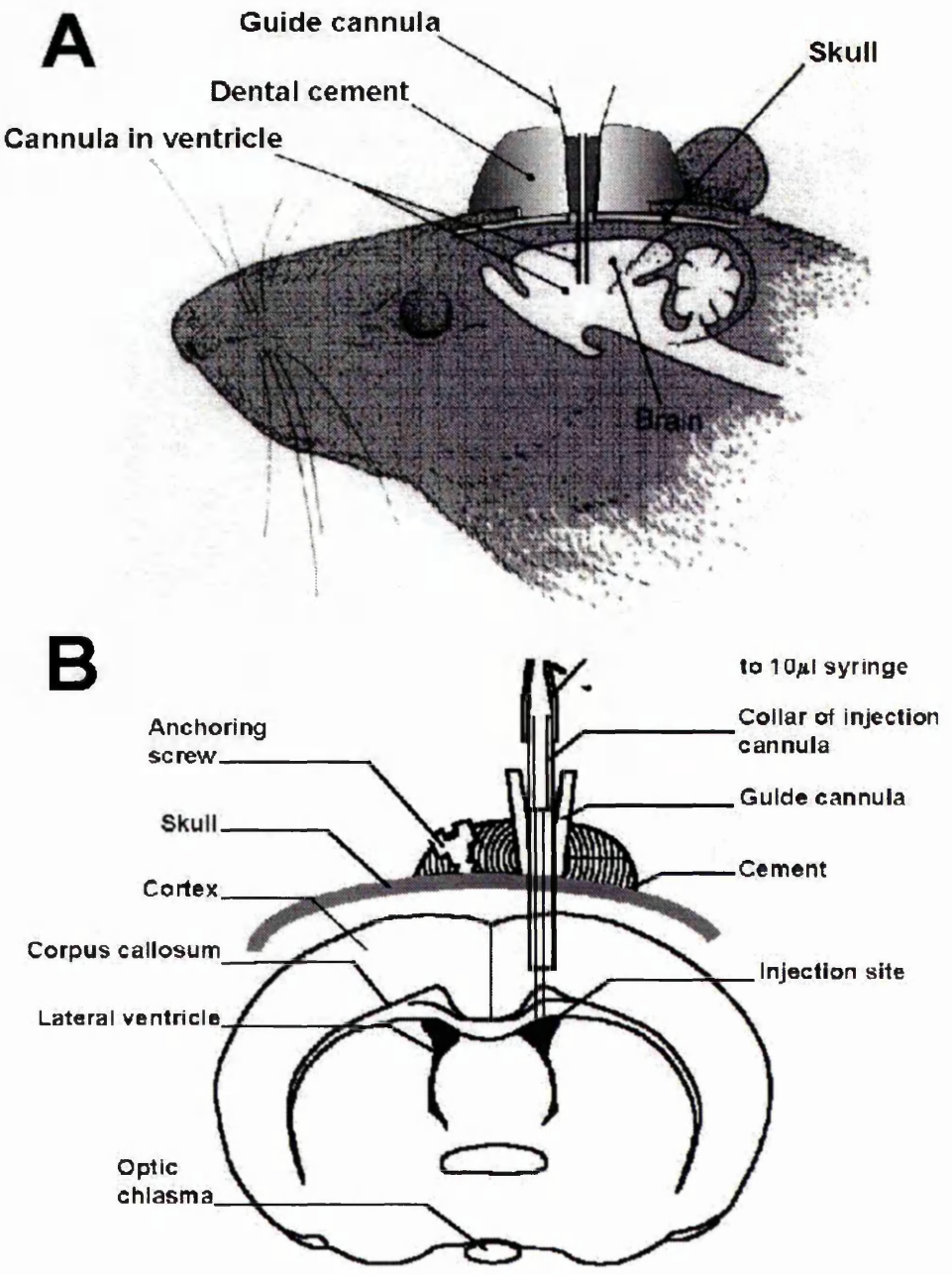

Figure 2.4.1.1. Schematic diagram representing (A) a sagital section of a guide cannula for (B) icv injection (shown coronally).

Whilst the cement was setting, it was possible to perform (if required) implantation of an intraperitoneal radiotransmitter (**Section 2.4.3**). When the cement had hardened sufficiently, the vertical arm of the frame was raised leaving the cannula in place, and the scalp was sutured.

2.4.2 Implantation of Lateral Ventricle Guide Cannulae in Mice

Mice were anaesthetised as above (**Section 2.4**), and positioned in a stereotaxic frame specially adapted for mice. The implantation procedure was performed as above (**Section 2.4.1**) using 0.9x1.7 mm screws and guide cannulae constructed (cut to 1.5 mm length) from sterile 0.6x30 mm hypodermic needles (Microlance 3, BD Ltd., UK). Co-ordinates from bregma for implanting guide cannulae were 0.3 mm caudal, 1.5 mm lateral (right) and 1.5 mm ventral (length of guide cannula).

2.4.3 Implantation of Temperature-Sensitive Radiotransmitters in Rats

An area (approx. 4x4 cm) was shaved in the abdomen on one side of the mid-line and swabbed with iodine solution. A small incision was made in the midline of the skin and underlying musculature to expose the peritoneal cavity. A temperature-sensitive radiotransmitter (Data Sciences International, USA), previously sterilised by soaking overnight in a 2% gluteraldehyde solution (Sigma, UK) before being washed in distilled water was then inserted into the cavity and the incised layers of tissue were sutured. Transmitters were calibrated previously by the manufacturer.

2.5 EXPERIMENTAL PROCEDURES

2.5.1 Housing

All animals were allowed one week to recover from surgery before being housed individually 24 h before the start of the experiment. Rats were housed in cages with sawdust bedding, whereas mice were housed in wire bottomed cages (to allow for measurement of food spillage).

2.5.2 Injections

Injections were performed at 10.00 h for daytime measurements, and at 18.00 h during evening experiments.

Drugs were administered directly into the brain of free-moving rats (**Figure 2B**), or lightly restrained mice by injection into the right lateral cerebral ventricle (icv) via a previously implanted guide cannula (**Sections 2.4.1 and 2.4.2**), using a 10 µl Hamilton syringe attached to a flexible injection cannula.

Injection cannulae for icv injection were made by inserting a length of 31 gauge stainless steel tubing into a 1 cm length of 23 gauge stainless steel tubing, and cementing the two together. The inner tubing was then neatly broken to achieve a length of 11.5 mm (rats) or 8 mm (mice). A 25 cm length of PE10 plastic tubing was glued to the proximal end of the cannulae to allow flexibility between the syringe and the cannula tip during injection into free-moving animals.

Volumes of 2-4 µl were injected over a period of 10-20 s, after which the injection cannula was left in place for a further 10 s to allow the expelled solution to diffuse into the ventricle.

Intraperitoneal (ip) injections (1 ml/kg) into hand-held animals were performed using sterile 0.5x16 mm hypodermic needles (Microlance 3, BD Ltd., UK).

Subcutaneous (sc) injections of 0.2 ml were administered via sterile 0.5x16 mm hypodermic needles to the dorsal skin fold of hand-held rats.

Intravenous (iv) injections were performed into rats restrained in a Perspex restraining tube. The tail (protruding from the tube) was immersed in warm water to vasodilate the tail vein, into which injections (1 ml/kg) were administered using sterile 0.5x16 mm hypodermic needles.

2.5.3 Measurement of Core Body Temperature in Rats

Core body temperature of animals was monitored for at least 1 h before injection to obtain constant baseline values.

2.5.3.1 Remote Radiotelemetry

Transmitters, previously implanted (**Section 2.4.3**) were activated magnetically, 24 h before injection. Each transmitter emitted a frequency that was proportional to the core body temperature of the animal. This output frequency was monitored by an antenna mounted in a receiver board situated beneath the cage of each animal. The information was then relayed to a peripheral processor (DataQuest IV, Data Sciences International, USA) connected to an IBM-PC (**Figure 2.5.3.1**) which sampled and recorded the temperature of each animal at 10 min intervals by converting the frequency information into degrees Celsius (°C). This system allowed continuous, undisturbed monitoring of the

freely moving conscious animals. Animals exhibiting a pre-injection temperature outside the range 36.9-37.3°C were excluded from the experiment.

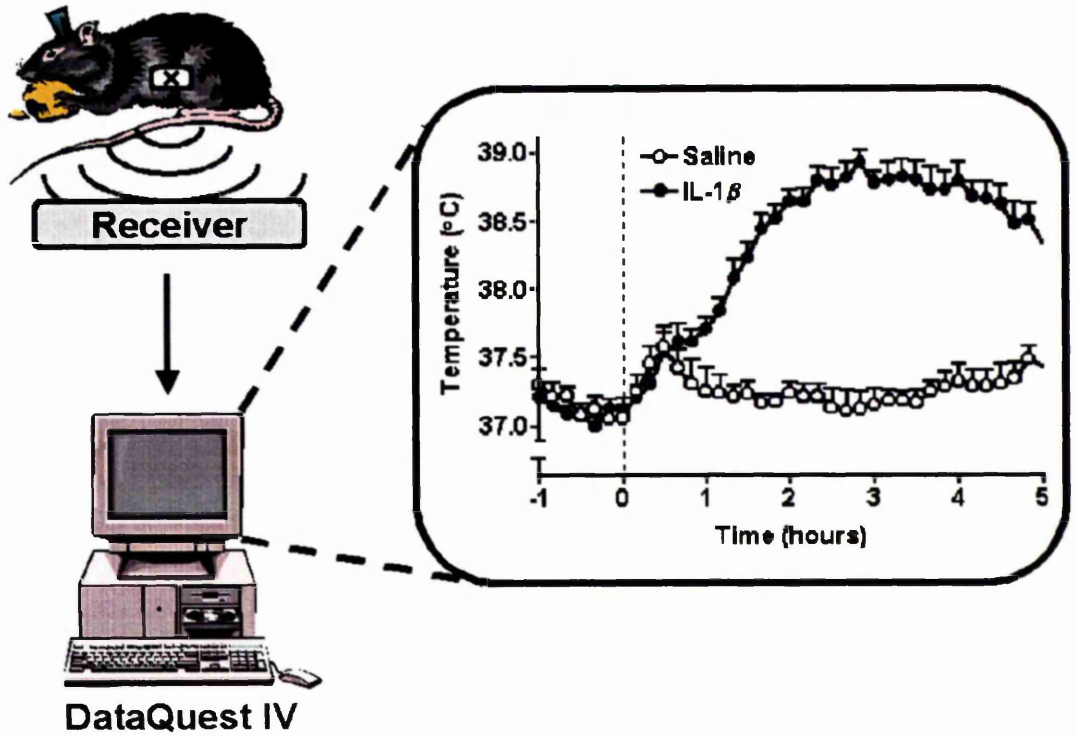

Figure 2.5.3.1. Schematic diagram showing measurement of core body temperature in rats, by remote radiotelemetry.

2.5.3.2 Colonic Probe

In selected experiments (**Chapter 5**), core body temperature was measured manually by colonic probe, in order to compare the accuracy of each method of temperature measurement, and to mimic published data.

Lightly-restrained rats were exposed daily to this method of temperature measurement during the one week recovery period after stereotaxic surgery, to

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familiarise them to the procedure and reduce stress during experiment. A plastic-coated thermocouple (Comark Electronics Ltd., UK) was greased with Vaseline and inserted approximately 5 cm beyond the anus of the lightly restrained rats. The probe was maintained in position for 20 s, or until the temperature reading remained constant, and then the temperature was recorded. This operation was repeated every 30 min, starting one hour before injection.

2.5.4 Measurement of Food Intake

Food for each animal (50 g/rat, 25 g/mouse) was weighed out (Mettler PJ360, DeltaRange; accurate to 0.1 g) at the time of injection (10.00 or 18.00 h). Food was weighed again at the beginning of the subsequent light phase (08.00 h), 22 or 14 h later respectively. Food spillage from mice was also collected at this time. Food intake was calculated by subtracting 22 or 14 h food weight, from food weight at injection (minus spillage for mice).

2.5.5 Measurement of Body weight

Rats were weighed using a balance accurate to 1 g (Lume-O-Gram balance, OHAUS). Mice were weighed with a balance accurate to 0.1 g (Mettler PJ360, DeltaRange). Measurements were performed in the same manner as for food intake (**Section 2.5.4**): firstly on injection, and again at the start of the subsequent light phase.

2.5.6 Immunohistochemistry

2.5.6.1 Tissue Preparation for Immunohistochemistry

Rats were injected with 0.5 ml sodium pentobarbitone (30 mg/ml, ip; Sagatal, Rhone Merieux Ltd., UK) for IL-1 immunohistochemistry. When animals were rendered unconscious, the ventral aspect of the rib cage was exposed and removed. A butterfly needle (0.6x19 mm, Abbott Laboratories Ltd., UK) was inserted into the left ventricle of the heart and the right atrium cut.

Saline (0.9%) cooled to 4°C was perfused into the heart at a rate of 40 ml/min for 8 min to remove all blood from the animal. The saline was then replaced by PBS / 4% paraformaldehyde (PFA) at 4°C for a further 12 min. The brain was removed and soaked overnight in fixative at 4°C before rinsing and storing in PBS. Sections were cut (50 μm thick) using a vibratome (Series 1000, The General Scientific Company Ltd., UK) and stored at 4°C in TBS.

2.5.6.2 Staining for Immunoreactive IL-1β

Sections at injection site and hypothalamic levels were transferred to Costars (1 section per well) and stained for immunoreactive (ir) IL-1β using:

i. 0.1% hydrogen peroxide (H_2O_2) / tris-buffered saline (TBS).

ii. Normal donkey serum (NDS) in 0.1% BSA / TBS (1:4).

iii. Primary antibody (sheep anti-rat IL-1 Ab) kindly donated by Dr Steve Poole (NIBSC, UK), diluted 1:750 in 0.1% BSA / TBS.

iv. Secondary antibody (biotinylated donkey anti-sheep Ab, Sigma, UK) diluted 1:1500 in 0.1% BSA / TBS.

v. 3% avidin peroxidase / TBS.

vi. 3% DAB / 0.2% H_2O_2 / TBS (10 min).

2.5.7 Immunoassays for Immunoreactive IL-1β and IL-6

Immunoassays were performed using enzyme-linked immunosorbent assays (ELISA) specific for rat IL-6 and rat IL-1β (kindly provided by Dr S. Poole, NIBSC, UK). Three hours after injection animals were anaesthetised (**Section 2.4**) and blood plasma was sampled by cardiac puncture, and stored at 4°C in heparin (50 µl Monoparin, CP Pharmaceuticals Ltd. / 5 ml blood). The animals were killed by cervical dislocation, and the hypothalami removed. The hypothalami were placed in PBS, homogenised and centrifuged (6000 rpm for 30 min at 4°C). Supernatant and blood plasma were assayed for immunoreactive IL-1β and IL-6 by ELISA, using immunoaffinity-purified polyclonal antibodies (2 µg/ml):

i. Sheep anti-rat IL-1β (S1002BH) or sheep anti-rat IL-6 (S206B1).

ii. Recombinant rat IL-1β (1.9-2000 pg/ml) or recombinant rat IL-6 (1.9-4000 pg/ml) reference standards.

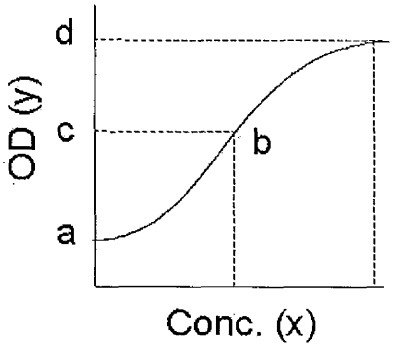
iii. Biotinylated immunoaffinity-purified polyclonal antibodies from sheep anti-rat IL-1β or sheep anti-rat IL-6 serum (1:1000).

iv. Avidin-horseradish peroxidase (Sigma, 1:5000), and colour reagent (OPD, Sigma). The optical densities of the wells were read at 490 nm using an MRX Microplate reader (Dynatech, UK).

Cytokine concentrations were calculated from the optical density (OD) using Rodbard's four-part equation (**Figure 2.5.7.1**).

$$y = \frac{[a-d]}{[1+(\frac{x}{c})^b]} + d$$

$$x = c \sqrt[b]{\frac{[a-d]}{[y-d]} - 1}$$



a = minimum response
b = shape factor
c = midway response (= $\frac{d-a}{2}$)
d = maximum response

Figure 2.5.7.1. Derivation of protein concentration from OD, using Rodbard's four-part equation

2.6 LIMULUS AMOEBOCYTE LYSATE (LAL) TEST

Leptin and CRF antagonists were assayed semi-quantitatively for Gram-negative associated endotoxin contamination by Pyrogent® gel-clot LAL kit (BioWhttaker, UK).

2.7 Data Manipulation and Statistics

All data are expressed as the mean ± SEM. Number (n) of animals in each experimental group ranged from 4-8 in all except immunohistological experiments, where n=2. Statistical analyses were performed following consultation with Dr Val Hillier (Department of Medical Biophysics, University of Manchester). Analyses that revealed a probability of less than 5% ($p<0.05$) were considered significant.

2.7.1 Analysis of Multiple Time Point Data

Body temperatures of animals were statistically analysed for significant differences at half-hour intervals over the time course of the experiment by multiple analysis of variance (MANOVA) using SPSS software. The following syntax (**Figure 2.7.1.1**) was written to perform this analysis.

```
MANOVA
t1 to t7  BY
treat(1,4)
/WSFACTORS time(7)
/CONTRAST (treat)=simple(1)
/ERROR WITHIN+RESIDUAL
/PRINT HOMOGENEITY(BARTLETT COCHRAN BOXM)
/design = treat(1), treat(2), treat(3)
/PRINT SIGNIF( MULT AVERF HF GG ).
```

Figure 2.7.1.1. SPSS syntax to perform MANOVA

This syntax instructs the programme to perform MANOVA on treatment groups 1-4 (altered depending on number of groups) over the 6 h time course after injection (time points `t1 to t7`), and then to compare the profile of treatment group 1 with that of the other three treatment groups. Repetition of the analysis after changing '`simple(1)`' (line 4) to '`simple(2)`' and '`simple(3)`' allows all groups to be compared against each other.

Where MANOVA revealed significant difference ($p<0.05$), individual time points of interest were further analysed (see below).

2.7.2 Analysis of single time point data

For experiments where data were obtained from a single time point, or where a point of interest warranted further investigation (as revealed by MANOVA, data from two treatment groups were analysed by unpaired student's t-test. Single time point data from more than two treatment groups were analysed by one-way analysis of variance (ANOVA) followed by Neuman-Keuls multiple comparisons test.

Chapter 3

Leptin and Cytokines

3.1 INTRODUCTION

It has been reported extensively that leptin reduces food intake and body weight in animals that possess normal leptin receptors. Most studies investigating the responses to injection of leptin have been performed on mice, since this was the species in which leptin was first identified (Zhang *et al.*, 1994), and because of the availability of genetically obese mice such as *ob/ob* and *db/db* (Coleman, 1973; Coleman, 1978).

The first studies reporting the effects of leptin on food intake and body weight were all published in the same edition of *Science* in 1995. Pelleymounter *et al.* described how ip injection of a high dose (10 mg/kg/day for 28 days) of leptin, lowered body weight, body fat, food intake, and serum glucose and insulin in *ob/ob*, but not normal mice (Pelleymounter *et al.*, 1995). In addition, metabolic rate, body temperature, and activity levels of *ob/ob* mice were increased by this treatment (although *ob/ob* mice normally exhibit lower body temperature and metabolic rate than lean animals) (Pelleymounter *et al.*, 1995). None of these parameters was altered beyond the level observed in lean controls, suggesting that leptin normalised the metabolic status of the *ob/ob* mice. Halaas *et al.* demonstrated similar effects in response to ip administration of leptin (5 mg/kg/day for 33 days) in *ob/ob* but not *db/db* mice (Halaas *et al.*, 1995). Furthermore, Campfield *et al.* injected a much lower dose (6 μg/mouse/day, equivalent to approx. 0.12 mg/kg/day) of leptin for 10 days, which still reduced food intake and body weights of *ob/ob* mice (Campfield *et al.*, 1995). This study also reported that icv injection of leptin (1 μg/mouse) significantly reduced food

intake and body weight in both *ob/ob* and lean, but not *db/db* mice after overnight fasting. Other studies have reported similar effects in response to acute icv injection of leptin (Van Heek *et al.*, 1997). Chronic infusion (icv) of leptin (3 ng/h for 30 days) elicited complete depletion of visible adipose tissue (Halaas *et al.*, 1997). Some studies have reported normalised core body temperatures of *ob/ob* mice in response to ip injection of leptin (10, 42 μg/day) (Pelleymounter *et al.*, 1995; Harris *et al.*, 1998). In addition, icv (1 μg) injection of leptin increased energy expenditure in *ob/ob* mice (Hwa *et al.*, 1996).

Although fewer studies have been performed in rats, similar responses to icv injection of leptin have been observed. Schwartz *et al.* described how icv injection of 3.5 μg leptin at the onset of nocturnal activity significantly reduced food intake of normal Long-Evans rats by 50% over 1 h, and by 42% over 4 h after injection (Schwartz *et al.*, 1996). Intraperitoneal injection of the same dose of leptin did not affect food intake. In a separate study, twice-daily injections of leptin (0.05 μg) for 3 days into the ventromedial hypothalamus (VMH) of normal Sprague-Dawley rats caused reduced food intake (56%) and 5% body weight loss (Jacob *et al.*, 1997). In this study, animals fully recovered from leptin-induced effects within 3 days (Jacob *et al.*, 1997).

The obese (*fa/fa*) Zucker rat is similar to the *db/db* mouse since they both possess defective leptin receptors. It has been demonstrated that food intake is reduced in both fed and food deprived lean (*Fa/?*), but not obese (*fa/fa*) Zucker rats in response to icv injection of leptin at doses of 3.5 μg/rat (Seeley *et al.*, 1996). Furthermore, Cusin *et al.* described how lean, but not obese Zucker rats

exhibited reduced food intake and body weight gains that persisted for 2 and 6 days respectively, in response to injection of 3 μg/rat leptin (Cusin *et al.*, 1996).

N-terminal (but not C-terminal) peptide fragments of the leptin molecule have also been shown to induce anorexic effects on food intake in response to icv injection (1 μg/rat) in normal Sprague Dawley rats (Samson *et al.*, 1996), indicating that satiety effects of leptin reside in the N-terminal region. However, a study by Fruhbeck *et al.* reported that ip injection (1 mg of each fragment per kg) of a pool of five 20-amino acid peptide fragments derived from the C-terminal region of the leptin protein produced a statistically significant reduction in body weight gain in Wistar rats, while rectal temperature showed a statistically significant increase (Fruhbeck *et al.*, 1998).

The studies described in this chapter were performed to describe in detail, the responses to icv injection leptin on food intake and body weight, in normal Sprague-Dawley rats, fed *ad lib.* Considering that leptin (Zhang *et al.*, 1997) and its receptor (Tartaglia *et al.*, 1995; White & Tartaglia, 1996; Nakashima *et al.*, 1997; Tartaglia, 1997) have been reported to possess similar structural properties to cytokines, further studies were performed to compare actions of leptin and proinflammatory agents.

3.2 Effects of Leptin from Different Sources on Food Intake and Body Weight in Rats

Many studies involving rats have used murine leptin (e.g. (Seeley *et al.*, 1996; Wang *et al.*, 1997; al-Barazanji *et al.*, 1997; Widdowson *et al.*, 1997)), since there is 96% homology of the molecule between species (Murakami & Shima, 1995), and with the assumption that injection of leptin induces the same effects in both rodent species. The objective of the first experiment described was to investigate whether murine and rat leptin indeed elicit the same effects on food intake and body weight in rats.

3.2.1 Experimental Design

Food intake and body weights were measured over 22 h after injection (at 10:00 h) of vehicle, or leptin from three different sources: rat (R&D Systems); murine (Insight Biotechnology); murine (Zeneca).

A dose (4 μg) similar to that used in published work (Schwartz *et al.*, 1996) was injected into the lateral cerebral ventricle (icv) of normal Sprague-Dawley rats, and the food intake and body weights monitored as described previously (**Section 2.5.4-5**).

3.2.2 Results

Vehicle-treated animals consumed 27.7±0.6 g rat chow over the experimental time course (**Figure 3.2.1**). Injection of leptin from all sources significantly (ANOVA: $p<0.001$ vs Vehicle) reduced food intake by 48% (rat, R&D), 45%

(murine, Insight), and 43% (murine, Zeneca). There was no significant difference in responses to leptin from different sources (ANOVA).

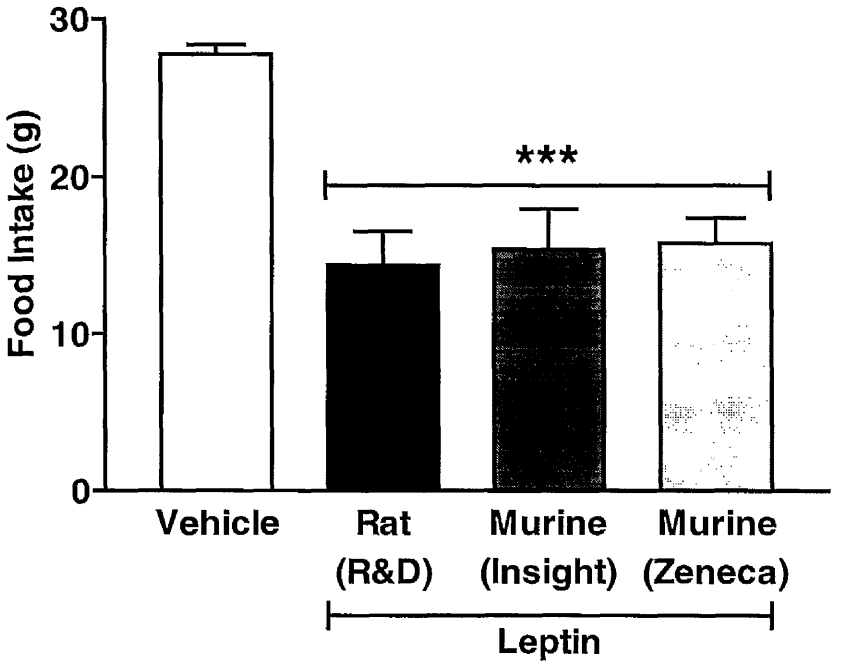


Figure 3.2.1. Food intake over 22 h in response to injection of leptin (4 μg, icv) from three different sources: rat leptin (R&D Systems), and murine leptin from Insight Biotechnology or Zeneca

(ANOVA: *** $p<0.001$ vs Vehicle)

Body weights of these animals were also measured (**Figure 3.2.2**). Vehicle-treated rats gained 7±1 g body weight over the 22 h after injection. Administration of leptin from each source induced significant loss of body weight over the experimental time course (ANOVA: $p<0.001$ vs Vehicle). Rat leptin (R&D Systems) reduced body weight by 12±4 g, whereas murine leptin obtained from Insight Biotechnology or Zeneca elicited weight loss of 13±4 and

16±3 g respectively. There was no significant difference in responses to leptin from different sources (ANOVA).

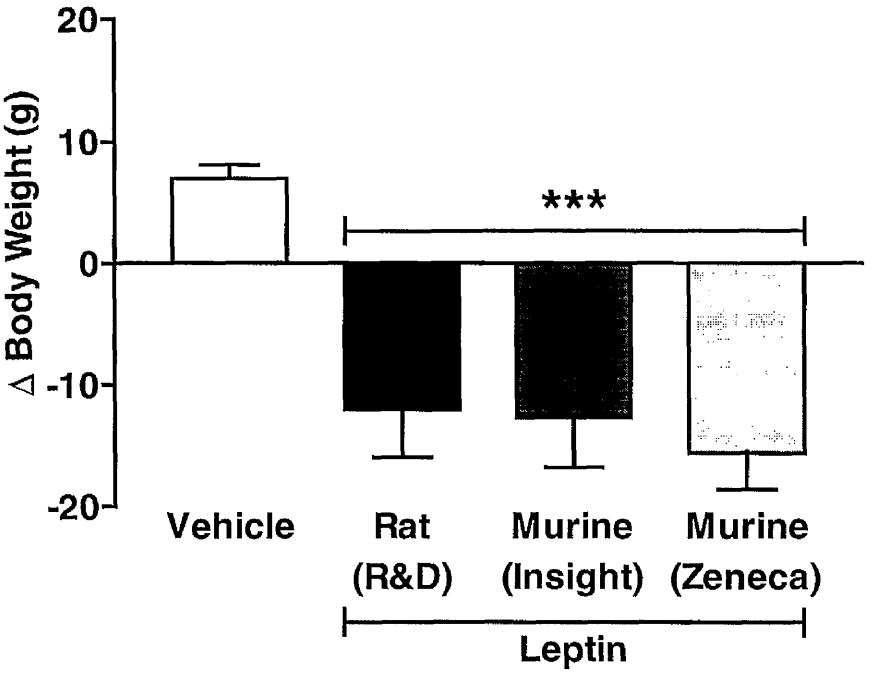


Figure 3.2.2. Body weight over 22 h in response to injection of leptin (4 μg, icv) from three different sources: rat leptin (R&D Systems), and murine leptin from Insight Biotechnology or Zeneca

(ANOVA: *** $p<0.001$ vs Vehicle)

3.3 Leptin Dose-Response Study on Food Intake and Body Weight (a.m. Injection)

In most of the subsequent studies presented in this thesis, murine leptin purchased from Insight Biotechnology was used, as this was the most readily available source. From the results described in the previous section, this leptin did not appear to differ in its effects on food intake and body weight compared

with leptin from other sources. Therefore the actions of murine leptin (Insight Biotechnology) were characterised further by performing a dose-response study on food intake and body weight gain in rats.

3.3.1 Experimental Design

Doses of leptin (0.4, 1, and 4 μg), or vehicle were injected (icv) at 10:00 h in normal rats. Food intake and body weight gains were measured over 22 h after injection.

3.3.2 Results

Vehicle-treated animals consumed 27.0±1.0 g food over the 22 h after injection at 10:00 h (**Figure 3.3.1**). All rats injected with leptin exhibited significantly attenuated food intake compared to the control group (ANOVA). Administration of 0.4 μg leptin inhibited food intake by 15% ($p<0.05$), whereas 1 μg and 4 μg attenuated consumption by 26% ($p<0.01$) and 40% ($p<0.001$) respectively, compared to vehicle-treated rats.

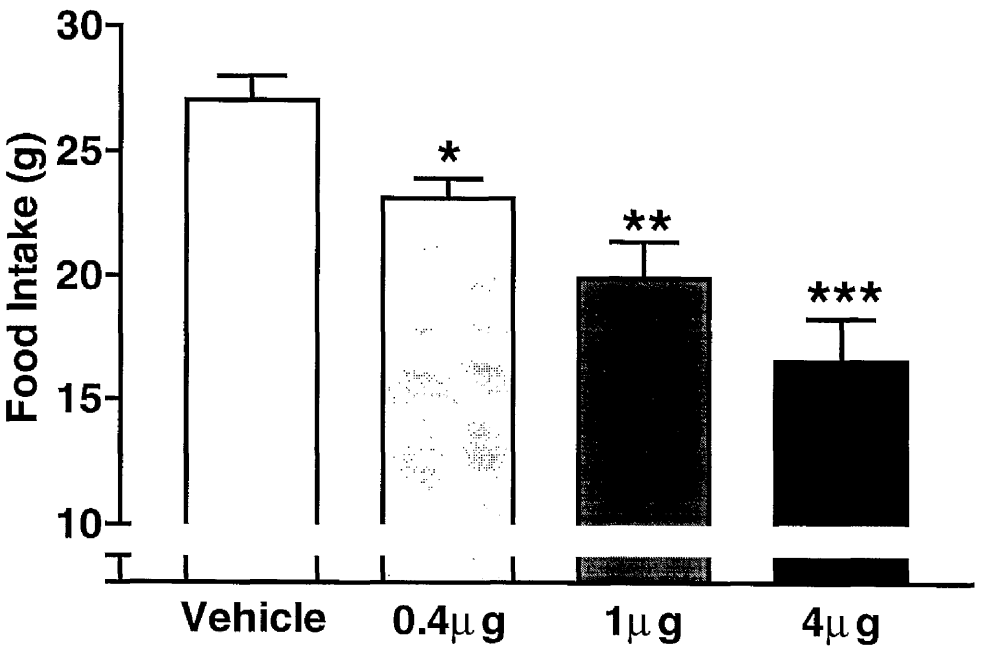


Figure 3.3.1. Dose-response study on the action of leptin (0.4, 1, and 4 μg, icv) on food intake over 22 h after injection at 10:00 h

(ANOVA: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs Vehicle)

Animals injected with vehicle gained 12±1 g body weight over the 22 h after injection at 10:00 h (**Figure 3.3.2**). The lowest dose of leptin (0.4 μg) limited body weight gain to only 1±1 g (ANOVA: $p<0.05$ vs Vehicle). Injection of 1 μg leptin caused weight loss of 5±4 g ($p<0.01$ vs Vehicle), whereas the highest dose of leptin (4 μg) elicited a reduction in body weight of 9±3 g ($p<0.001$ vs Vehicle). The weight loss caused by injection of 4 μg leptin was also significantly different compared to the effect of the lowest dose (0.4 μg) of leptin on body weight (ANOVA: $p<0.05$).

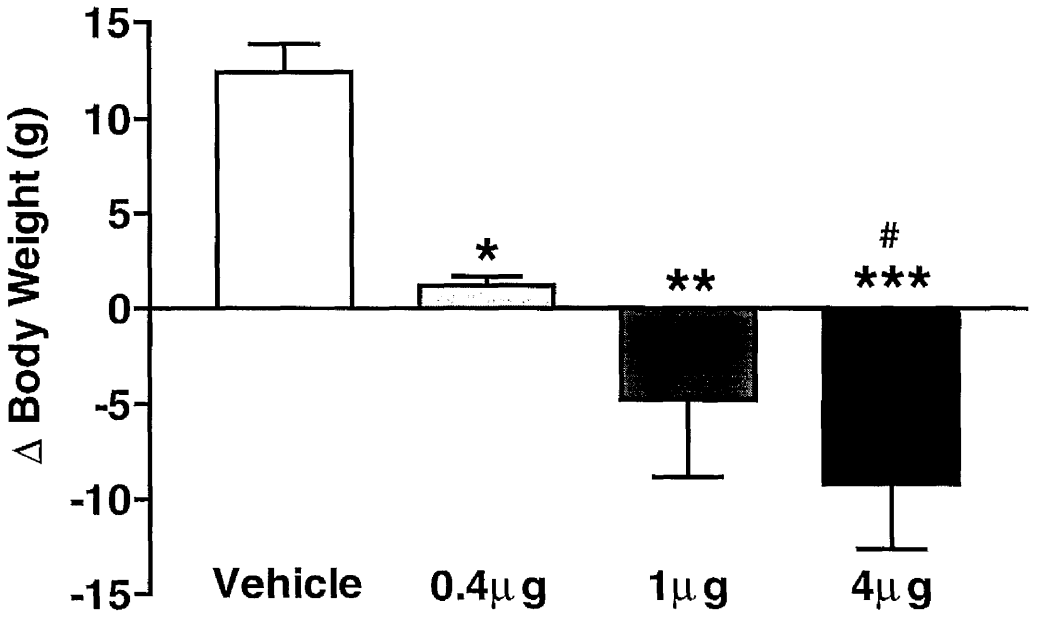


Figure 3.3.2. Dose-response study on the action of leptin (0.4, 1, and 4 μg, icv) on body weight gain over 22 h after injection at 10:00 h

(ANOVA: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs Vehicle; # $p<0.05$ vs 0.4 μg)

3.4 Leptin Dose-Response Study on Food Intake and Body Weight (P.M. Injection)

Rats are nocturnal animals, and so eat mainly during the dark phase. Thus it was proposed that central injection of leptin just before the dark phase, might elicit greater effects on food intake and body weight than exhibited by animals receiving leptin at the beginning of the light phase as described in the previous section.

3.4.1 Experimental Design

Doses of leptin (0.4, 1, 4 μg), or vehicle were injected (icv) at 18:00 h, and food intake and body weight gain measured for 14 h over the subsequent dark phase (20:00-08:00 h).

3.4.2 Results

Vehicle-treated animals consumed 24.3±0.8 g food over 14 h after injection at 18:00 h (**Figure 3.4.1**). All rats injected with leptin (0.4, 1 or 4 μg) exhibited significantly attenuated food intake (ANOVA). Administration of 0.4 μg leptin inhibited food intake by 19% ($p<0.05$). Injection of 1 and 4 μg leptin attenuated consumption by 45% ($p<0.001$) and 47% ($p<0.001$) respectively. Both higher doses (1 and 4 μg) elicited significantly greater inhibition of food intake than that induced by 0.4 μg leptin (ANOVA: $p<0.01$).

Animals injected with vehicle gained 13±1 g body weight over 14 h after injection at 18:00 h (**Figure 3.4.2**). The lowest dose of leptin (0.4 μg) limited body weight gain to 7±1 g (ANOVA: $p<0.05$ vs Vehicle). Injection of 1 μg leptin inhibited body weight gain to 2±2 g ($p<0.01$ vs Vehicle), whereas the highest dose of leptin (4 μg) elicited a weight loss of 5±3 g ($p<0.001$ vs Vehicle). This weight loss was also significantly different compared with changes in body weight elicited by the lower doses (0.4 and 1 μg) of leptin (ANOVA: $p<0.001$, $p<0.01$ respectively).

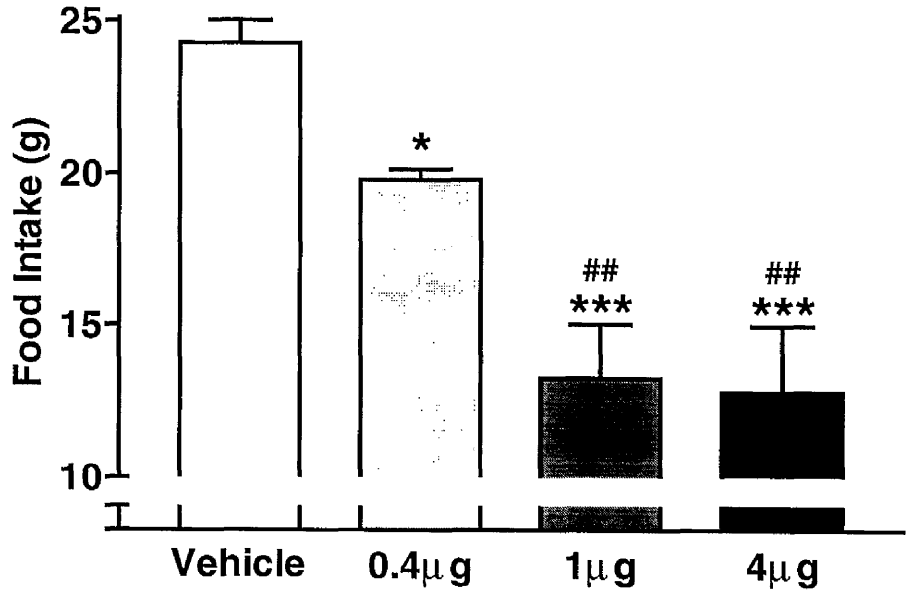


Figure 3.4.1. Dose-response study on the action of leptin (0.4, 1, and 4 μg, icv) on food intake over 14 h after injection at 18:00 h

(ANOVA: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs Vehicle; ## $p<0.01$ vs 0.4 μg)

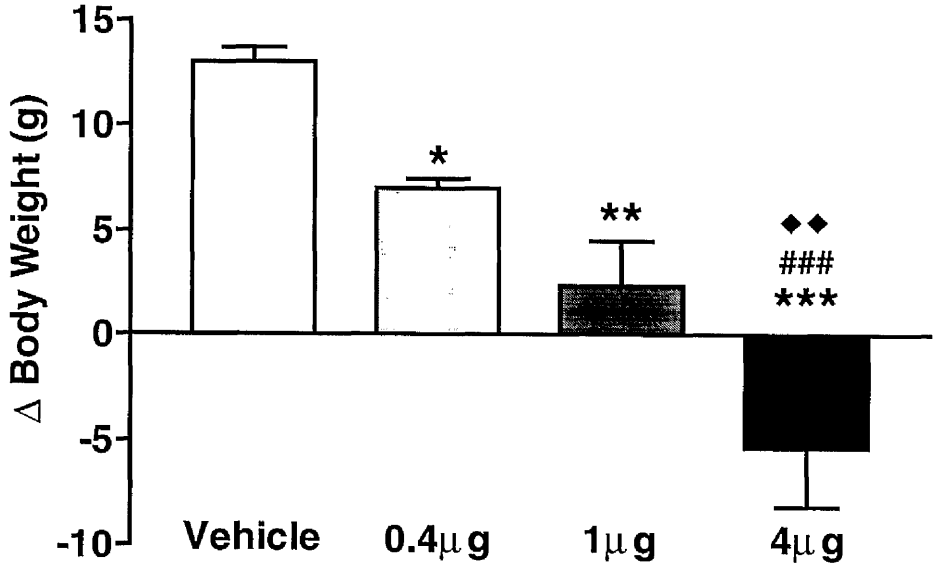


Figure 3.4.2. Dose-response study on the action of leptin (0.4, 1, and 4 μg, icv) on body weight gain over 14 h after injection at 18:00 h

(ANOVA: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs Vehicle; ### $p<0.001$ vs 0.4 μg; ◆◆ $p<0.01$ vs 1 μg)

3.5 Time Course of Actions of Leptin on Food Intake and Body Weight Gain (p.m. Injection)

The studies described in the last two sections were performed over different time periods, after injection at different times (**Section 3.3**, 10:00 h injection, 22 h duration; **3.4**, 18:00 h injection, 14 h duration), but both measured nocturnal food intake over one dark phase. In the experiment described here food intake and body weight were measured acutely and chronically, to elucidate the influence of leptin effects in response to injection just before the dark phase.

3.5.1 Experimental Design

Rats received injections of vehicle, or 4 μg leptin at 19:00 h, just before the beginning of the dark phase (20:00 h). Food intake was then measured 1, 2, 3, 12, 24, 36, 48, 60 and 72 h after injection. Body weight was measured 12, 24, 36, 48, 60 and 72 h after injection.

3.5.2 Results

Cumulative food intake was not significantly attenuated over the first three time points measured (0-3 h) after injection of leptin (**Figure 3.5.1A**). However, leptin administration significantly attenuated cumulative food intake compared to vehicle-treated animals at each subsequent time point. Total food intake at the 12 and 24 h time points (28.7±0.7 and 31.4±1.2) was inhibited by 37 and 39% ($p<0.001$) respectively. Animals did not compensate for this reduction in food intake, such that at the 36, 48, 60 and 72 h time points, cumulative food intake

was still attenuated (36 h, 58.3±2.7 g, 32%; 48 h, 60.8±3.4 g, 29%; 60 h, 87.9±3.7 g, 21%; and 72 h, 90.3±4.1 g, 23%; $p<0.01$).

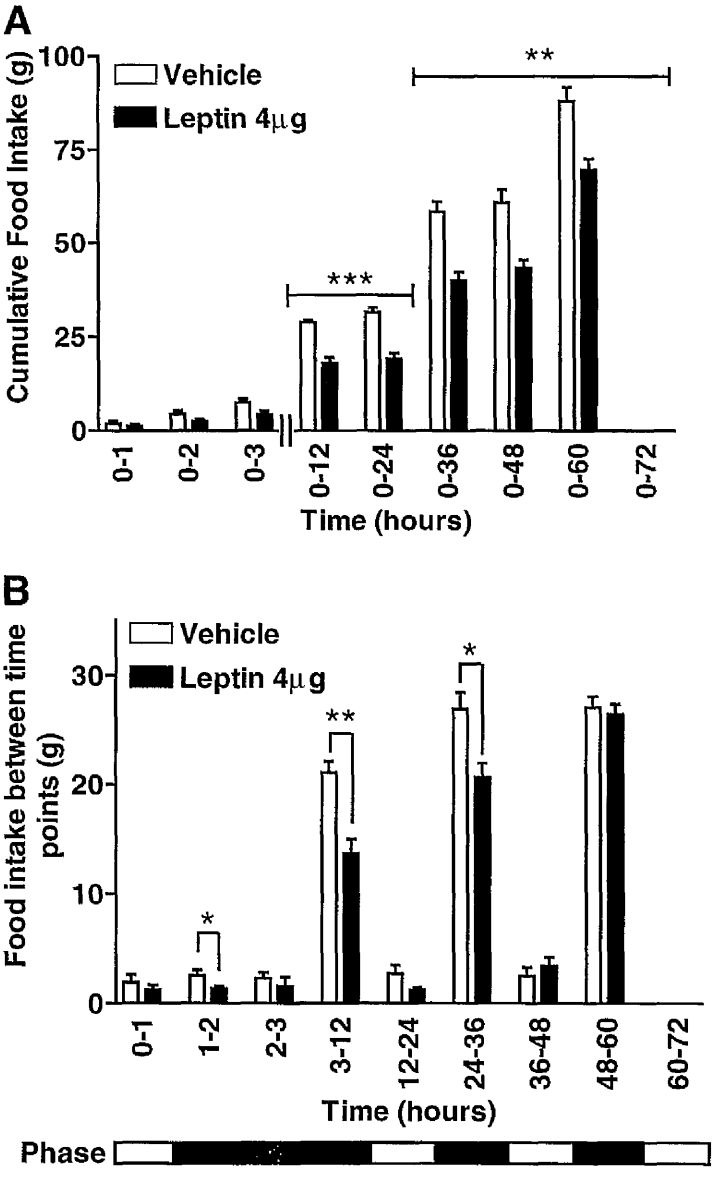


Figure 3.5.1. Time course of food intake in rats injected (icv) with leptin (4 μg) at 19:00 h. Data are displayed as (A) cumulative consumption and (B) food intake between each time point

(t-test: *$p<0.05$, ** $p<0.01$, *** $p<0.001$ vs Vehicle at each respective time point. Light and dark phases are indicated by the bar underneath graph B)

Analysis of food intake between each time point (**Figure 3.5.1B**) revealed that injection of leptin significantly inhibited consumption (1.3±0.2 g; t-test: $p<0.05$) by 47% in the first hour of the dark phase (1-2 h) compared to vehicle-treated animals (2.5±0.5 g). Leptin also attenuated food intake (13.6±1.3 g) by 35%, between 3-12 h after injection (t-test: $p<0.01$) compared to vehicle-treated animals (21.1±1.0 g). Hypophagic response to leptin persisted (t-test: $p<0.05$) during the subsequent dark phase (24-36 h), when leptin-treated animals exhibited food intake (20.7±1.3 g) 23% less than consumption by vehicle-treated animals (26.9±1.5 g). Food intakes of leptin and vehicle-treated animals were not significantly different between subsequent time points (t-test).

Cumulative body weight gain (**Figure 3.5.2A**) measured over the first dark phase (12 h) after injection was significantly attenuated (to 4±1 g) by injection of leptin compared to vehicle-treated animals (17±2 g; $p<0.01$). Over 24 h, animals treated with leptin exhibited significant body weight loss (8±1 g) compared to rats injected with vehicle which gained 6±1 g (t-test: $p<0.001$). Leptin did not induce a significant change in total body weight gain over 36 h after injection (t-test). However, measurements at all subsequent time points (48, 60 and 72 h) revealed that body weight gain of leptin-treated animals (3±2, 24±2 and 13±2 g) was significantly attenuated (t-test) compared to that exhibited by animals injected with vehicle (15±2, $p<0.01$; 34±2 and 23±3 g, $p<0.05$).

Over the first 2 h after injection, body weights did not change significantly in either treatment group (**Figure 3.5.2B**). However between the 2 and 12 h time points (dark phase), animals injected with leptin exhibited significantly

attenuated (t-test: $p<0.01$) body weight gain (4±1 g) compared to vehicle-treated animals (17±2 g). Animals injected with either vehicle or leptin lost and gained body weight alternatively between consecutive light and dark phase measurements, but were not significantly different to each other (t-test).

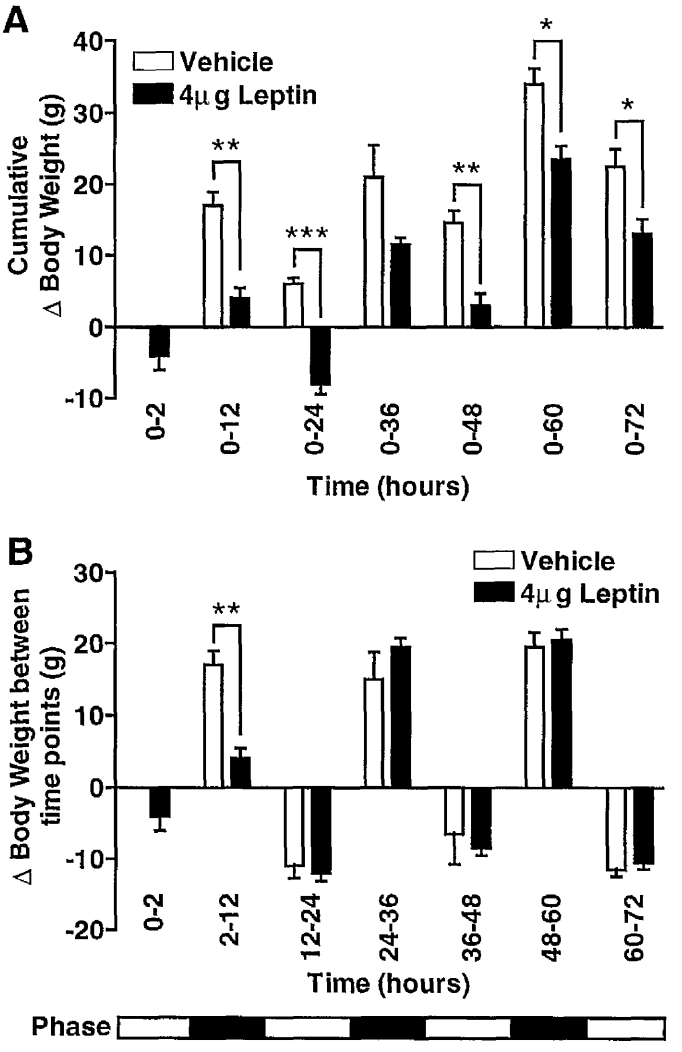


Figure 3.5.2. Time course of body weight gain in rats injected (icv) with leptin (4 μg) at 19:00 h. Data are displayed as (A) cumulative weight gain and (B) weight gain between each time point

(t-test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs Vehicle at each respective time point. Light and dark phases are indicated by the bar underneath graph B)

3.6 Comparison of Actions of LPS, IL-1β and Leptin on Food Intake and Body Weight

The host defence responses to infection are mimicked by peripheral administration (ip) of LPS (Martich *et al.*, 1993; Redl *et al.*, 1993). These responses are mediated in the brain by the actions of cytokines such as IL-1 (Dinarello, 1996; Ilyin *et al.*, 1998). LPS, IL-1, and other proinflammatory agents have been shown to induce synthesis and release of leptin from adipose tissue both *in vitro* and *in vivo* (Grunfeld *et al.*, 1996; Janik *et al.*, 1997; Faggioni *et al.*, 1998; Finck *et al.*, 1998). Indeed, the effects of leptin on food intake and body weight gain described in previous sections are similar to reported responses to injection of infectious stimuli such as LPS (McCarthy *et al.*, 1986) or proinflammatory cytokines such as IL-1 (Uehara *et al.*, 1989; Mrosovsky *et al.*, 1989). The subsequent investigation was designed to verify this observation by comparing actions of leptin, LPS and IL-1β on food intake and body weight gain.

3.6.1 Experimental Design

Food intake and body weight were measured over 22 h after injection (at 10:00 h) of vehicle (ip and icv), LPS (100 μg/kg, ip), IL-1β (5 ng, icv) or leptin (4 μg, icv). Doses injected have been shown previously to induce hypophagic responses in rats (McCarthy *et al.*, 1986; Schwartz *et al.*, 1996; Luheshi *et al.*, 1996). LPS was administered systemically (ip) to mimic a peripheral infection. IL-1β was injected centrally (icv) because the defence responses to infectious stimuli such as LPS, are mediated by IL-1β in the brain (Dinarello, 1996).

3.6.2 Results

Vehicle-treated animals consumed 26.3±1.8 g rat chow over the 22 h after injection (**Figure 3.6.1**). Peripheral administration of LPS attenuated this food intake by 22% (ANOVA: $p<0.05$). Injection of IL-1β (icv) reduced control food intake by 33% (ANOVA: $p<0.01$). Leptin administration induced hypophagia (52% reduction in food intake, ANOVA: $p<0.001$ vs Vehicle) that was significantly greater than that caused by LPS or IL-1β (ANOVA: $p<0.01$, $p<0.05$ respectively).

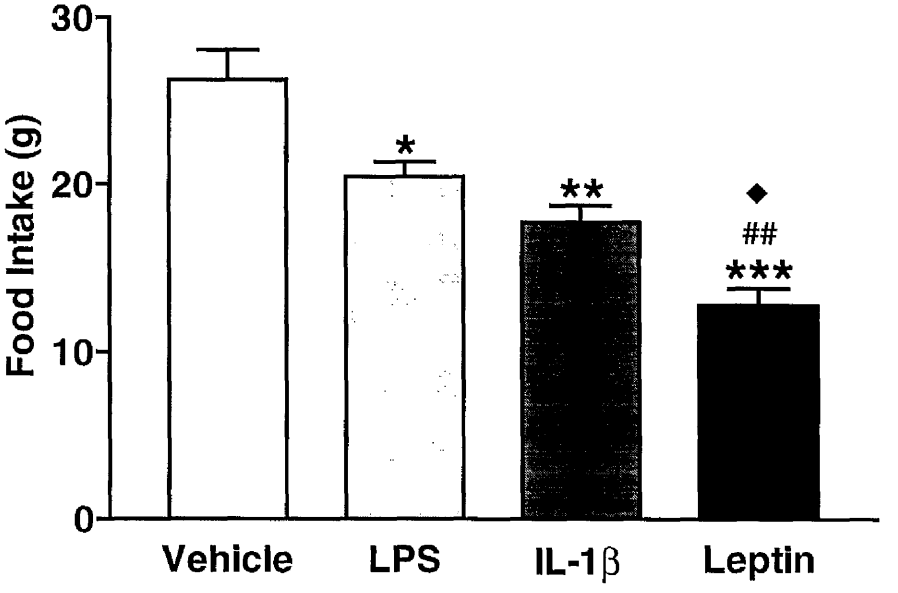


Figure 3.6.1. Food intake over 22 h after injection of LPS (100 μg/kg, ip), IL-1β (5 ng, icv) or leptin (4 μg, icv)

(ANOVA: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs Vehicle; $^{\#\#}p<0.01$ vs LPS; $^{\blacklozenge}p<0.05$ vs IL-1β)

Animals injected with vehicle gained 7±1 g body weight over 22 h after injection (**Figure 3.6.2**). LPS administration (ip) prevented any gain in body weight

(ANOVA: $p<0.05$ vs Vehicle), and central injection of IL-1β elicited a significant decrease in body weight (2±1 g, ANOVA: $p<0.05$) compared to vehicle-treated animals. Leptin administration (icv) decreased body weight (by 9±1 g) - a response that was significantly different from the weight change observed in the control group (ANOVA: $p<0.001$).

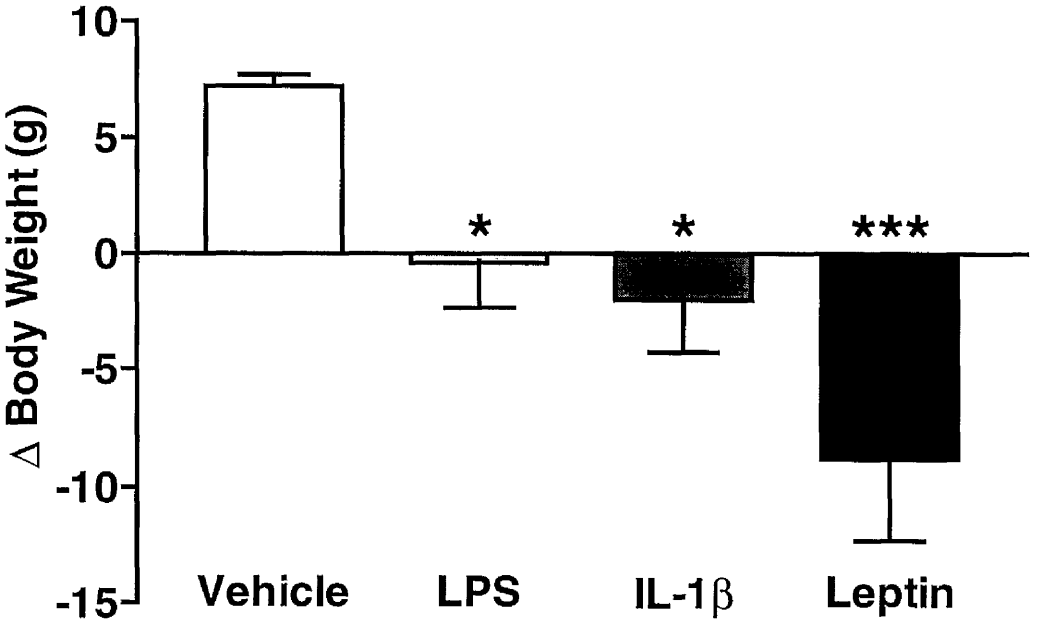


Figure 3.6.2. Body weight gain over 22 h after injection of LPS (100 μg/kg, ip), IL-1β (5 ng, icv) or leptin (4 μg, icv)

(ANOVA: *** $p<0.001$, ** $p<0.01$ vs Vehicle)

3.7 Comparison of Actions of LPS, IL-1β and Leptin on Core Body Temperature

In addition to reduced food intake and loss of body weight, perhaps the most widely recognised response to infection is fever. Both LPS and IL-1β have been

reported extensively to induce fever in rats (Busbridge *et al.*, 1989; Dascombe *et al.*, 1989; Luheshi *et al.*, 1996) and mice (Kozak *et al.*, 1994; Kluger *et al.*, 1998). Given the similarities described in the previous section between actions of LPS, IL-1β and leptin on food intake and body weight gain, and the fact that leptin has been reported to increase energy expenditure in mice (Hwa *et al.*, 1996; Hwa *et al.*, 1997; Harris *et al.*, 1998), leptin may therefore also affect core body temperature in rats. This study investigated whether, like the proinflammatory agents LPS and IL-1β, leptin affects core body temperature in rats.

3.7.1 Experimental Design

Core body temperature was measured for 10 h, under the same conditions as demonstrated earlier (**Section 3.6**). Injections of vehicle (ip and icv), LPS (100 μg/kg, ip), IL-1β (5 ng, icv) or leptin (4 μg, icv) were all performed at 10:00 h (0 h).

3.7.2 Results

Core body temperatures of vehicle-treated rats remained between 36.9-37.4°C for 9 h after injection, before rising sharply to 37.9°C at the 10 h time point (beginning of the dark phase).

Administration of LPS (**Figure 3.7.1A**) elicited a significant and biphasic increase in body temperature (MANOVA: $p<0.001$ vs Vehicle) that began to rise 1.5 h after injection and peaked at the 2.5 and 5 h time points (1.8°C and 1.0°C above control; ANOVA: $p<0.001$ and $p<0.01$ respectively vs Vehicle).

Temperatures of animals treated with LPS returned to control levels approximately 8.5 h after injection.

Animals injected with IL-1β exhibited an acute and monophasic increase in core body temperature (MANOVA: $p<0.001$ vs Vehicle) within the first 0.5 h after injection (**Figure 3.7.1B**). This response reached a peak at the 2.5 h time point (2.3°C above control; ANOVA: $p<0.001$) before declining and returning to control temperatures 6.5 h after injection.

Injection of leptin (**Figure 3.7.1C**) also elicited a monophasic increase in core body temperature. Temperatures began to rise within 0.5 h after injection, and like the responses to LPS and IL-1β, peaked at the 2.5 h time point (1.7°C above control; ANOVA: $p<0.001$). Body temperature of leptin-treated animals remained elevated until 9.5 h after injection, when control temperatures rose to similar levels at the beginning of the dark phase. The temperature response induced by leptin was significantly greater than the response to injection of LPS (MANOVA: $p<0.01$) and varied significantly with time compared with all groups (MANOVA: $p<0.01$ vs LPS and IL-1β, $p<0.05$ vs Vehicle).

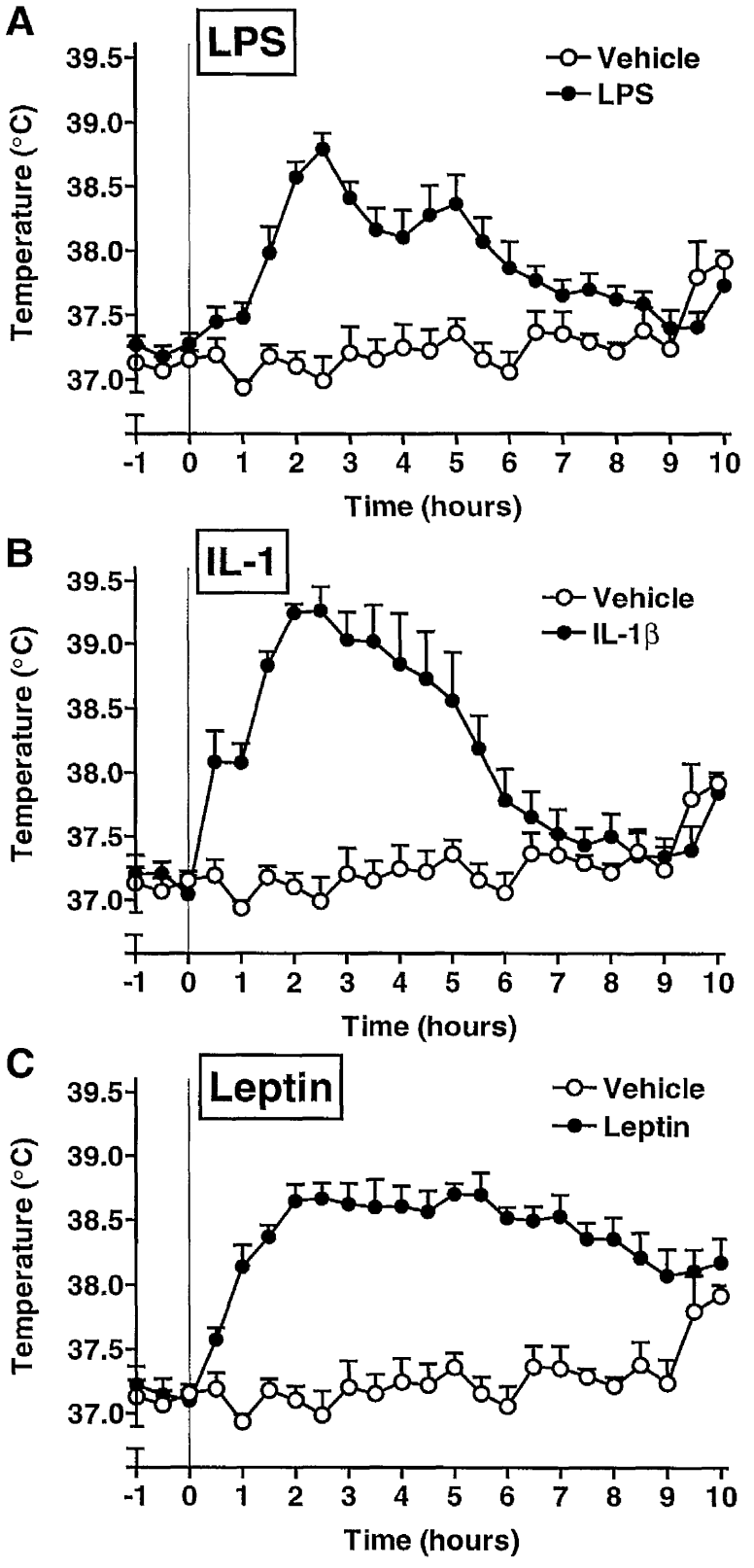


Figure 3.7.1. Core body temperature in response to injection of (A) LPS (100 μg/kg, ip) (B) IL-1β (5 ng, icv) or (C) leptin (4 μg, icv)

3.8 Effects of Leptin from Different Sources on Core Body Temperature

Although the murine leptin used in these investigations had similar effects on food intake and body weight to the responses to injection of leptin obtained from alternative sources (**Section 3.2**), the temperature response described in the previous section may be an effect specific to murine leptin from Insight Biotechnology. Therefore, core body temperature was monitored in rats injected with leptin from three different sources, to investigate whether comparable temperature responses were induced.

3.8.1 Experimental Design

Injections (icv) of vehicle, or 4 μg of either rat leptin purchased from R&D Systems, murine leptin purchased from Insight Biotechnology, or murine leptin obtained from Zeneca, were performed at 10:00 h (0 h). Core body temperature was monitored for 10 h, until the end of the light phase.

3.8.2 Results

Core body temperatures of vehicle-treated rats remained between 36.9-37.5°C for 9 h after injection, before rising sharply to 37.9°C at the 9.5 h time point (beginning of the dark phase).

Injection of leptin from all sources, induced significant increases in core body temperature compared with vehicle-treated animals (MANOVA: $p<0.001$).

Administration of rat leptin (**Figure 3.8.1A**) elicited increased body temperatures by the 1.5 h time point, and that peaked 5.5 h after injection (1.4°C above

control, ANOVA: $p<0.001$). Temperatures of leptin-treated animals returned to control levels at the 9.5 h time point.

Rats injected with murine leptin obtained from Insight Biotechnology (**Figure 3.8.1B**) exhibited elevated body temperatures after the 0.5 h time point, and that rose to a peak (1.4°C above control, ANOVA: $p<0.001$) 3.5 h after injection. Temperatures subsequently declined, reaching control levels at approximately the 7 h time point. Core body temperatures rose again between 8 and 10 h after injection, along with those of the control group.

Murine leptin obtained from Zeneca (**Figure 3.8.1C**) induced increased temperatures 2 h after injection. This response was maximal (1.3°C above control, ANOVA: $p<0.001$) at the 5 h time point. Temperatures persisted until 9.5 h after injection when control temperatures rose at the end of the light phase.

In summary, injection of leptin from each source elicited significant (MANOVA: $p<0.001$ vs Vehicle) and similar increases in core body temperature which were not significantly different from each other over the 10 h after injection (MANOVA).

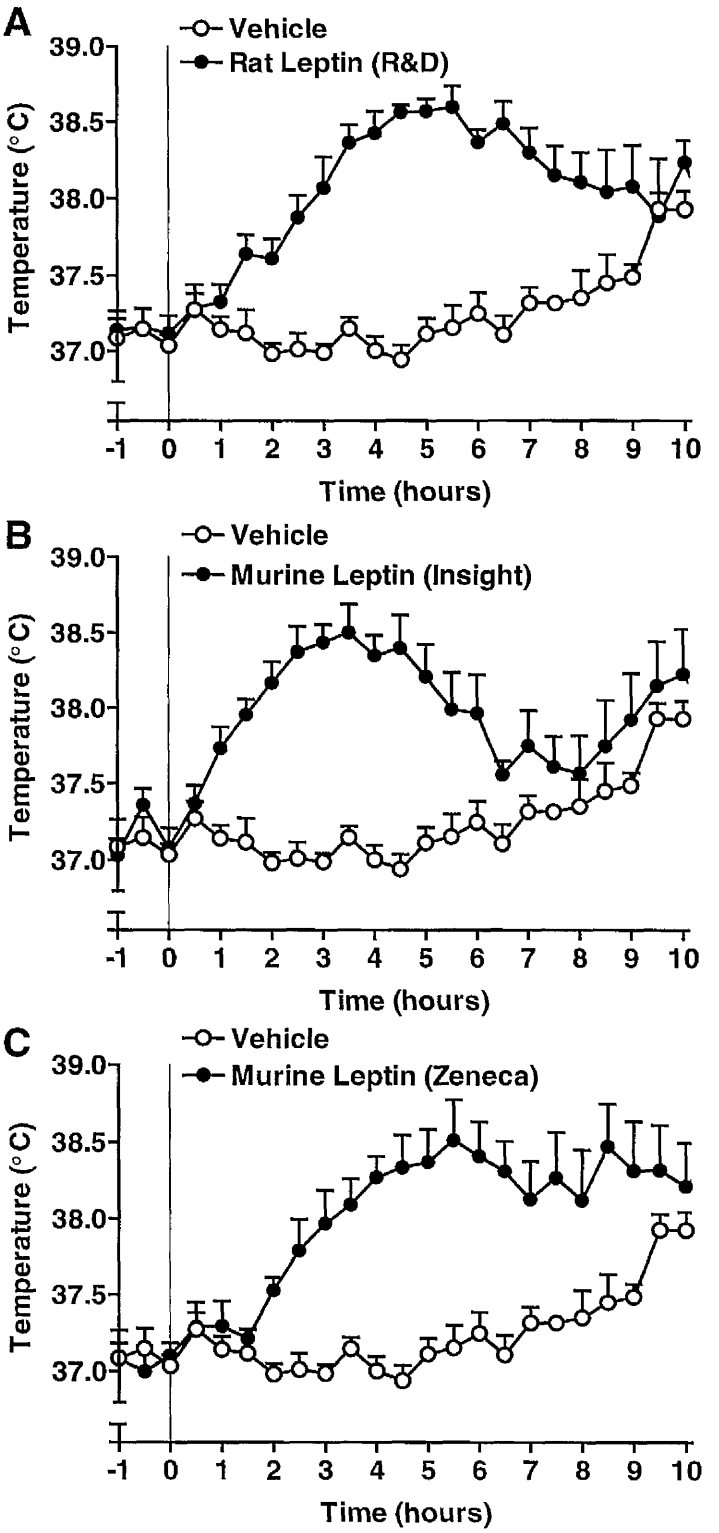


Figure 3.8.1. Core body temperature in response to icv injection of 4 μg (A) rat leptin (R&D Systems), or murine leptin obtained from (B) Insight Biotechnology or (C) Zeneca

3.9 Leptin Dose-Response Study on Core Body Temperature (a.m. Injection)

The observation in the previous section that central injection of leptin from each of the three sources elicited increases in body temperature led to the proposal that, like the effects of leptin on food intake and body weight (**Section 3.3**), effects on body temperature were also dose-dependent. This study investigated the effects of increasing doses of leptin on core body temperature.

3.9.1 Experimental Design

Three doses of leptin (0.4, 1, and 4 μg), or vehicle were injected (icv) at 10:00 h (0 h) in rats. Core body temperatures were monitored for 10 h after injection.

3.9.2 Results

Core body temperatures of vehicle-treated rats remained between 36.9-37.3°C for 9 h after injection, before rising to 37.7°C at the 9.5 h time point (beginning of the dark phase).

Animals injected with the lowest dose of leptin (0.4 μg) exhibited body temperatures which although were slightly elevated after the 5 h time point, did not deviate significantly from those receiving vehicle (MANOVA) (**Figure 3.9.1**).

Injection of 1 μg leptin induced a significant increase in core body temperature (MANOVA: $p<0.01$ vs Vehicle) that began to rise 1 h after injection, and remained constant between the 2.5 and 5.5 h time points, 1.0°C above temperatures of vehicle-treated animals (ANOVA: $p<0.01$).

The highest dose of leptin (4 μg) also elicited a significant increase in core body temperature (MANOVA: $p<0.001$ vs Vehicle) that began to rise 1 h after injection, and peaked at the 5 h time point (1.7°C above control; ANOVA: $p<0.001$). Temperatures of animals injected with either 1 or 4 μg leptin declined steadily from 5.5 or 5 h after injection respectively, until the 9.5 h time point when the body temperatures of vehicle-treated rats rose to similar levels at the beginning of the dark phase.

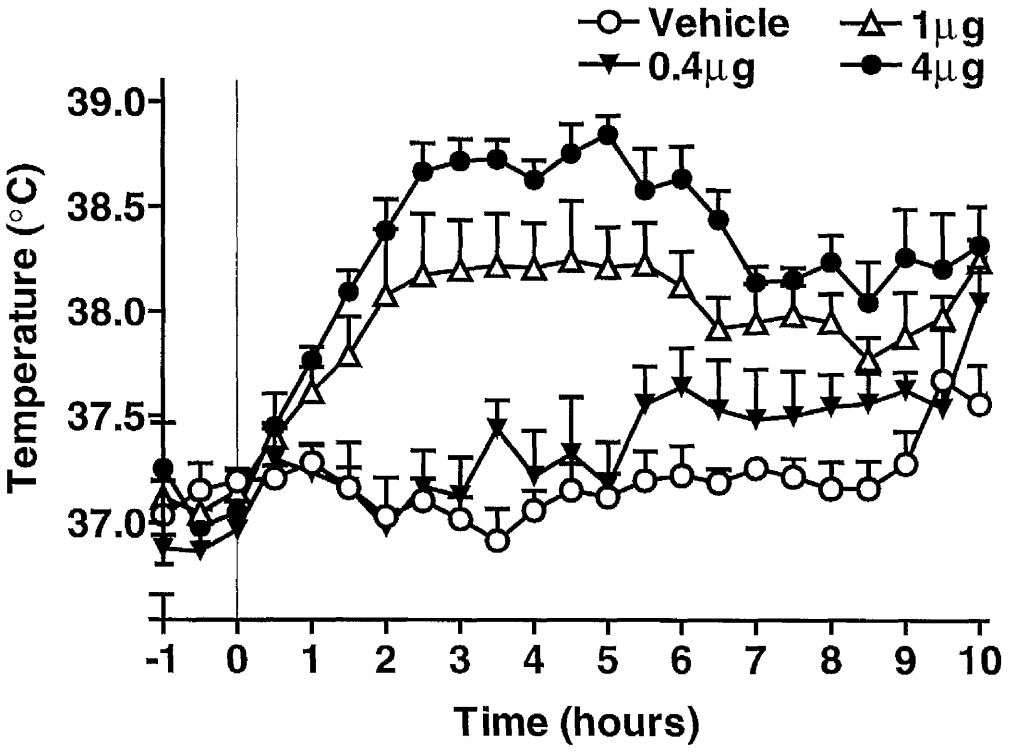


Figure 3.9.1. Effects of injection of leptin (0.4, 1, and 4 μg, icv) at 10:00 h on core body temperature

3.10 Leptin Dose-Response Study on Core Body Temperature (p.m. Injection)

Basal core body temperatures of rats are elevated during the dark phase, corresponding with the period when animals are most active and are feeding. It was postulated that these physiological and behavioural changes might alter the body temperature response induced by injection of leptin. Furthermore, levels of endogenous leptin show a circadian pattern (Simon *et al.*, 1998) and are increased during the dark phase in nocturnal animals such as the rat, in response to feeding (Saladin *et al.*, 1995; Pickavance *et al.*, 1998). This fluctuation might also affect the actions of leptin on body temperature. The influence of nocturnal conditions on the temperature response to leptin were investigated in this study by injecting three doses of leptin (as in **Section 3.9**) just before the beginning of the dark phase.

3.10.1 Experimental Design

Three doses of leptin (0.4, 1, and 4 μg), or vehicle were injected (icv) at 18:00 h in rats. Core body temperatures were monitored for 10 h after injection.

3.10.2 Results

Vehicle-treated animals exhibited core body temperatures that rose by 0.8°C over the first 3.5 h after injection, peaking at 21:30 h - 1.5 h into the dark phase (**Figure 3.10.1**). Core body temperatures remained elevated (37.9-38.3°C) for the remainder of the experiment.

Animals injected with either 0.4 or 1 μg leptin exhibited body temperatures that did not deviate significantly from those receiving vehicle (MANOVA) over the 10 h after injection.

The highest dose of leptin (4 μg) elicited a significant rise in core body temperature (MANOVA: $p<0.01$ vs Vehicle; $p<0.05$ vs 0.4 μg) that peaked 3 and 5 h after injection (0.8 and 0.6°C above control; ANOVA: $p<0.01$). Temperatures subsequently declined to control levels at the 7.5 h time point.

Although injection of 1 μg leptin did not induce a significant response over the whole time course, further analysis revealed that after the 4 h time point, temperatures of leptin treated animals were significantly greater than temperatures in rats injected with vehicle (MANOVA: $p<0.05$).

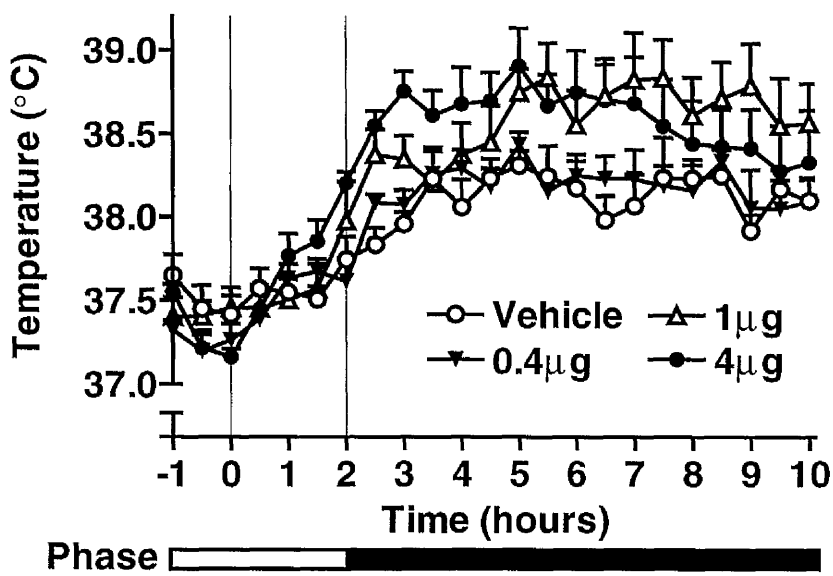


Figure 3.10.1. Effects of injection of leptin (0.4, 1, and 4 μg, icv) at 18:00 h on core body temperature

(Light and dark phases are indicated by the bar underneath the graph)

3.11 Effects of Heat Denaturation on Biological Activity of Leptin

The results described in the previous four sections (**3.7-3.10**) show for the first time that central injection of leptin elicits increases in core body temperature in normal rats. Further studies were therefore required to verify that these responses were induced by leptin, and not by endotoxin contamination. Such contamination is possible since leptin from each source used in these studies was expressed in *E.Coli* bacteria, although data supplied by the manufacturers quoted levels of endotoxin to be as low as <0.1 ng/μg leptin (Insight Biotechnology and R&D Systems). Nevertheless, the following study was performed to investigate the effects of injection of leptin that had been inactivated by heat denaturation. If the responses to leptin administration described in previous sections persisted after heat denaturation, then they were probably induced by a contaminant such as LPS.

Injections in all subsequent studies that measured core body temperature were performed during the light phase (from 10:00 h), when vehicle-treated animals display constant temperatures that allow effective comparison with animals in other treatment groups.

3.11.1 Experimental Design

A sample of leptin (2 μg/μl) was heated to 95°C for 30 min to denature the protein. Vehicle, leptin (4 μg/rat), or the equivalent quantity of heat-treated leptin were injected at 10:00 h. Core body temperature was monitored for 7 h after injection. Food intake and body weights were measured over 22 h (until the beginning of the subsequent light phase).

3.11.2 Results

Vehicle-treated animals consumed 26.5±1.0 g food over 22 h after injection (**Figure 3.11.1A**). This food intake was significantly reduced (by 39%) in animals injected with leptin (ANOVA: $p<0.001$ vs Vehicle). Heat-treatment of leptin abolished this response (ANOVA: $p<0.001$ vs leptin) such that animals injected with denatured leptin consumed similar quantities of food (25.6±0.3 g) to vehicle-treated rats (ANOVA).

Animals injected with vehicle gained 11±2 g body weight over the same 22 h time period (**Figure 3.11.1B**). Leptin administration induced a significant (ANOVA: $p<0.001$ vs Vehicle) body weight loss (4±1 g). This leptin-induced response was also abolished by heat-treatment of the protein ($p<0.001$).

Animals injected with vehicle showed core body temperatures that remained between 37.0-37.3°C over 7 h after injection (**Figure 3.11.1C**). Injection of leptin induced a significant increase in core body temperature (MANOVA: $p<0.001$) compared to vehicle-treated animals. This increase was first apparent 2.5 h after injection, and rose to a peak (1.6°C above control, ANOVA: $p<0.001$) at the 6.5 h time point. Heat-treated leptin failed to evoke a temperature response (MANOVA: $p<0.001$ vs Leptin) such that animals exhibited temperatures that were not significantly different from temperatures of vehicle-treated rats (MANOVA).

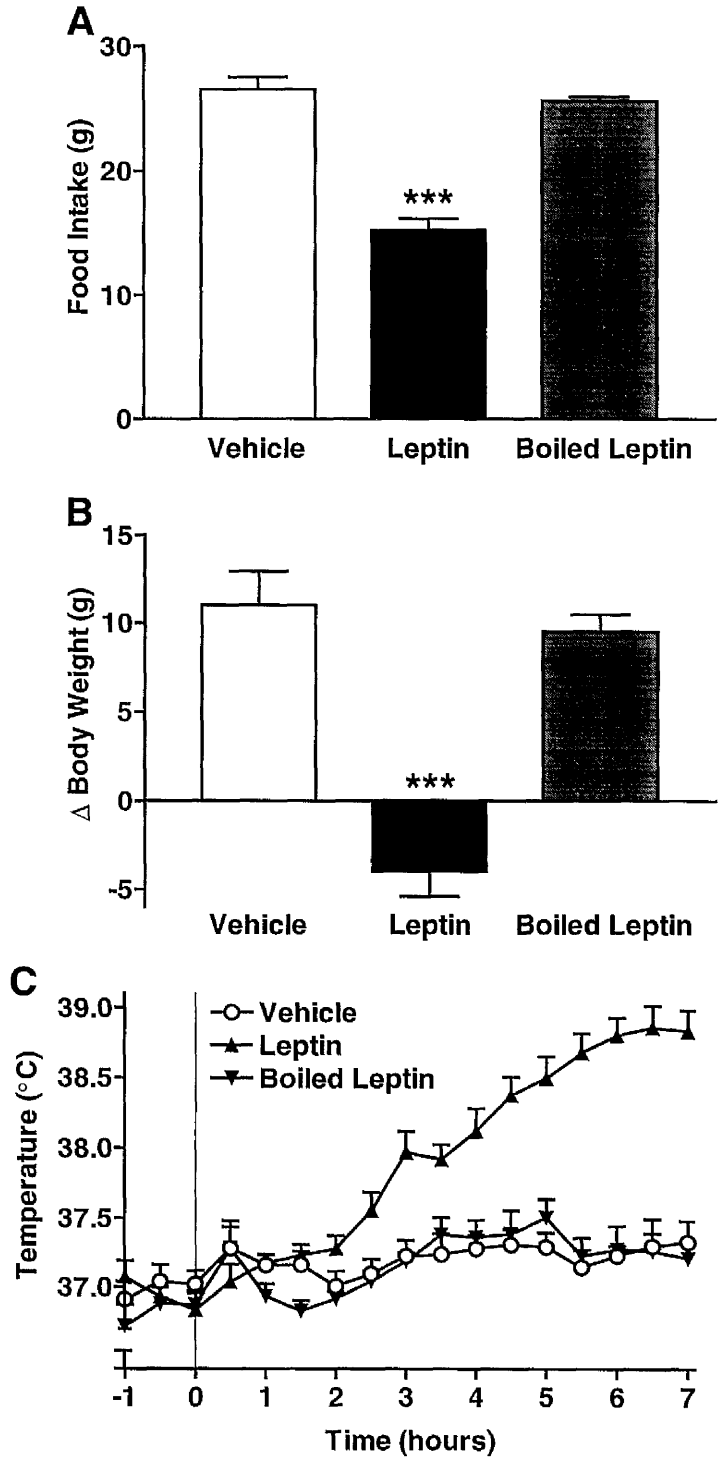


Figure 3.11.1. Effects of heat treatment (95°C for 30 min) on actions of leptin (4 μg, icv) on (A) food intake, (B) body weight, and (C) core body temperature

(ANOVA: *** $p<0.001$ vs Vehicle)

3.12 Effects of Leptin in Lean and Obese Zucker Rats

The data described in the previous section indicate that the changes in food intake, body weight and body temperature are specific actions of leptin, and are not a result of contamination by endotoxin or other heat resistant substances. The leptin receptor has been reported to belong to the class I family of cytokine receptors (Tartaglia *et al.*, 1995; Tartaglia, 1997), and possesses similar signal transduction properties to those of IL-6 receptors (Baumann *et al.*, 1996; Nakashima *et al.*, 1997). It may be possible therefore, that the responses observed here were induced by the interaction of leptin with other cytokine receptors involved in hypophagia and pyrogenic responses. This study was designed to investigate whether these responses were specific to the action of leptin on the leptin receptor.

Currently there are no commercially available leptin receptor antagonists that block leptin receptor stimulation. Therefore in this experiment, genetically obese Zucker rats were used, which possess a defective leptin receptor (Iida *et al.*, 1996; Phillips *et al.*, 1996), and therefore should have an attenuated response to injection of leptin (Cusin *et al.*, 1996). These animals should however still exhibit anorexic and febrile responses to proinflammatory agents such as PGs that mediate responses to LPS and IL-1β (Blatteis & Sehic, 1998; Milton, 1998).

3.12.1 Experimental Design

Lean (*Fa/?*) and obese (*fa/fa*) Zucker rats were injected (icv) with vehicle, PGE_2 (500 ng) or leptin (4 μg) at 10:00 h. Core body temperatures were monitored for 6 h; food intake and body weight were monitored over 22 h after injection.

3.12.2 Results

Obese Zucker rats consumed slightly, but not significantly more food (27±2 g) than lean animals (24±1 g) over 22 h, after injection of vehicle (**Figure 3.12.1**). Injection of PGE_2 attenuated food intake by 17% ($p<0.05$) in lean animals, and by 22% ($p<0.05$) in obese rats (ANOVA). Comparison of food consumed by lean and obese Zucker rats treated with PGE_2 revealed no significant difference (ANOVA). Administration of leptin inhibited food intake by 50% in lean Zucker rats (ANOVA: $p<0.001$ vs Lean-Vehicle), but did not significantly affect food intake (28±2 g) in obese animals (ANOVA vs Obese-Vehicle).

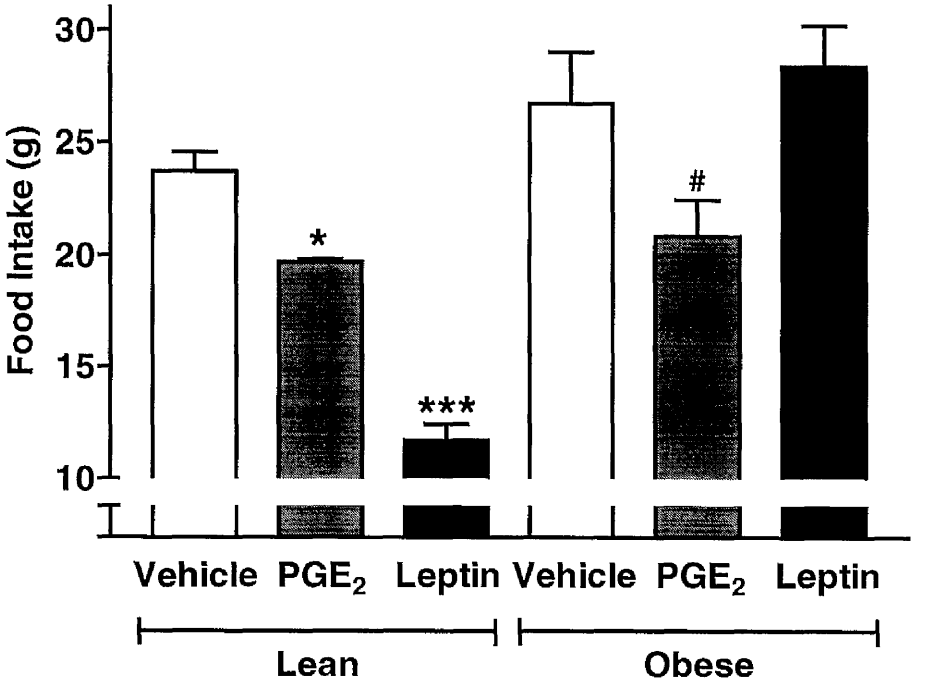


Figure 3.12.1. Food intake over 22 h in lean and obese Zucker rats in response to injection (icv) of PGE_2 (500 ng) or leptin (4 μg)

(ANOVA: * $p<0.05$, *** $p<0.001$ vs Lean-Vehicle; # $p<0.05$ vs Obese-Vehicle)

In the same animals, body weight gain was measured over the 22 h period after injection. At the beginning of the experiment, obese Zucker rats had significantly greater (t-test: $p<0.001$) body weights (530±15 g) than lean animals (320±5 g). Body weight gains (**Figure 3.12.2**) in lean (11±1 g) and obese animals (12±1 g) treated with vehicle were not significantly different (ANOVA). Injection of PGE_2 significantly inhibited body weight gain in lean and obese rats to 3±2 and 2±1 g respectively (ANOVA: $p<0.001$ vs Lean and Obese-Vehicle). Similarly, body weight gain was significantly attenuated (to 2±1 g) in lean animals treated with leptin (ANOVA: $p<0.001$ vs Lean-Vehicle), but was not significantly affected (ANOVA vs Obese-Vehicle) in leptin-treated obese rats (9±2 g).

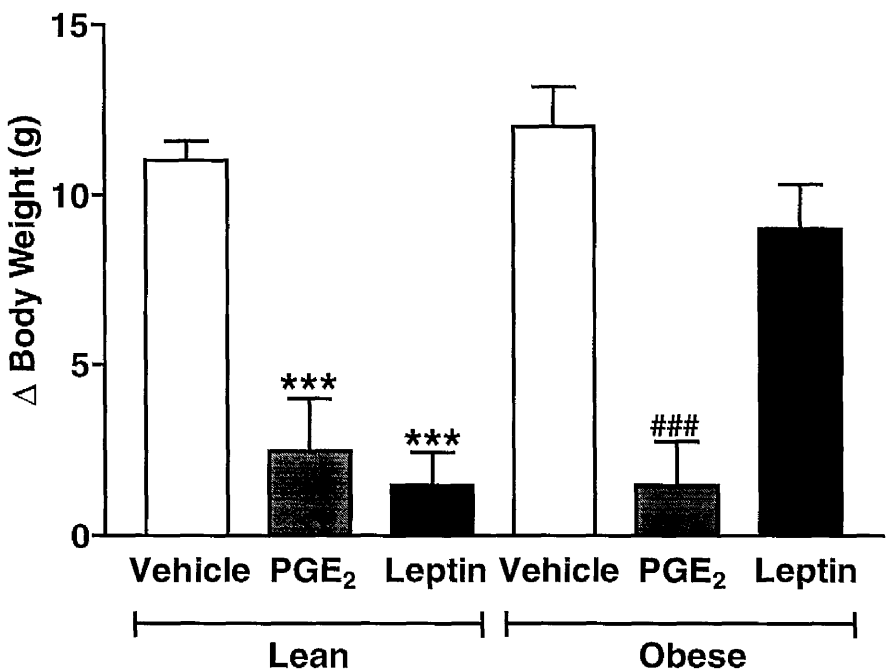


Figure 3.12.2. Body weight gain over 22 h in lean and obese Zucker rats in response to injection of PGE_2 (500 ng, icv) or leptin (4 μg, icv)

(ANOVA: *** $p<0.001$ vs Lean-Vehicle; ### $p<0.001$ vs Obese-Vehicle)

Core body temperature was also measured in these animals. Lean rats injected with vehicle exhibited core body temperatures that remained between 36.9-37.3°C over the first 6.5 h after injection. Temperatures subsequently began to rise, peaking (38.0±0.2°C) at the 10 h time point (at the beginning of the dark phase). Over the first 6 h after injection, vehicle-treated obese Zucker rats exhibited temperatures between 36.9-37.2°C, after which temperatures also rose to peak (37.9±0.2°C) at the 10 h time point.

Administration of PGE_2 induced a rapid and significant increase in core body temperature in both lean and obese animals compared to vehicle-treated rats (MANOVA: $p<0.001$) (**Figure 3.12.3A**). Temperatures peaked at the 0.5 h time point (3°C above controls, ANOVA: $p<0.001$) before declining sharply, returning to control values 2 h after injection.

Lean Zucker rats exhibited increased core body temperatures (**Figure 3.12.3B**) 2.5 h in response to injection of leptin, which peaked at the 3 h time point (1°C above control, ANOVA: $p<0.001$). Elevated temperatures persisted for approximately 9 h (MANOVA: $p<0.01$) before temperatures of vehicle-treated rats rose to similar levels at the beginning of the dark phase. In contrast, core body temperatures of obese animals treated with leptin increased slightly, but not significantly compared to those injected with vehicle (MANOVA).

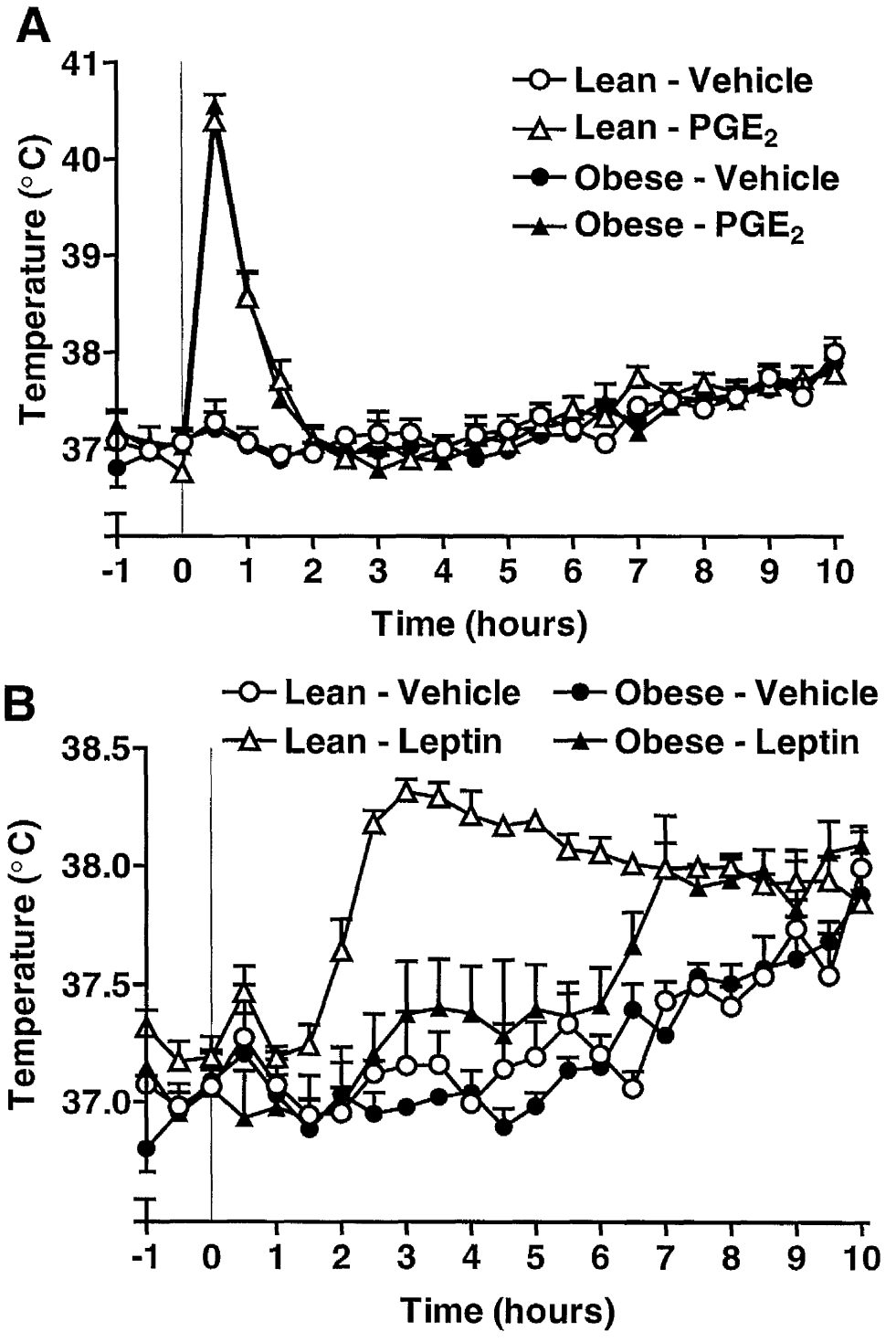


Figure 3.12.3. Core body temperatures of lean and obese Zucker rats in response to injection of (A) PGE_2 (500 ng, icv) or (B) leptin (4 μg, icv)

3.13 EFFECTS OF A CYCLO-OXYGENASE INHIBITOR ON RESPONSES TO LEPTIN

The results described in the previous section indicate that PG (but not leptin) mediated pathways are still active in the obese Zucker rat. PGs mediate many actions of proinflammatory agents such as LPS and IL-1, including fever and anorexia (Hellerstein *et al.*, 1989; Rothwell, 1990; Milton, 1998). Given the similar food intake and body temperature responses to injection of LPS, IL-1 and leptin (**Section 3.6-3.7**), effects of leptin in animals possessing normal functioning leptin receptors, may also be mediated by PG-dependent pathways. PGs are produced from arachadonic acid, by cyclo-oxygenase enzymes (Kaufmann *et al.*, 1997). Therefore this study was designed to investigate whether inhibiting PG generation (by administration of a cyclo-oxygenase inhibitor) blocks responses to leptin on food intake and core body temperature, by inhibiting PG synthesis.

3.13.1 Experimental Design

Animals were injected (icv) at 10:00 h with vehicle or leptin (4 μg), and with the cyclo-oxygenase inhibitor flurbiprofen (1 mg/kg, ip), or its vehicle. Core body temperature was monitored for 10 h after injection - until the end of the light phase. Food intake and body weight gain were measured 22 h after injection, at the end of the subsequent dark phase.

3.13.2 Results

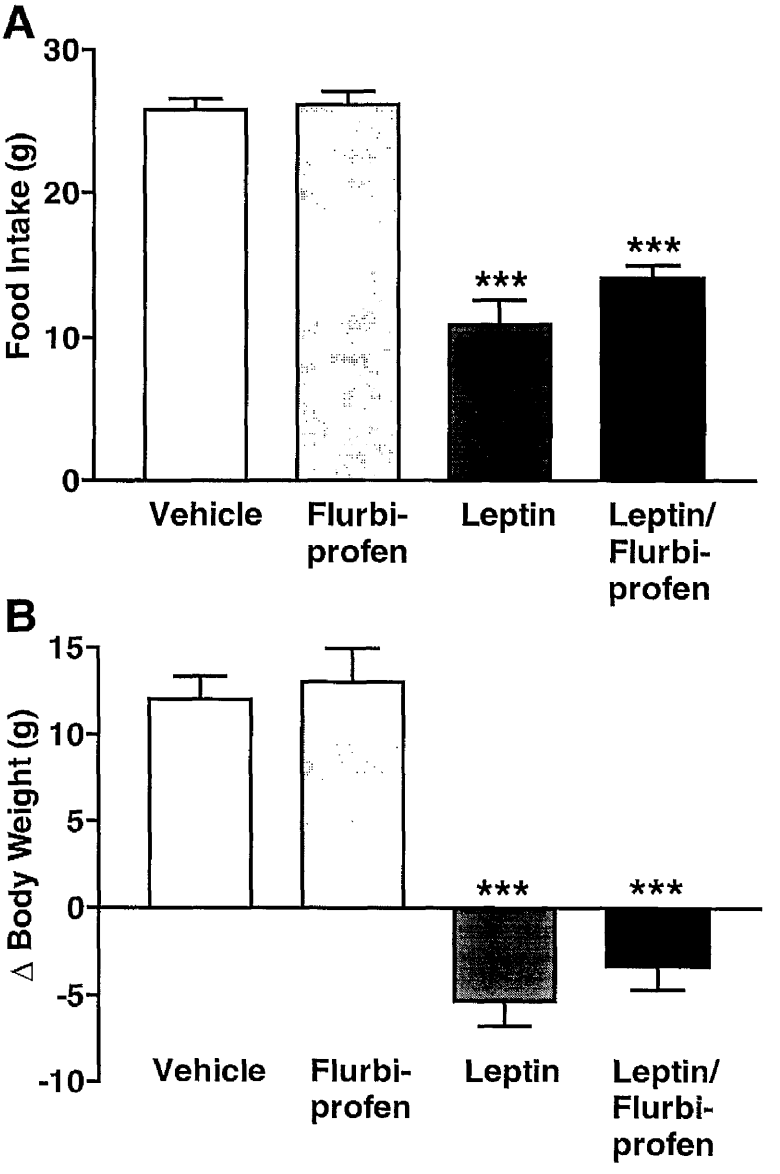
Vehicle-treated animals consumed 25.8±0.8 g food over 22 h after injection (**Figure 3.13.1A**). Injection of flurbiprofen elicited food intake (26.2±0.9 g) that was not significantly different from that after treatment with vehicle (ANOVA).

Leptin administration induced a significant reduction (by 58%) in food intake (ANOVA: $p<0.001$ vs Vehicle). Animals co-administered with leptin and flurbiprofen exhibited food intake (14.1±0.9 g) that was not significantly different from those treated with leptin alone (10.9±1.7 g, ANOVA), but was still significantly attenuated (by 45%) compared to food intake of vehicle-treated rats (ANOVA: $p<0.001$).

Animals injected with vehicle gained 12±1 g body weight over the same 22 h time period (**Figure 3.13.1B**). Injection of flurbiprofen elicited body weight gain (13±2 g) that was not significantly different from that exhibited by vehicle-treated animals (ANOVA). Leptin administration induced a significant (ANOVA: $p<0.001$ vs Vehicle) body weight loss (5±1 g). This leptin-induced body weight loss (3±1 g) was not significantly affected (ANOVA) in animals treated with both leptin and flurbiprofen, and was still significantly different compared to the weight gain observed in vehicle-treated animals (ANOVA: $p<0.001$).

Animals injected with vehicle exhibited core body temperatures that remained between 36.8-37.4°C over the first 9 h after injection (**Figure 3.13.1C**). Temperatures subsequently rose, peaking (at 37.9±0.1°C) at the 10 h time point. Administration of flurbiprofen did not significantly affect this temperature response (MANOVA). Injection of leptin induced a significant increase in core body temperature (MANOVA: $p<0.001$ vs Vehicle) that began to rise 1.5 h after injection, and peaked (1.7°C above control, ANOVA: $p<0.001$) at the 6.5 h time point. Elevated temperatures declined slightly and then persisted (38.6-38.7°C) from the 8 h time point to the end of the 10 h time course. Co-administration of

flurbiprofen completely abolished the increase in core body temperature elicited by injection of leptin (MANOVA: $p<0.001$) such that temperatures were not significantly different from those exhibited by vehicle-treated animals (MANOVA).



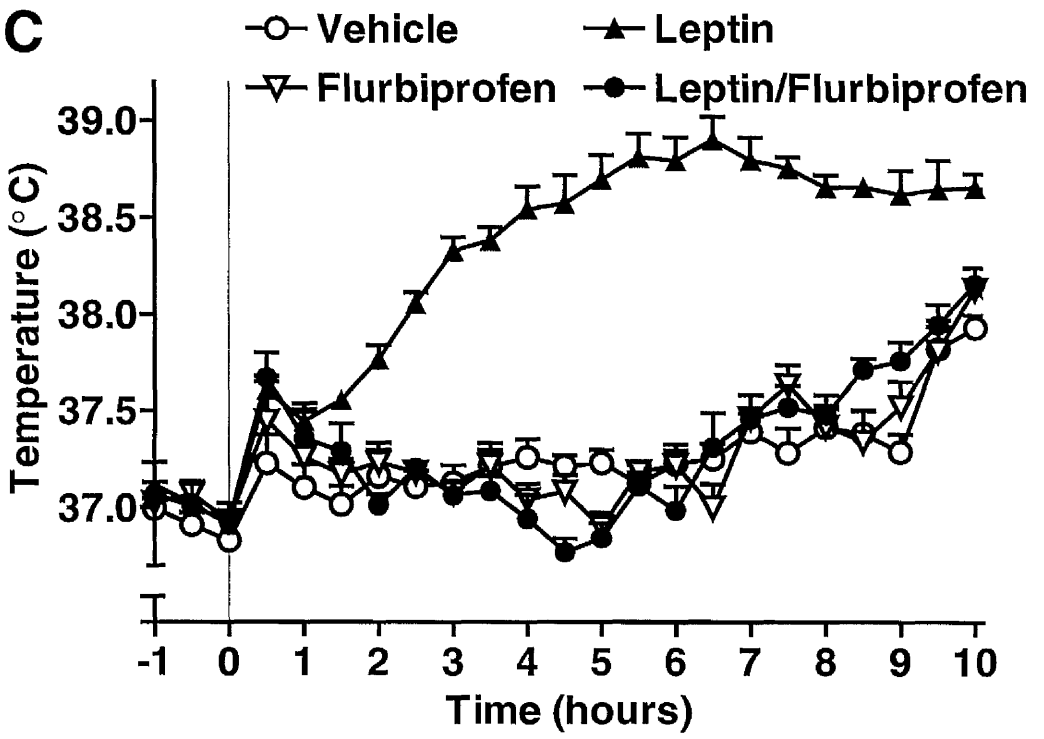


Figure 3.13.1. Effects of cyclo-oxygenase inhibitor flurbiprofen (1 mg/kg, ip) on (A) food intake, (B) body weight gain, and (C) core body temperature responses to icv injection of leptin (4 μg)

3.14 Summary

The results described here indicate that injection of leptin (rat or murine) into the rat brain not only causes dose and time-dependent inhibition of food intake and body weight (**Section 3.2-3.5**), but also increases core body temperature (**Section 3.8-3.10**) - effects that are similar to those induced by the proinflammatory agents LPS and IL-1β (**Section 3.6 and 3.7**). This is the first time such effects on core body temperature have been reported in response to injection of leptin in normal rodents. The leptin injected appears to be free of endotoxin contamination (**Section 3.11**) and mediates its effects by the direct action of leptin on its receptor (**Section 3.12**). Furthermore, the data indicate that leptin-induced responses on core body temperature, but not food intake and body weight, are mediated by PGs (**Section 3.13**).

3.15 Discussion

The results of experiments described in **Section 3.2**, performed over 22 h after injection of leptin at 10:00 h, confirm previous findings (Seeley *et al.*, 1996; Schwartz *et al.*, 1996; Wang *et al.*, 1997; al-Barazanji *et al.*, 1997; Widdowson *et al.*, 1997) that murine leptin inhibits both food intake (**Figure 3.2.1**) and body weight gain (**Figure 3.2.2**) in rats. These responses are similar for murine and rat leptin - a finding that may be expected since murine and rat leptin molecules have 96% sequence homology (Murakami & Shima, 1995). Because of the similarity between responses to murine and rat leptin, and the availability of murine leptin, most subsequent studies were performed using the latter.

The results presented in **Sections 3.3** and **3.4** demonstrate that the inhibitory effects of murine leptin on both food intake and body weight gain are dose-dependent when injected in the morning and measured over 22 h (**Figure 3.3.1-2**), or evening and measured over 14 h (**Figure 3.4.1-2**). More potent effects on these parameters were elicited by evening injection of leptin compared to morning injection, probably because the nocturnal feeding behaviour of rats is more likely to be affected by injection of leptin directly prior to this period.

The data described in **Section 3.5** show that cumulative food intake was not significantly affected until 3 h after injection of leptin (**Figure 3.5.1A**), although consumption between 1 and 2 h was significantly reduced in response to leptin (**Figure 3.5.1B**). Conversely, Schwartz *et al.* reported significant effects of leptin over these early time points in free-fed rats, but did not observe effects 24 h after injection (Schwartz *et al.*, 1996). In the study described here however, the greatest inhibition of food intake in response to injection of leptin was observed over the 3-12 and 24-36 h periods of nocturnal feeding behaviour, after which, normal feeding was resumed. These results are comparable to published data stating that normal food intake of animals is recovered by the third day after leptin administration (Cusin *et al.*, 1996; Jacob *et al.*, 1997). However, the leptin-induced suppression of food intake shown here was not compensated for by increased consumption in subsequent days.

In normal rats, body weight showed the expected diurnal variation, as the animals underwent phases of feeding (**Figure 3.5.2B**). The inhibitory effects of leptin injection on food intake were reflected in the resulting attenuation of body weight gain, such that weight gain was reduced over the first 12 h after injection

(**Figure 3.5.2B**). However, although food intake was reduced between 24 and 36 h after injection (**Figure 3.5.1B**), body weight gain was unaffected (**Figure 3.5.2B**). Furthermore, like the food intake of these leptin-treated animals, the reduced body weight gain was not compensated for over the remainder of the experiment (**Figure 3.5.2A**).

The effects of leptin on food intake and body weight reported here were similar to those seen in response to injection of proinflammatory cytokines (Mrosovsky *et al.*, 1989) or LPS (McCarthy *et al.*, 1986). Moreover, the structural similarities between leptin and cytokines (Zhang *et al.*, 1997) and their receptors (Tartaglia *et al.*, 1995; White & Tartaglia, 1996; Baumann *et al.*, 1996; Tartaglia, 1997) prompted a direct comparison of the effects of icv injection of leptin, IL-1β and LPS on food intake and body weight (**Section 3.6**). Indeed, injection of LPS and IL-1β elicited similar reductions in food intake (**Figure 3.6.1**) and body weight gain (**Figure 3.6.2**). Although these results do not confer any relationship between leptin and these proinflammatory agents, the doses of LPS and IL-1β injected are commonly used to induce fever in experimental animals (Busbridge *et al.*, 1990; Strijbos *et al.*, 1992; Luheshi *et al.*, 1996; Larson *et al.*, 1996). This observation suggested that leptin may also have effects on core body temperature in normal rats. Indeed, data presented in **Section 3.7-3.10** are the first to report convincingly that injection (icv) of leptin (murine or rat) induces significant dose-dependent increases in core body temperature in freely-fed rats.

A separate study has reported modest increases body temperatures in response to injection of leptin in rats (Fruhbeck *et al.*, 1998). Injection (ip) just prior to the dark phase, of a pool of 20 amino acid peptide fragments of the leptin molecule (1-5 mg/kg each peptide) increased core body temperatures 75 min after injection. However, the high basal temperatures (>38°C) reported in this study (Fruhbeck *et al.*, 1998), and the timing of injections (just prior to the increase in nocturnal activity) may have limited any significant changes in core body temperature.

It may be argued that the actions of leptin observed in this study were not specific to leptin receptors, and that the leptin injected may be stimulating similar receptors, such as the IL-6 receptor (Baumann *et al.*, 1996; Nakashima *et al.*, 1997). The absence of a commercially available leptin receptor antagonist discounted the possibility of proving pharmacologically that responses on food intake, body weight gain, and core body temperature observed in this study were induced by direct action of leptin on the leptin receptor. However, it has been reported that effects on body weight gain of endogenous leptin are reversed by injection of a mutant form of leptin, R128Q (Verploegen *et al.*, 1997), or specific leptin peptide fragments (Grasso *et al.*, 1997). Furthermore, icv injection of rabbit anti-mouse leptin antibodies that recognise a major epitope in the C-terminal region of the leptin molecule increased food intake during the first hour after injection (which was not compensated during the following 19 h period) in lean, but not obese Zucker rats (Brunner *et al.*, 1997). These molecules may be of great future importance in both laboratory and

clinical investigations, for the therapeutic treatment of wasting disorders such as anorexia and cachexia.

The profile of the temperature response to injection of leptin shared some characteristics with LPS and IL-1β fever, but was not identical (**Figure 3.7.1A-C**). Whereas leptin-induced hyperthermia was monophasic, like the response to injection of IL-1β, it was of similar duration to the LPS-induced response. Therefore it appears that leptin induces a unique temperature profile, which may be associated with a steady, prolonged increase in thermogenesis. Although the temperature responses to injection of leptin obtained from different sources (**Section 3.8**) were all of similar pattern (**Figure 3.8.1**), the duration of responses in this experiment differed slightly (murine, Zeneca > rat > murine, Insight), but not significantly.

The data presented in **Section 3.9** show that the increase in core body temperature of rats injected icv with leptin is dose-dependent (**Figure 3.9.1**). Injection of leptin was also found to increase body temperature when injected just prior to the nocturnal phase (**Section 3.10**), although only the highest dose of leptin elicited a significant response (**Figure 3.10.1**), probably because of the higher basal temperature. Endogenous leptin levels are naturally circadian (Simon *et al.*, 1998) and are increased during the dark phase in rats, in response to nocturnal feeding behaviour (Saladin *et al.*, 1995; Pickavance *et al.*, 1998). These variations in plasma leptin are also reported to be inversely related to levels of adrenocorticotrophic hormone (ACTH) and cortisol (Licinio *et al.*, 1997), and mirrored by plasma glucose, insulin and importantly, temperature (Simon *et al.*, 1998). However, the effects of increased nocturnal levels of

endogenous leptin and injection of exogenous leptin are not additive. Visual comparison of the temperature profiles of morning (**Figure 3.9.1**) and evening (**Figure 3.10.1**) injections of leptin reveal few differences. Nevertheless, the presence of a temperature response elicited by the highest dose of leptin suggests that the circadian clock does not gate effects of endogenous leptin on temperature i.e. nocturnal physiology does not inhibit the responses to injection of leptin.

Most subsequent experiments involving actions of leptin on core body temperature were performed using leptin injected at 10:00 h, since significant increases in body temperature were observed above constant basal temperatures of animals in the control group. In addition, significant effects on food intake and body weight were still observed.

The experiment described in **Section 3.11** showed that heat denatured leptin failed to induce effects on food intake, body weight gain or core body temperature (**Figure 3.11.1**). These data support the suggestion that the effects described here on food intake, body weight and body temperature were due to the action of leptin. Furthermore, the temperature response to injection of leptin was different from the biphasic fever elicited by injection of LPS (**Figure 3.7.1**).

Genetic models of obesity have been used extensively as tools for investigating actions of leptin, most notably *ob/ob* and *db/db* mice. This study however investigated actions of leptin in rats. Therefore the obese Zucker rat (*fa/fa*) which possesses a defective leptin receptor, was used to test the specificity of the actions observed in normal rats in response to injection of leptin (**Section 3.12**). Lean Zucker rats (*Fa/?*) exhibited similar food intake, body weight gain

and body temperature responses to leptin as those seen previously in Sprague-Dawley rats (**Sections 3.2-3.11**). However, leptin failed to induce significant changes in any of these factors in obese Zucker rats (**Figure 3.12.1-3**). These data indicate that responses to leptin administration reported here were induced by direct action of leptin on leptin receptors.

Previous studies have also reported that obese Zucker rats fail to respond to injection of leptin (Seeley *et al.*, 1996). However, injection of large doses of leptin have been shown to attenuate body weight gain (Cusin *et al.*, 1996; al-Barazanji *et al.*, 1997), supporting the proposal that obese Zucker rats still possess limited leptin receptor signalling (Yamashita *et al.*, 1997). In spite of this, obese *fa/fa* Zucker rats responded normally to central injection of PGE_2 (**Section 3.12**). Nevertheless, the high plasma levels of glucocorticoids that have been reported to affect febrile responses to cytokines in obese animals (Busbridge *et al.*, 1990) may also attenuate leptin-induced temperature changes. Therefore to fully verify that the effects of leptin on food intake, body weight and temperature are specific to action on leptin receptors, further experiments should be performed using adrenalectomised Zucker rats.

It may also be argued that due to the greater body weight of obese Zucker rats, injection of leptin may have a proportionally reduced effect, compared to responses in lean animals. Thereby, a 4 μg injection (icv) of leptin relates to a 12.5 μg/kg ratio in lean (320 g) animals, but only a 7.5 μg/kg ratio in obese (530 g) rats, which may result in reduced leptin effects in the obese animals. However, although this relationship may be relevant to peripheral injection of leptin where the distribution volume is obviously greater in the obese rat, icv

injection, where leptin is confined largely to the brain, probably corrects for this discrepancy.

Earlier studies reported that leptin normalises the attenuated body temperatures of obese *ob/ob* mice (Pelleymounter *et al.*, 1995; Harris *et al.*, 1998) which are deficient in endogenous leptin. However, neither study reported acute effects of leptin since temperature was measured after 3 weeks (Pelleymounter *et al.*, 1995) or four days (Harris *et al.*, 1998) of leptin treatment. Temperature responses to other pyrogens (e.g. IL-1) are rapidly lost (within 3 days) due to tolerance (Plata-Salaman *et al.*, 1996), and this may also be the case for leptin. The experiments described in these publications were performed at relatively low ambient temperatures for mice (approximately 26°C). Injection of other pyrogens such as IL-1 fail to elicit fever in mice, below ambient temperatures of approximately 28°C (Kozak *et al.*, 1994). Therefore any increases in heat production may have been masked.

Prostaglandins, which are activated by the actions of cyclo-oxygenase enzymes, are important in mediating fever (Kaufmann *et al.*, 1997). It has already been shown in this chapter that food intake, body weight and body temperature responses to injection of leptin are similar to those induced by the pyrogenic proinflammatory agents LPS and IL-1β (**Section 3.6-3.7**). These molecules induce fever via PG-dependent mechanisms (Coceani *et al.*, 1988; Rothwell, 1989; Saper & Breder, 1992; Cao *et al.*, 1997). It was suggested therefore that PGs also mediate leptin-induced increase in body temperature. Indeed, administration of the cyclo-oxygenase inhibitor flurbiprofen, which blocks the effects of both type 1 and type 2 cyclo-oxygenases (Riendeau *et al.*,

1997), and inhibits febrile responses to injection of cytokines (Rothwell *et al.*, 1991), abolished the effects of icv injection of leptin on body temperature, but did not affect changes in food intake or body weight (**Section 3.13**). These data suggest that the hypophagic and hyperthermic responses to icv injection of leptin are mediated by separate PG-independent and PG-dependent pathways, respectively.

The fact that the increased core body temperatures observed in response to injection of leptin in normal rats are mediated by PGs suggests that leptin may be inducing *fever* (Kluger, 1991; Kluger et al., 1995). However, although these temperature changes are PG-dependent, to elucidate whether leptin indeed induces 'true' fever, a course of behavioural studies should be performed to investigate whether the preferred ambient temperature of the animals is altered. If leptin *is* acting on the hypothalamic set-point to increase core body temperature, animals that are housed in a temperature gradient and injected with leptin may be expected to migrate to an area of higher ambient temperature than vehicle-treated animals so as to facilitate a febrile response.

The similarities described in this chapter between the effects of icv injection of leptin on food intake, body weight and core body temperature in rats, and those induced by injection of the cytokine IL-1, and the fact that like IL-1, leptin 'fever' is mediated by PGs, suggests that there may be a causal relationship between the actions of leptin and cytokines.

Chapter 4

Involvement of Cytokines in Actions of Leptin

4.1 INTRODUCTION

Leptin has been implicated in the control of energy balance by suppressing food intake and increasing energy expenditure as described in **Chapter 3**, and in previous studies (Zhang *et al.*, 1994; Hwa *et al.*, 1996), thereby mimicking some aspects of a host response to disease or infection. This hypothesis is supported further by evidence linking proinflammatory agents with the synthesis and release of leptin.

Administration of bacterial lipopolysaccharide (LPS) in rodents, to generate a host defence response, upregulates gene expression and serum protein levels of leptin (Grunfeld *et al.*, 1996; Sarraf *et al.*, 1997; Berkowitz *et al.*, 1998). LPS is also a potent inducer of cytokines both *in vivo* and *in vitro* (Rothwell & Hopkins, 1995), and induction of leptin in response to LPS appears to be mediated via release of the cytokines IL-1 and TNF (Sarraf *et al.*, 1997; Faggioni *et al.*, 1998; Finck *et al.*, 1998). Indeed, both IL-1 and TNF have been shown to directly increase leptin mRNA expression and serum leptin concentration in rodents (Grunfeld *et al.*, 1996; Sarraf *et al.*, 1997; Berkowitz *et al.*, 1998). Conversely, exogenous leptin has been demonstrated to upregulate LPS-induced phagocytosis and proinflammatory cytokines (TNF, IL-6, IL-12) in *ex vivo* macrophages from mice (Loffreda *et al.*, 1998). In addition, obese leptin-deficient (*ob/ob*) mice, and obese Zucker (*fa/fa*) rats possessing a defective leptin receptor show attenuated levels of serum TNF and IL-6 in response to LPS administration (Loffreda *et al.*, 1998). Obese Zucker rats also exhibit reduced immune responsiveness (Plotkin *et al.*, 1996). This study reported that the bacteria colonies were established at a greater rate and to a greater extent

in obese compared to lean Zucker rats. Furthermore, peritoneal polymorphonuclear leucocytes, resident macrophages and thioglycollate-elicited macrophages from lean Zucker rats displayed a significantly higher ability to kill ingested yeast cells than analogous cell populations from obese Zucker rats (Plotkin *et al.*, 1996). These findings suggest that leptin is linked to the actions of proinflammatory cytokines, and may be involved in host defence responses to infection in the periphery. This suggestion is supported by studies performed in humans. Injection (iv) of IL-1α in cancer patients has been reported to dose-dependently increase serum leptin concentrations (Janik *et al.*, 1997). However, despite continued administration of IL-1α, serum leptin concentrations of patients in this study returned to pre-treatment levels after 5 days of therapy. Patients with acute sepsis exhibit increased plasma leptin (by three-fold), IL-6 and cortisol, and reduced ACTH levels (Bornstein *et al.*, 1998). The control patients in this study exhibited a diurnal fluctuation in plasma leptin levels (23:00 h peak) whereas septic patients maintained high leptin levels throughout. This suggests that leptin is involved in host defence responses to infection in both animals and humans.

The anti-inflammatory cytokine TGF-β has also been reported to increase leptin mRNA expression *in vitro* (Granowitz, 1997). Conversely, treatment of 3T3-L1 adipocytes with IL-1β, IL-6, IL-11, or TNF-α in this study resulted in a decrease in leptin message. Since other studies report that IL-1 and TNF-α induce leptin expression, these results suggest that pro-inflammatory cytokines induce leptin synthesis *in vivo* via secondary mediators such as TGF-β.

The aforementioned studies demonstrate strongly a relationship where proinflammatory mediators such as cytokines stimulate the synthesis and release of leptin. However, such mediators (cytokines) are known to be highly involved in positive and negative feedback loops with other cytokine molecules (Hopkins & Rothwell, 1995). The structure of leptin and its receptor have been likened to cytokines, and the experiments presented in the previous chapter demonstrate cytokine-like effects of leptin on food intake, body weight and core body temperature. Therefore, it was suggested that leptin may have a similar relationship with cytokines, such that the hypophagic and febrile effects of leptin in the brain described in **Chapter 3**, are mediated via the release of cytokines such as IL-1 and IL-6.

4.2 Influence of IL-1ra on Actions of Centrally Administered Leptin

Studies described in **Chapter 3** showed that leptin induces both hypophagia and increased body temperature - responses that mimic effects of proinflammatory agents such as LPS and the cytokine IL-1 (**Section 3.6-3.7**). The data also indicated that leptin actions on temperature, but not food intake or body weight, are mediated by PGs - molecules that mediate many responses to cytokines such as IL-1 (Hellerstein *et al.*, 1989; Rothwell, 1990; Milton, 1998). This objective of this experiment was to address the hypothesis that IL-1 is directly involved in mediating effects of central injection of leptin on food intake, body weight gain, and core body temperature. This was achieved by the use of the naturally occurring receptor antagonist to IL-1 receptors, IL-1ra.

4.2.1 Experimental Design

Animals were injected (icv) at 10:00 h (0 h) with vehicle or leptin (4 μg), and with previously determined dose (200 μg) of IL-1ra (Luheshi et al., 1996a; Miller *et al.*, 1997) or vehicle (icv). Animals were treated again with IL-1ra (200 μg, icv) at the 1 h time point. Core body temperature was monitored for 10 h after injection - until the end of the light phase. Food intake and body weight gains were measured over 22 h - until the end of the subsequent dark phase.

4.2.2 Results

Food intake (**Figure 5.2.1**) was significantly reduced by 55% (ANOVA: $p<0.001$) over 22 h in normal rats in response to icv injection of leptin, compared to vehicle-treated animals (26.8±0.1 g). Central injection of IL-1ra at the 0 and 1 h

time points, alone did not significantly affect food intake (ANOVA). Co-administration of leptin and IL-1ra however, attenuated the suppressed food intake observed in leptin-treated rats (ANOVA: $p<0.001$), returning consumption to within 60% of normal (ANOVA: $p<0.001$).

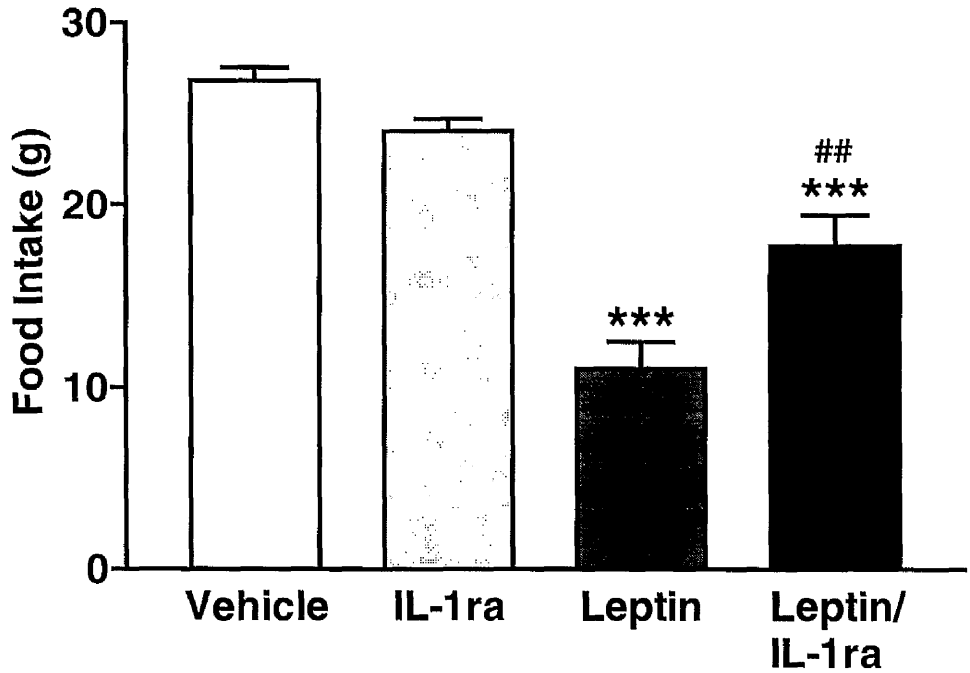


Figure 4.2.1. Food intake over 22 h in response to injection (icv) of leptin (4 μg) and IL-1ra (200 μg, 0 and 1 h)

(ANOVA: *** $p<0.001$ vs Vehicle; ## $p<0.01$ vs Leptin)

In the same animals, body weight (**Figure 5.2.2**) was measured over 22 h after icv injection of leptin and IL-1ra. Leptin significantly reduced (ANOVA: $p<0.001$) body weight (by 7±2 g) in comparison to the weight gain observed in vehicle-treated rats (12±1 g). This weight loss induced by central injection of leptin was

markedly inhibited (ANOVA: $p<0.001$) by co-administration of IL-1ra. Injection of IL-1ra alone did not significantly affect body weight (ANOVA).

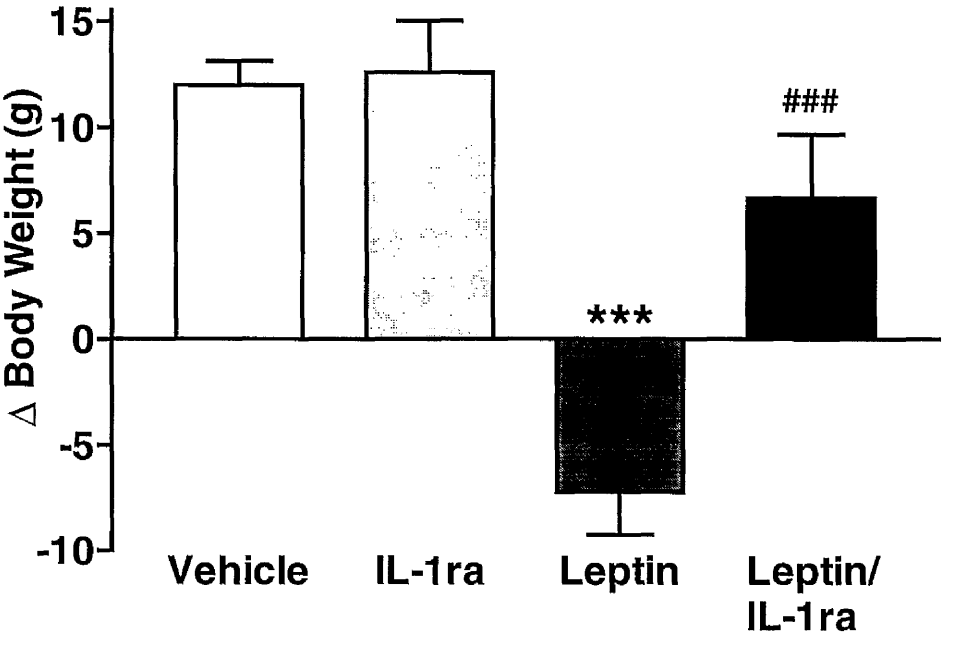


Figure 4.2.2. Changes in body weight in response to injection (icv) of leptin (4 μg) and IL-1ra (200 μg, 0 and 1 h)

(ANOVA: *** $p<0.001$ vs Vehicle; ### $p<0.001$ vs Leptin)

Central administration of IL-1ra did not significantly affect core body temperature (**Figure 4.2.3**) compared to vehicle-treated rats (ANOVA). Injection (icv) of leptin induced a significant increase in core body temperature (MANOVA: $p<0.001$ vs Vehicle). Body temperatures of leptin-treated animals rose after the 1.5 h time point, and peaked (1.7°C above control) 6.5 h after injection (ANOVA: $p<0.001$ vs Vehicle). Temperatures remained elevated for

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the remainder of the 10 h study. This leptin-induced response was completely abolished (MANOVA: $p<0.001$) by co-administration of IL-1ra.

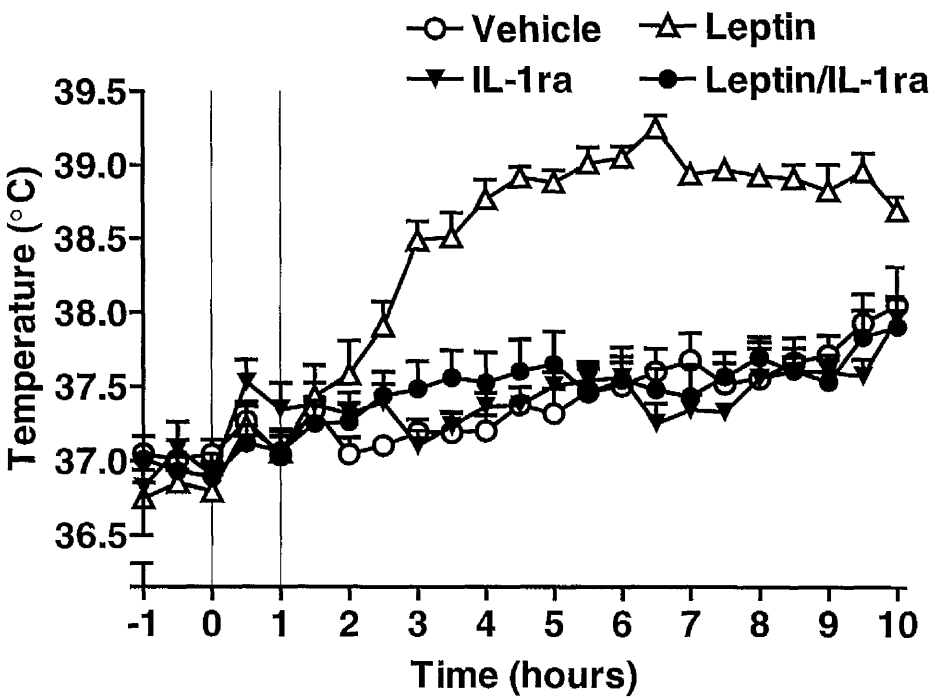


Figure 4.2.3. Core body temperature in response to injection (icv) of leptin (4 μg) and IL-1ra (200 μg, 0 and 1 h)

4.3 Influence of IL-1ra on Actions of Peripherally Administered Leptin

All studies described in this thesis have involved injection of leptin into the brain of rats, where leptin elicits many of its actions on food intake and energy balance (Campfield *et al.*, 1996; Considine & Caro, 1997; Auwerx & Staels, 1998). However, leptin is synthesised and released from white adipose tissue in the periphery, and must cross the BBB via a saturable mechanism (Banks *et al.*, 1996), to exert actions in the brain. Therefore leptin levels in the brain after icv injection may exceed those that occur naturally after entry from the periphery.

Therefore this study was performed to investigate whether peripheral administration of leptin elicited similar responses to those observed in response to icv injection; and whether any effects seen were inhibited by IL-1ra.

4.3.1 Experimental Design

Animals were injected (ip) with leptin (3.4 mg/kg, approx. 1 mg/rat) or vehicle at 10:00 h (0 h), and with a dose (200 μg, icv) of IL-1ra or its vehicle. Animals were treated again with IL-1ra (200 μg, icv) at the 1 h time point. Core body temperature was monitored for 6 h after injection. Food intake and body weight gains were measured over 22 h - until the end of the subsequent dark phase.

4.3.2 Results

Peripheral injection of leptin significantly reduced food intake (**Figure 4.3.1**) in rats (by 26%) over 22 h compared with vehicle-treated animals (31.5±0.5 g, ANOVA: $p<0.001$). Central injection of IL-1ra alone did not affect food intake, as shown in the previous section (**4.2**). However co-administration of IL-1ra significantly (ANOVA: $p<0.01$) attenuated (by 60%) the hypophagia induced by leptin, such that food intake was not significantly different from control levels (ANOVA).

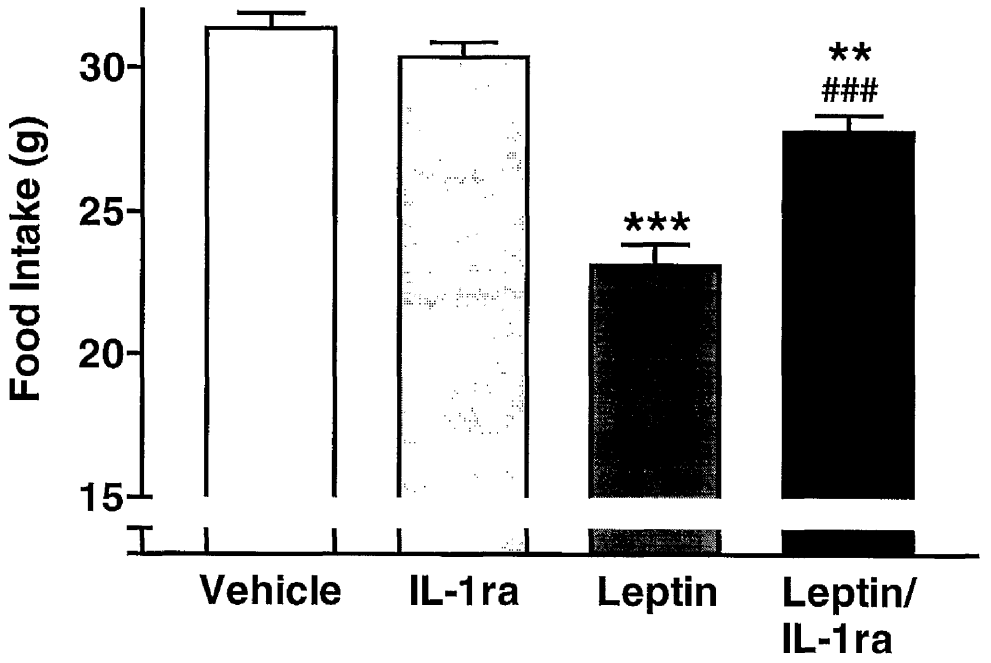


Figure 4.3.1. Food intake over 22 h after injection of leptin (1mg/rat, ip) and IL-1ra (200 μg, 0 and 1 h, icv)

(ANOVA: *** $p<0.001$, ** $p<0.01$ vs Vehicle; ### $p<0.01$ vs Leptin)

In the same animals, body weight was measured (**Figure 4.3.2**). Vehicle-treated animals gained 14±1 g body weight over the 22 h after injection. This gain in body weight was unaffected by central administration of IL-1ra. Peripheral injection of leptin significantly attenuated body weight gain to 3±1 g (ANOVA: $p<0.001$ vs Vehicle) - an effect that was inhibited (ANOVA: $p<0.01$) to within 70% of control levels by co-administration of IL-1ra.

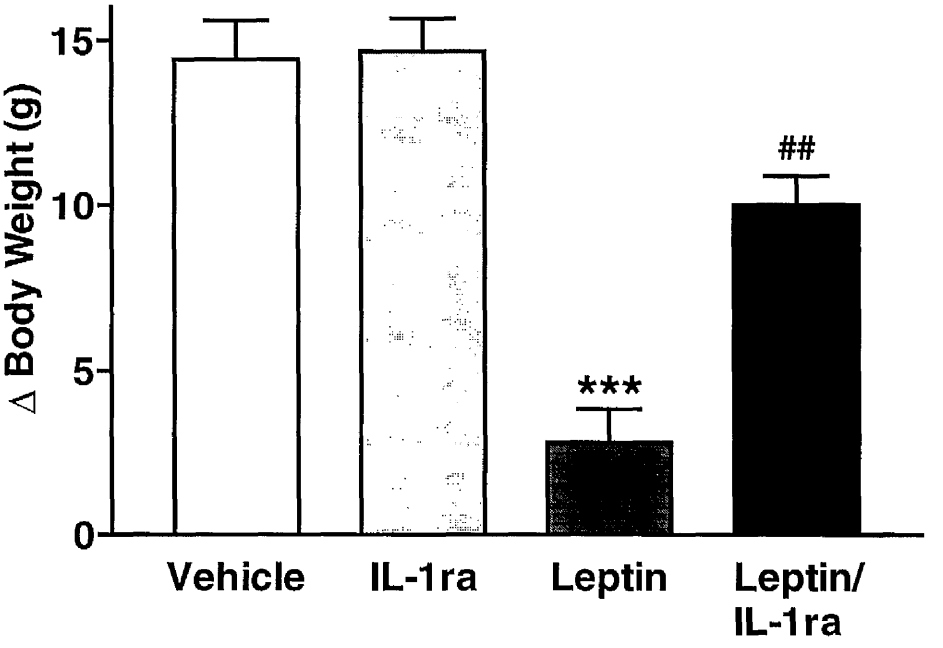


Figure 4.3.2. Body weight gain of rats over 22 h in response to injection of leptin (1 mg/rat, ip) and IL-1ra (200 μg, 0 and 1 h, icv)

(ANOVA: *** $p<0.001$ vs Vehicle; ### $p<0.001$ vs Leptin)

Core body temperatures were also monitored in these animals, for 6 h after injection (**Figure 4.3.3**). Central injection of IL-1ra did not significantly affect core body temperature compared with vehicle-treated animals (MANOVA). Peripheral injection of leptin induced a significant, although modest and transient increase in core body levels (MANOVA: $p<0.01$ vs Vehicle) after the 2.5 h time point. Temperatures peaked 3.5 h after injection (0.9°C above control, ANOVA: $p<0.01$) before returning to control temperatures at the 5.5 h time point. Co-administration of IL-1ra abolished this increase in core body temperature (MANOVA: $p<0.05$) to control levels.

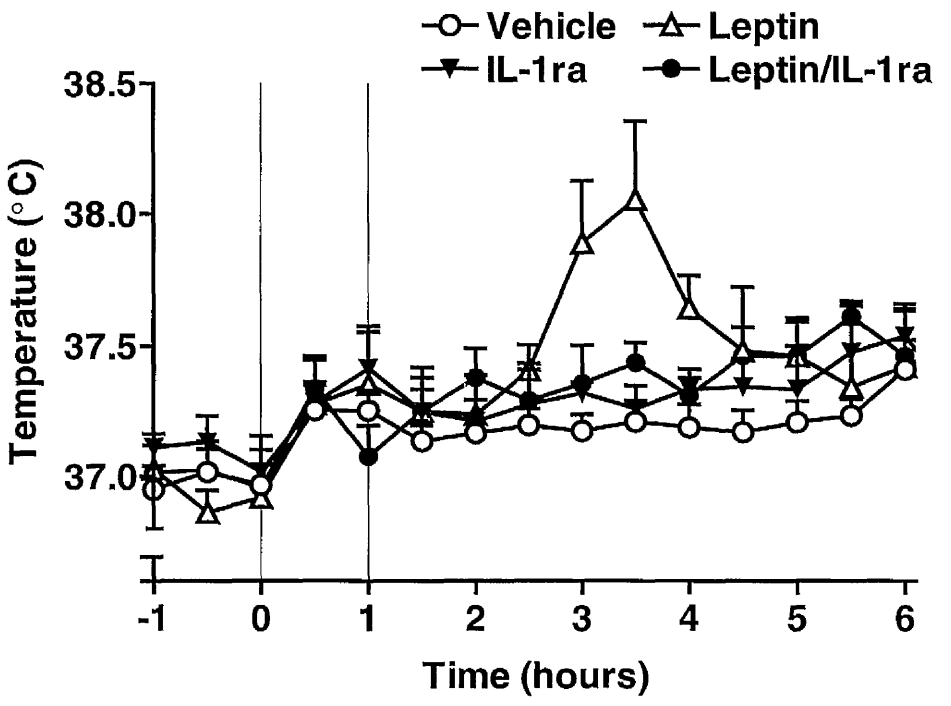


Figure 4.3.3. Core body temperature in response to injection of leptin (1 mg/rat, ip) and IL-1ra (200 μg, 0 and 1 h, icv)

4.4 IL-1RI (-/-) Mice are Insensitive to Leptin

The results presented above suggest that the effects of leptin on food intake, body weight and core body temperature are dependent on IL-1, since they are blocked by IL-1ra. In order to verify this proposal, the effects of leptin were investigated in transgenic IL-1RI (-/-) mice that have been produced with a genetically disrupted type I IL-1 receptor gene (Glaccum *et al.*, 1997). Because these mice are insensitive to IL-1, and previous experiments have shown effects of leptin to be dependent on IL-1, it may be expected that these IL-1RI (-/-) mice are also insensitive to leptin. It must be noted that most of the following experiments involving mice were preliminary, and were performed

using normal C57BL/6 mice rather than the more genetically relevant C57129 strain.

4.4.1 Experimental Design

IL-1RI (-/-) mice, or C57BL/6 mice used as controls were housed in wire-bottomed cages (to allow measurement of food spillage), and injected (icv) with leptin (4μg) or vehicle at 18:00 h. Food intake and body weights were measured 14 h after injection, at the beginning of the subsequent light phase. Unfortunately facilities were not adequately adapted for the accurate measurement of core body temperature in mice, and so this parameter was not monitored.

4.4.2 Results

Food intake of vehicle-treated C57BL/6 mice (3.9±0.2 g) over 14 h after injection (at 18:00 h) was significantly inhibited (ANOVA: $p<0.001$) by 75% in response to icv injection of leptin (**Figure 4.4.1**). IL-1RI (-/-) mice consumed 3.6±0.2 g food over 14 h after vehicle injection. Knockout mice treated with leptin exhibited food intake that was not significantly different compared to vehicle-treated mice (ANOVA).

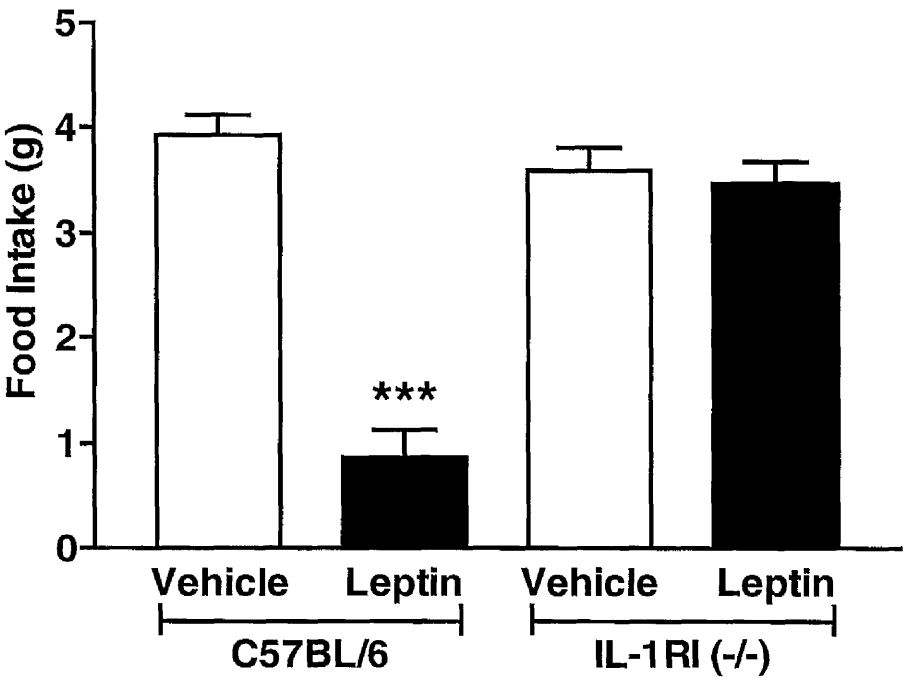


Figure 4.4.1. Food intake of IL-1RI (-/-) mice and their wild type controls (C57BL/6) in response to icv injection of leptin (4 μg)

(ANOVA: *** $p<0.001$ vs All)

Vehicle-treated C57BL/6 mice lost 0.3±0.1 g body weight over 14 h after injection (**Figure 4.4.2**). This weight loss was exacerbated (2.2±0.3 g) in response to leptin (ANOVA: $p<0.001$). Injection of vehicle or leptin in IL-1RI (-/-) mice induced body weight loss (0.9±0.1 and 0.9±0.2 g respectively) which did not differ significantly (ANOVA).

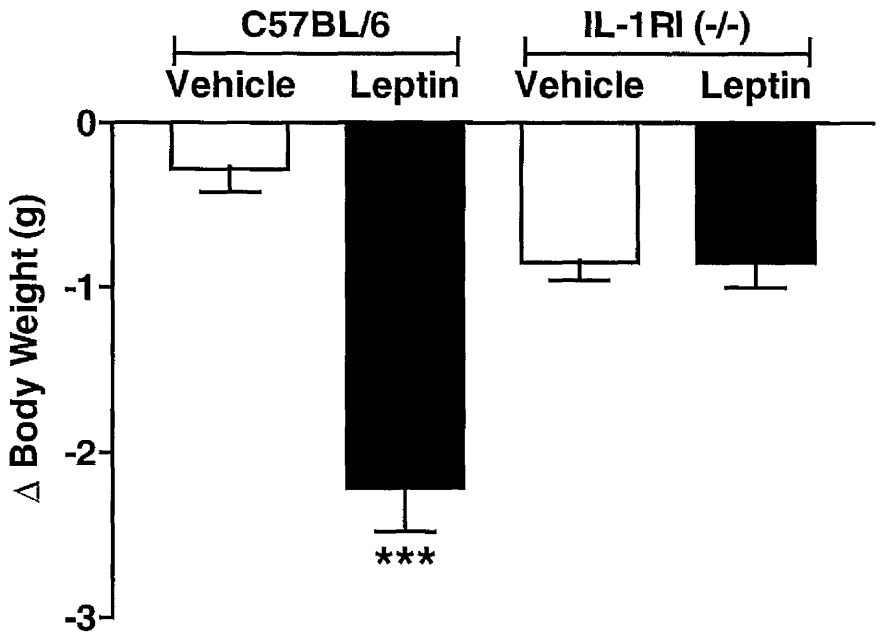


Figure 4.4.2. Changes in body weight of IL-1RI (-/-) mice and their wild type controls (C57BL/6) in response to icv injection of leptin (4 μg)

(ANOVA: *** $p<0.001$ vs All)

Pre-injection body weights of IL-1RI (-/-) mice (32.9±1.2 g) were significantly greater (by 11%) than the body weights (29.7±0.5 g) of C57BL/6 mice (ANOVA: $p<0.05$) (**Figure 4.4.3**).

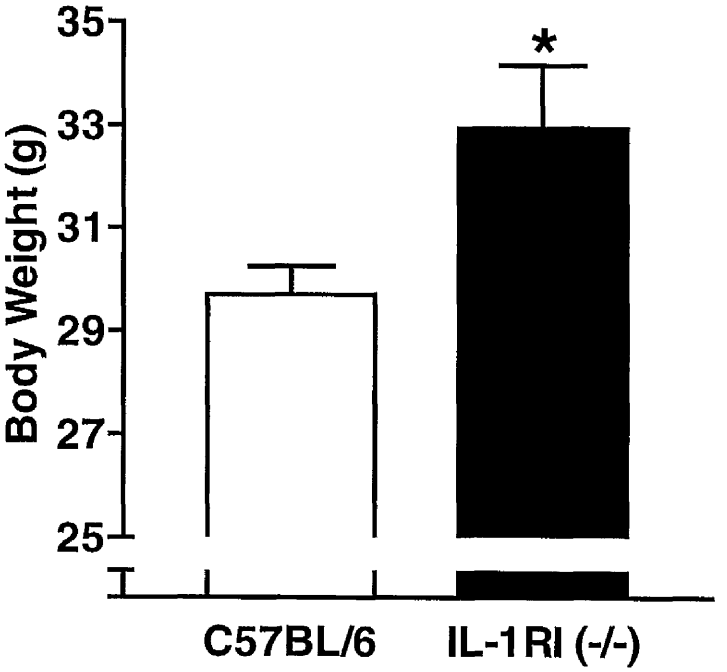


Figure 4.4.3. Pre-injection body weights of IL-1RI (-/-) mice and their wild type controls (C57BL/6)

(t-test: * $p<0.05$)

4.5 Effects of Leptin on Hypothalamic IL-1β and IL-6 Immunoreactivity

The results described previously (**Sections 4.2-4.4**) indicate that the effects on food intake, body weight gain and core body temperature induced by central or peripheral injection of leptin, are mediated by IL-1 in the brain. These effects on energy balance are most likely to be mediated in the hypothalamus - a region of the brain that controls most effects on energy balance (Campfield *et al.*, 1996; Wilding *et al.*, 1997). This hypothesis is supported by reports that the hypothalamus contains a high concentration of leptin receptors (Couce *et al.*, 1997; Elmquist *et al.*, 1998). The hypothalamus is also an area of the brain

where cytokines such as IL-1 and IL-6 play an important role in neuroimmune responses (Kluger *et al.*, 1995; Plata-Salaman, 1998; Blatteis & Sehic, 1998). Therefore, it was hypothesised that the effects of leptin on food intake, body weight and core body temperature have been shown to be mediated by cytokines (IL-1), may be associated with associated with increased expression of hypothalamic immunoreactive IL-1 and IL-6.

4.5.1 Experimental Design

Normal SD rats were injected (icv) with 4 μg leptin or vehicle at 10:00 h. Four hours after injection, animals were anaesthetised by halothane and blood samples taken by cardiac puncture. The animals were then sacrificed, the brains were removed and the hypothalami dissected out. Hypothalamic and plasma samples were latter assayed for the presence of immunoreactive IL-1β and IL-6 by ELISA.

4.5.2 Results

Administration of leptin (4 μg, icv) elicited a significant, five-fold increase (t-test: $p<0.001$) in the levels of immunoreactive IL-1β in the brains of normal rats (1800±30 pg/mg protein) compared to vehicle-treated animals (Vehicle, 300±100 pg/mg protein; detection limit, 90 pg/mg protein), 4 h after injection (**Figure 4.5.1**).

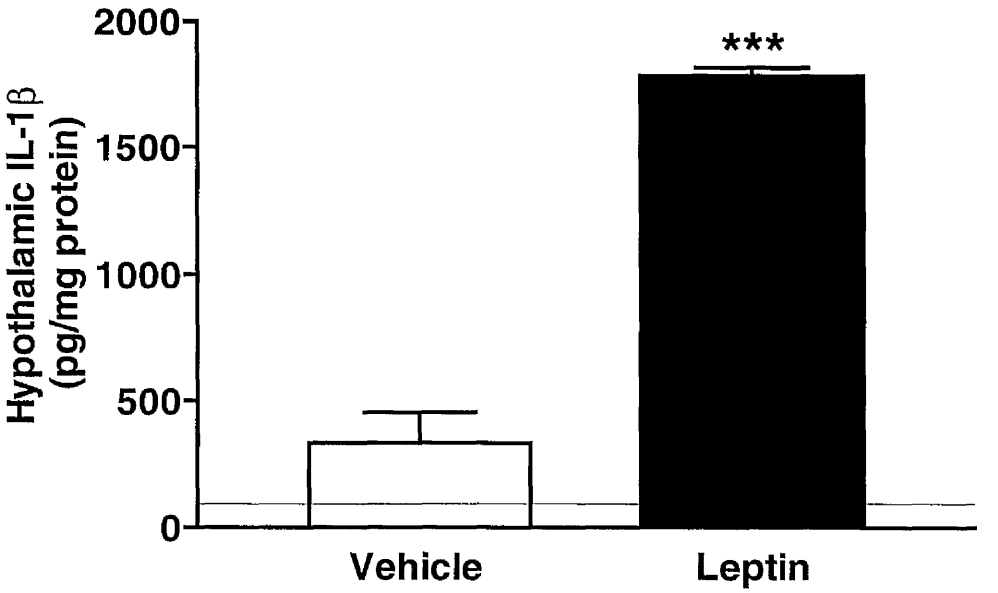


Figure 4.5.1. Levels of hypothalamic irIL-1β (measured by ELISA) 4 h after injection of leptin (4 μg, icv) in SD rats

(t-test: *** $p<0.001$)

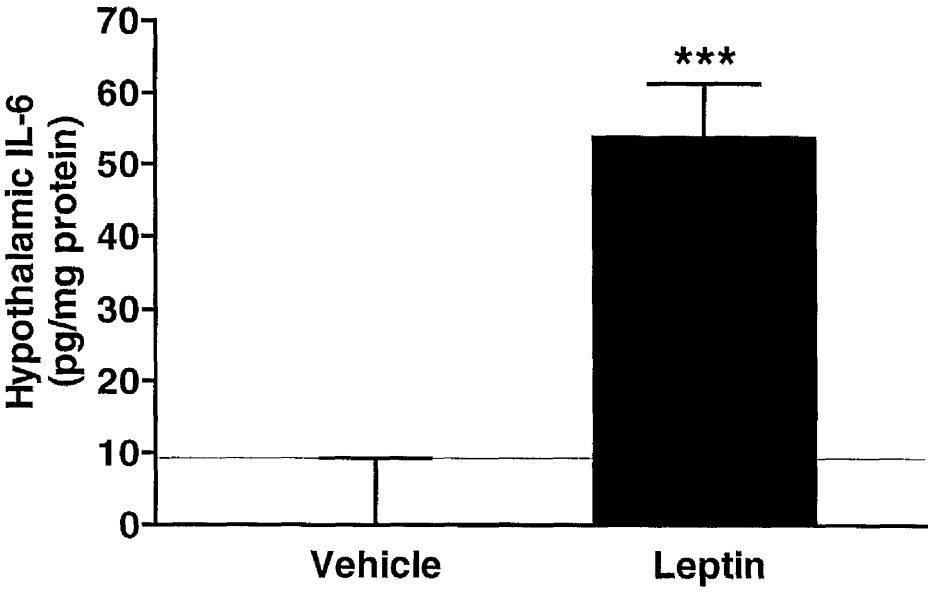


Figure 4.5.2. Levels of hypothalamic irIL-6 (measured by ELISA) 4 h after injection of leptin (4 μg, icv) in SD rats

(t-test: *** $p<0.001$)

Levels of hypothalamic IL-6 were also increased (although to a lesser extent) to 55±5 pg/mg protein (t-test: $p<0.001$). IL-6 levels in the hypothalami of vehicle-treated rats were below the detection limit of the assay (10 pg/mg protein).

Levels of IL-1β and IL-6 in the samples of blood plasma were below the detection limits of the assays (data not shown).

4.6 Effects of Leptin on Hypothalamic IL-1β and IL-6 Immunoreactivity in Zucker Rats

In normal animals, effects of icv injection of leptin on food intake, body weight gain, and core body temperature appear to be mediated by IL-1 (**Section 4.2-4.3**) (and possibly IL-6) released in the hypothalamus (**Section 4.5**). Results described in the **Chapter 3** indicate that obese Zucker rats, which possess a defective leptin receptor, are insensitive to effects of leptin on food intake, body weight and body temperature. Therefore if the increased levels of hypothalamic IL-1β and IL-6 observed in normal SD rats are induced by the direct action of leptin on its receptor, obese Zucker rats should fail to exhibit these increases.

4.6.1 Experimental Design

Both lean and obese Zucker rats were injected (icv) as in the previous section (**4.5**) with 4 μg leptin or vehicle at 10:00 h. Four hours after injection, animals were anaesthetised by halothane and blood samples taken by cardiac puncture. The animals were then sacrificed, the brains were removed and the

hypothalami dissected out. Hypothalamic and plasma samples were later assayed for the presence of immunoreactive IL-1β and IL-6 by ELISA.

4.6.2 Results

In lean Zucker rats (**Figure 4.6.1**), icv injection of leptin induced increase in levels of IL-1β (1800±50 pg/mg protein) which were similar to those induced in normal SD rats in the previous section (**4.5**). These levels were significantly greater (ANOVA: $p<0.001$) than in vehicle-treated lean animals (50±10 pg/mg protein) that were below the detection limit of the assay (150 pg/mg protein). Injection of leptin in obese Zucker rats also induced significant increases (ANOVA: $p<0.001$ vs Vehicle) in hypothalamic IL-1β (1100±50 pg/mg protein; Vehicle, 130±50 pg/mg protein) that were significantly attenuated compared to the sub-detection levels observed in leptin-treated lean rats (ANOVA: $p<0.001$).

Levels of IL-1β in the samples of plasma taken from both lean and obese Zucker rats were below the detection limits of the assays.

Levels of IL-6 were below the detection limit of the assay in hypothalamic and plasma samples taken from both lean and obese Zucker rats.

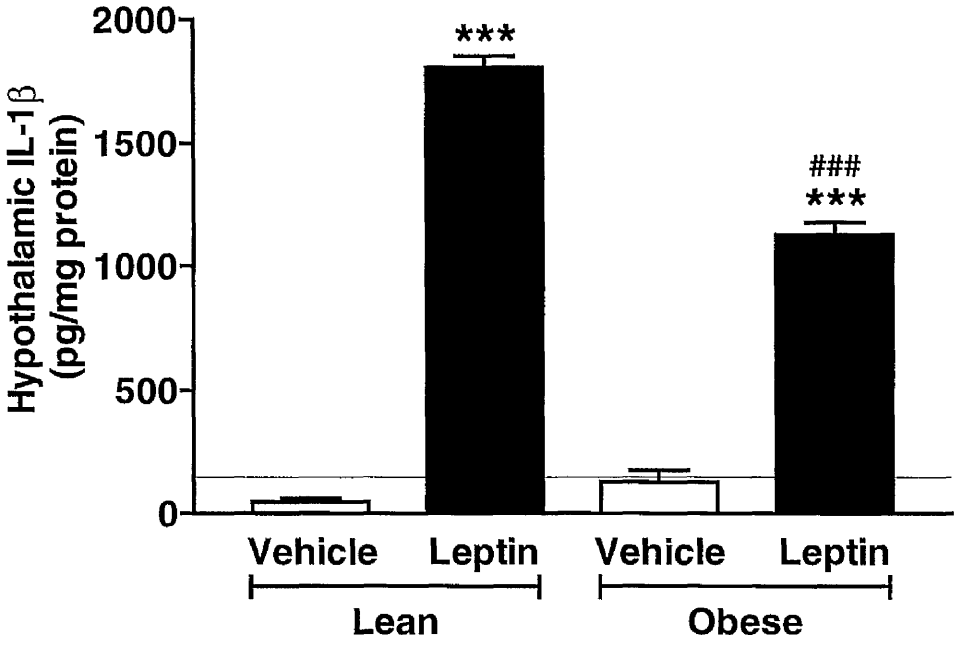


Figure 4.6.1. Hypothalamic irIL-1β (measured by ELISA) 4 h after injection of leptin (4 μg, icv) in lean (*Fa/?*) and obese (*fa/fa*) Zucker rats

(ANOVA: *** $p<0.001$ vs Vehicle; ### $p<0.001$ vs Lean-Leptin)

4.7 Leptin-Induced Expression of IL-1β in the Rat Brain

Previous experiments (**Sections 4.2-4.6**) have shown that leptin increases expression of hypothalamic IL-1, and that the responses to icv injection of leptin are mediated by IL-1β. This preliminary study was designed to localise expression of irIL-1β in response to icv injection of leptin.

4.7.1 Experimental Design

Rats were injected icv with vehicle or leptin (4 μg) at 10:00 h. Four hours after injection, at the time point when the temperature response to injection of leptin

was maximal (derived from experiments described in previous sections), animals received a lethal dose of anaesthetic (**Section 2.5.6**). Rats were perfused with PFA, the brains were removed, sectioned and stained immunohistochemically for irIL-1β, as described in **Chapter 2** (**Section 2.5.6**).

4.7.2 Results

Both vehicle and leptin-treated animals exhibited IL-1β expression at the site of injection (**Figure 4.7.1**). Brain sections from rats treated with leptin also exhibited IL-1β highly defined immunoreactive choroid plexus cells (especially in the cytoplasm) in the brain ventricles (**Figure 4.7.2**), in microglial cells in hypothalamic regions surrounding the third ventricle (**Figure 4.7.3**), and in meningeal macrophages (**Figure 4.7.4**). There was no expression of IL-1β observed in cells of the cerebral cortex.

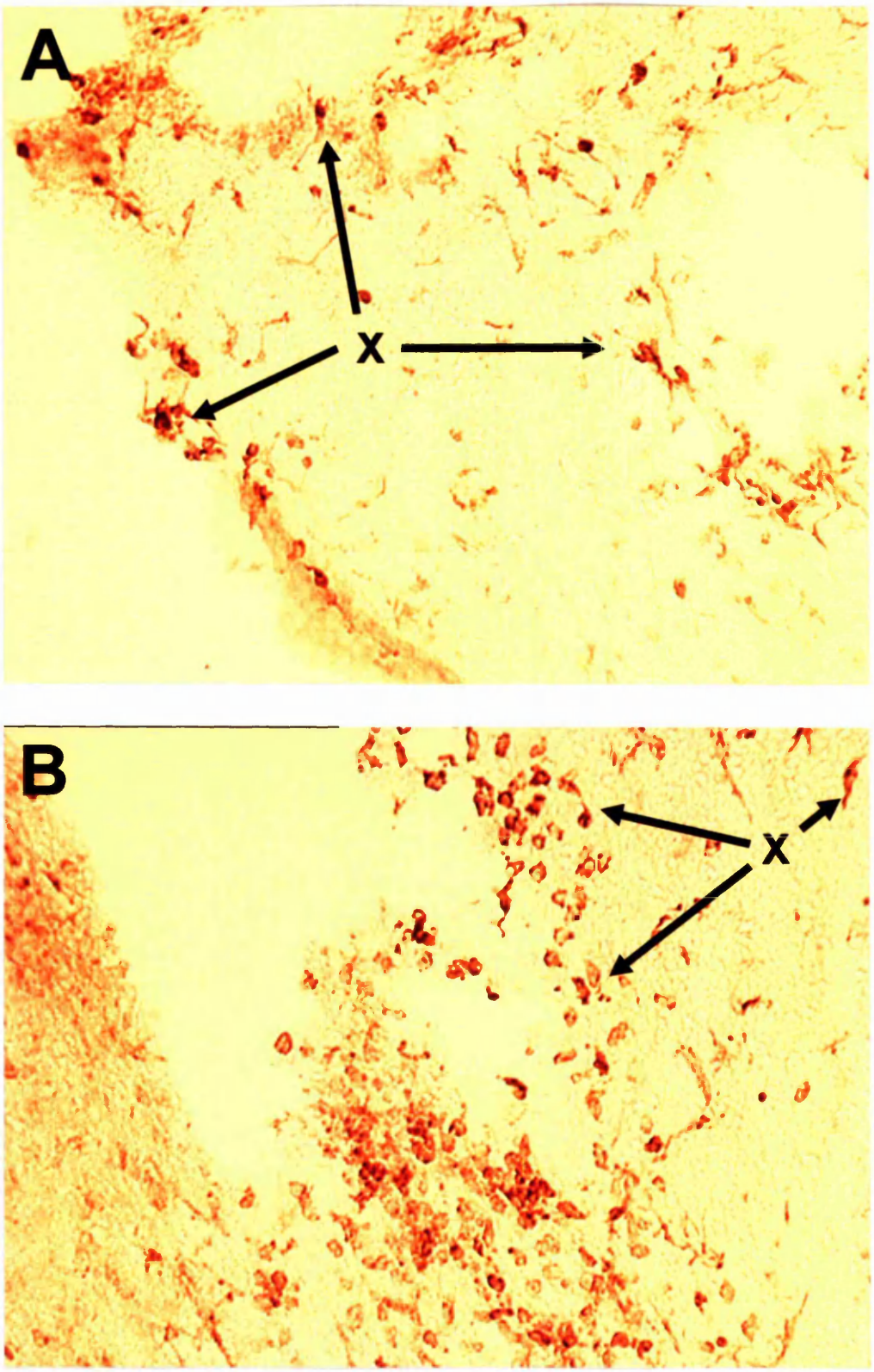


Figure 4.7.1. Expression of irIL-1β in macrophages (X) surrounding the injection site, 4 h after icv injection of vehicle (A) or 4μg leptin (B)

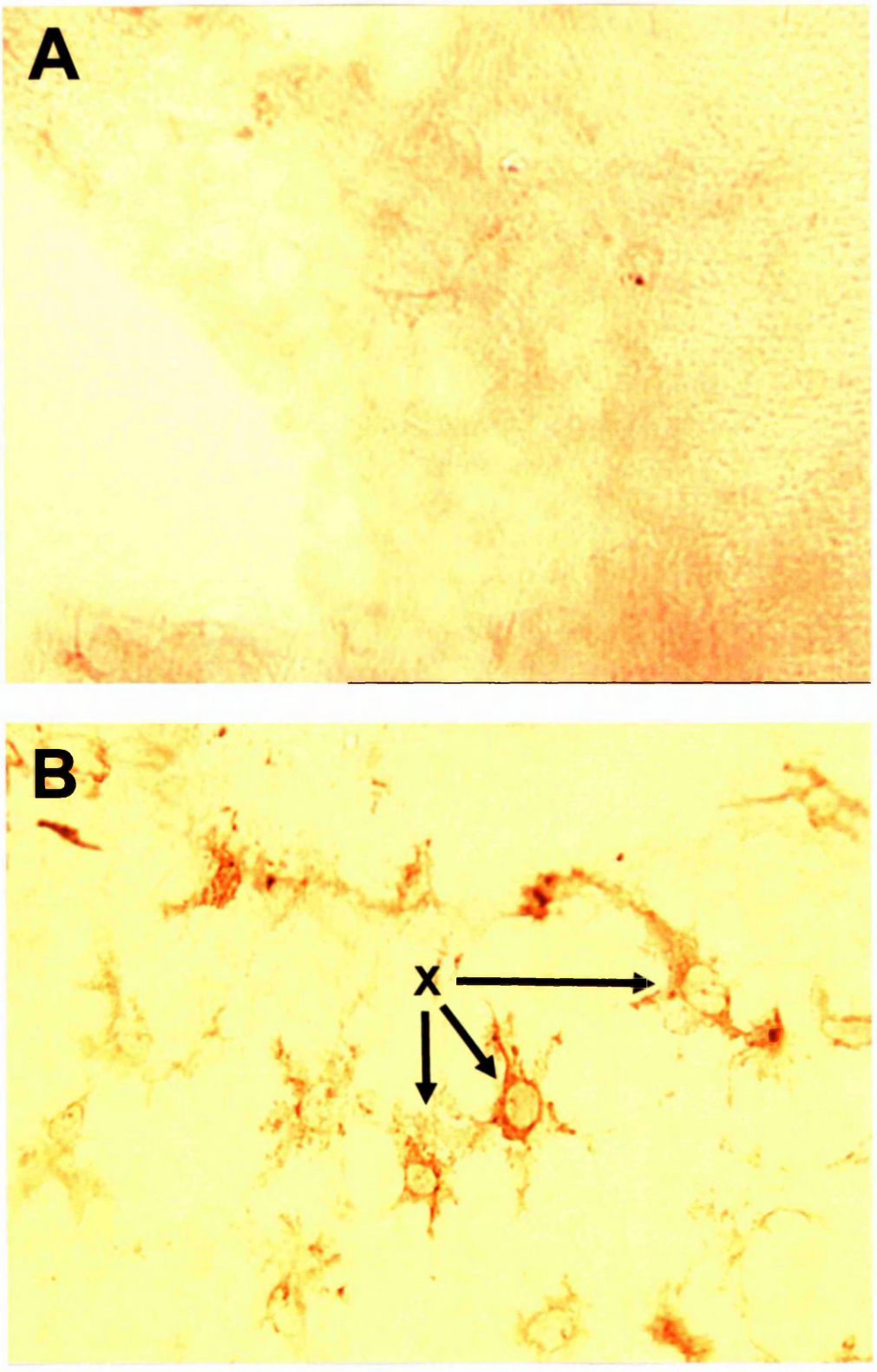


Figure 4.7.2. Expression of irIL-1β in choroid plexus cells (X), 4 h after icv injection of vehicle (A) or 4μg leptin (B)

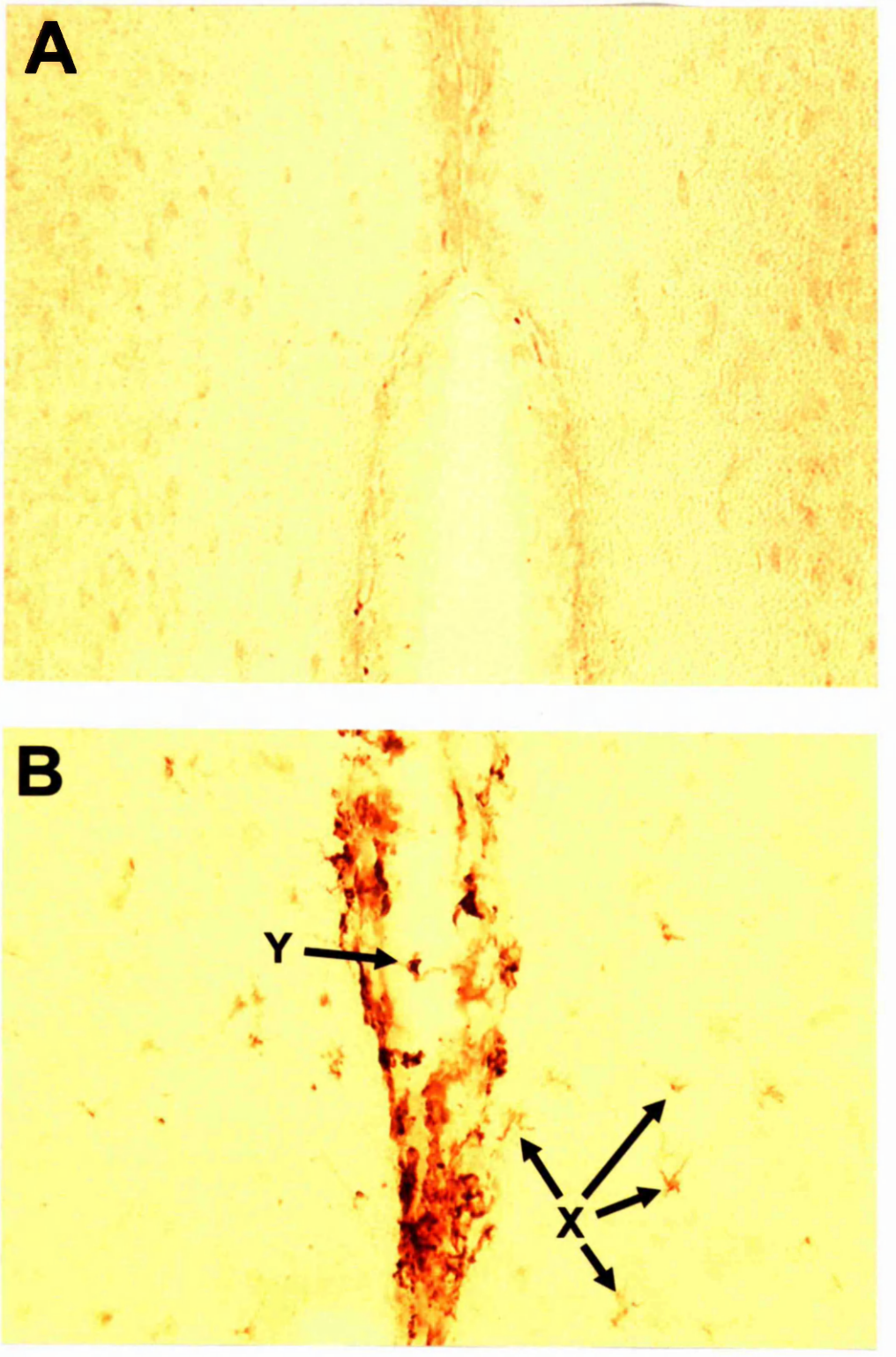


Figure 4.7.3. Expression of irIL-1β in hypothalamic macrophages (X) and third ventricle choroid plexus cells (Y) in the hypothalamus, 4 h after icv injection of vehicle (A) or 4μg leptin (B)

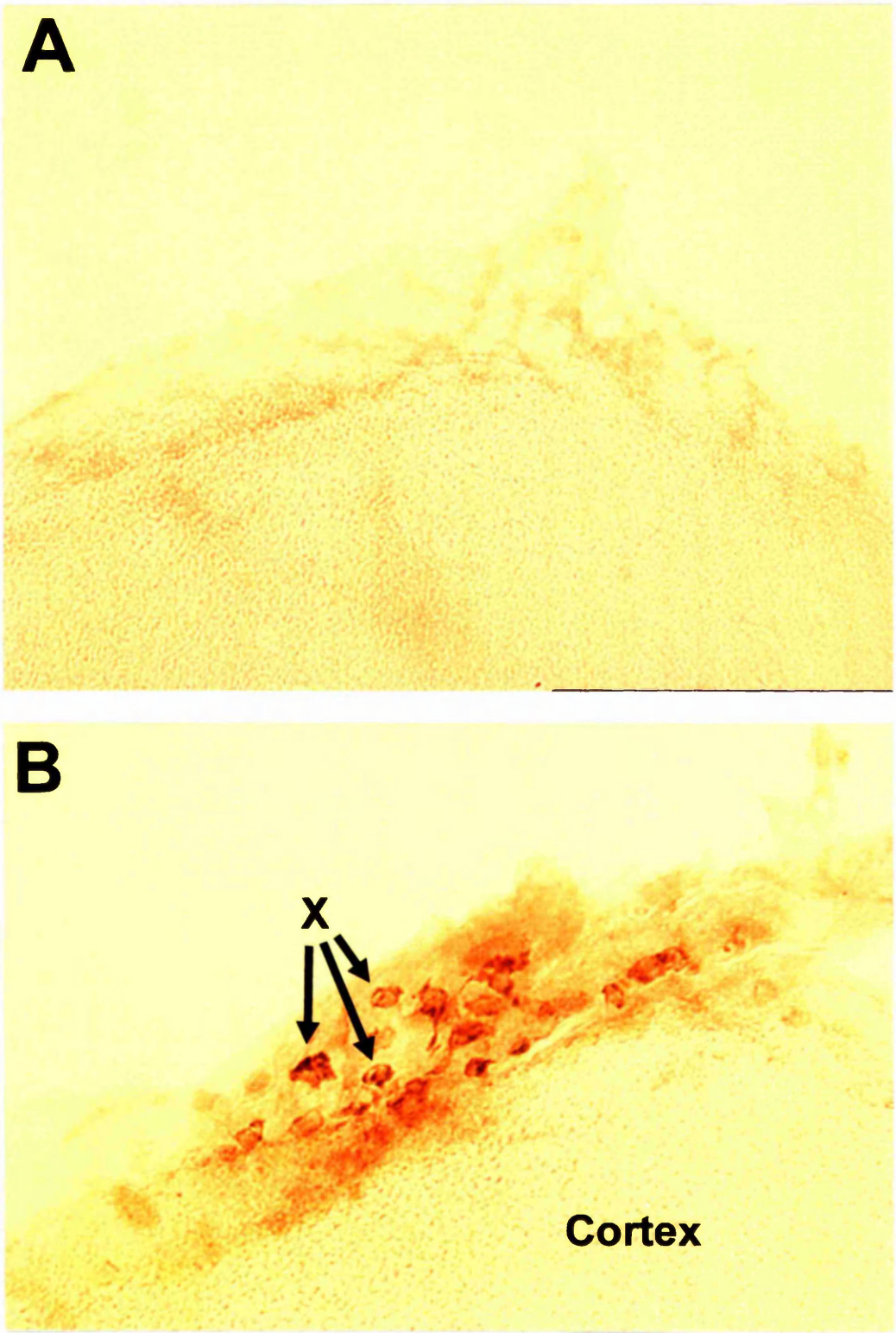


Figure 4.7.4. Expression of irIL-1β meningeal macrophages (X) surrounding the cerebral cortex, 4 h after icv injection of vehicle (A) or 4μg leptin (B)

4.8 Responses to Leptin in IL-6 (-/-) Mice

The evidence presented in this chapter strongly suggests that IL-1 mediates actions of leptin in both rats and mice. IL-1 actions are mimicked and sometimes mediated by IL-6. Therefore, IL-6 may also play a role in mediating leptin actions, as suggested by the increased hypothalamic levels of IL-6 in normal SD rats in response to icv injection of leptin (**Section 4.5**). There is no currently available IL-6 receptor antagonist that may be used to inhibit IL-6-mediated effects. Therefore the involvement of IL-6 in the actions of leptin was investigated in this preliminary experiment using IL-6 deficient mice - IL-6 (-/-). If IL-6 mediates actions of leptin, these mice, like the IL-1RI (-/-) mice, should also be insensitive to leptin.

4.8.1 Experimental Design

IL-6 (-/-) mice or the C57BL6 mice used as controls, were housed in wire-bottomed cages (to allow measurement of food spillage), and injected (icv) with leptin (4μg) or vehicle at 18:00 h. Food intake and body weights were measured 14 h after injection, at the beginning of the subsequent light phase. Core body temperatures were not monitored.

4.8.2 Results

Food intake of vehicle-treated C57BL6 mice (4.6±0.2 g) over 14 h after injection (at 18:00 h) was significantly inhibited (ANOVA: $p<0.001$) by 40% in response to icv injection of leptin (**Figure 4.8.1**). IL-6 (-/-) mice consumed 4.5±0.2 g food over 14 h after vehicle injection. Like C57BL6 mice, IL-6 (-/-) mice treated with leptin also exhibited reduced food intake (by 56%, ANOVA: $p<0.001$). In fact, IL-

6 (-/-) mice exhibited significantly greater sensitivity (40%) to the effects of injection of leptin on food intake compared to that of C57BL6 mice (ANOVA: $p<0.01$).

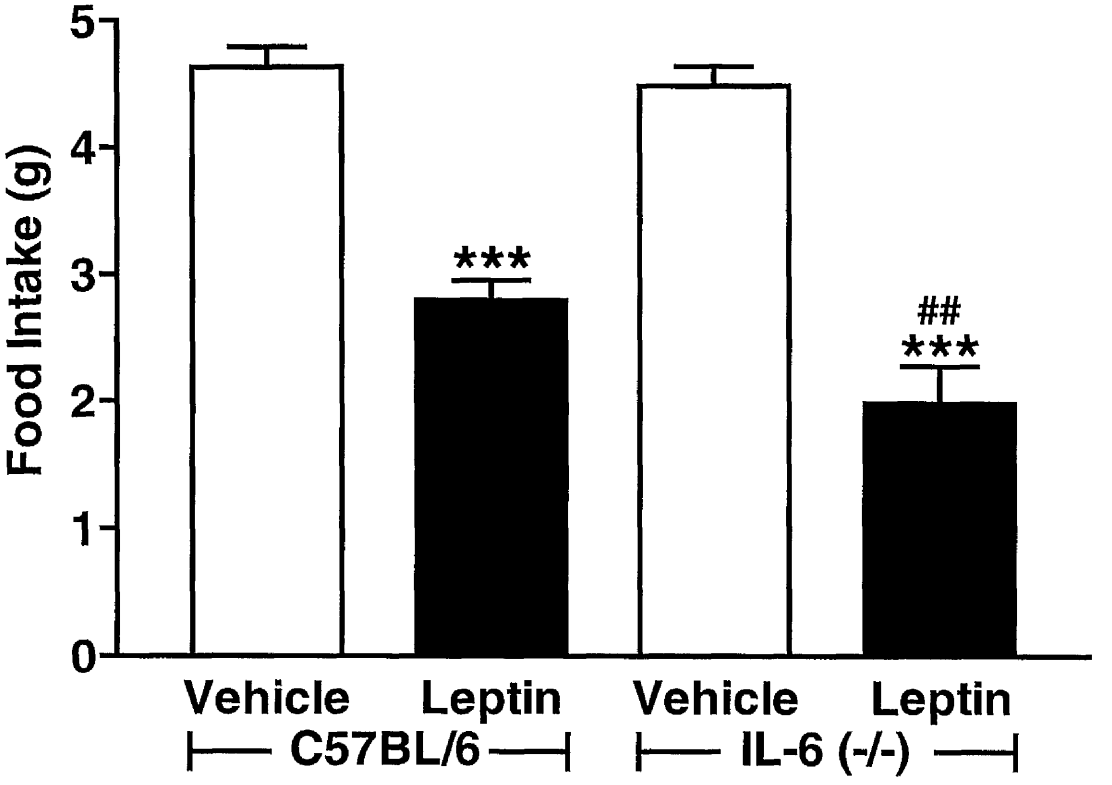


Figure 4.8.1. Food intake of IL-6 (-/-)and C57BL6 mice in response to icv injection of leptin (4 μg)

(ANOVA: *** $p<0.001$ vs Vehicle; ## $p<0.01$ vs C57-Leptin)

Vehicle-treated C57 mice gained 0.5±0.1 g body weight over 14 h after icv injection (**Figure 4.8.2**). Injection of leptin elicited significant (ANOVA: $p<0.001$ vs Vehicle) weight loss in these animals (0.7±0.1 g). IL-6 (-/-) mice gained 0.2±0.1 g body weight of 14 h after injection of vehicle. Administration of leptin

to IL-6 (-/-) mice induced significant (ANOVA: $p<0.001$ vs Vehicle) loss of body weight (1.3±0.2 g). This body weight loss induced by injection of leptin in IL-6 (-/-) mice was significantly greater (by 20%) than that exhibited by C57 mice (ANOVA: $p<0.01$).

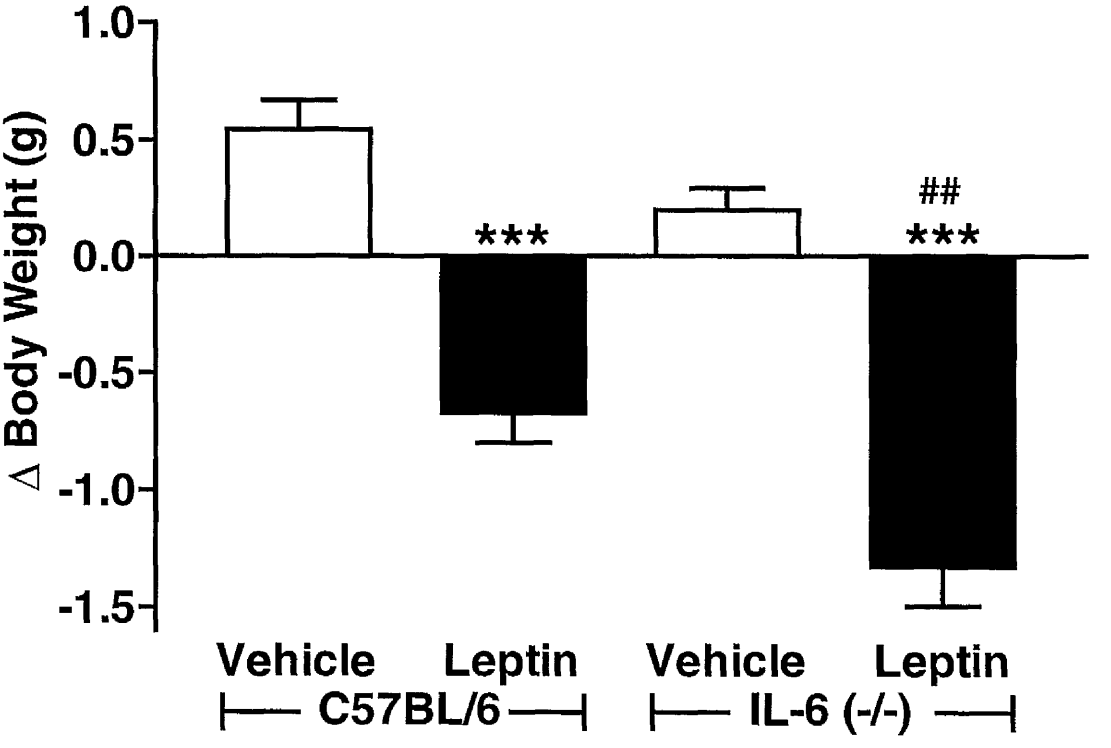


Figure 4.8.2. Changes in body weight of IL-6 (-/-) and C57BL6 mice in response to icv injection of leptin (4 μg)

(ANOVA: *** $p<0.001$ vs Vehicle; ## $p<0.01$ vs C57-Leptin)

Pre-injection body weights of IL-6 (-/-) mice (27.0±0.4 g) were slightly, but significantly less (by 5%) than the body weights (28.2±0.3 g) of C57BL/6 mice (ANOVA: $p<0.05$) (**Figure 4.8.3**).

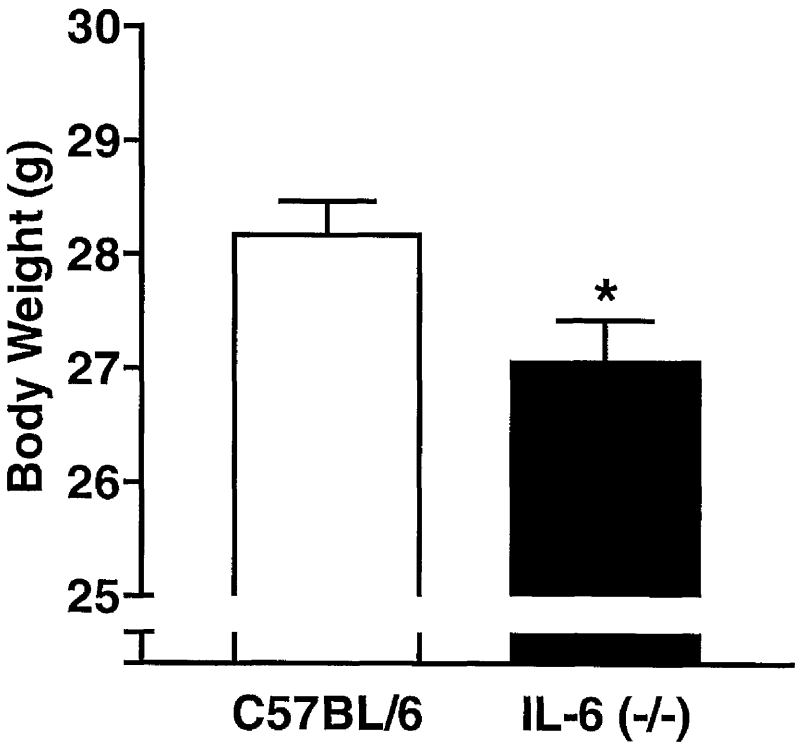


Figure 4.8.3. Pre-injection body weights of IL-6 (-/-)and C57BL6 mice

(t-test: * $p<0.05$)

4.9 SUMMARY

The data presented in this chapter demonstrate that peripheral injection of leptin elicits similar effects on food intake, body weight and body core temperature as central administration, and that these responses are inhibited by injection of IL-1ra to the brain (**Section 4.2-4.3**). Levels of IL-1β and IL-6 were both upregulated in the hypothalami (but not the circulation) of normal Sprague-Dawley rats in response to icv injection of leptin (**Section 4.5**). Administration of leptin (icv) also induced increased hypothalamic levels of irIL-1β in obese Zucker rats that were attenuated compared to levels in lean animals, although neither lean nor obese Zucker rats exhibited detectable IL-6 levels (**Section 4.6**). Immunohistochemistry revealed upregulation of irIL-1β protein expression in ventricular choroid plexus cells, hypothalamic microglia and meningeal macrophages in response to central administration of leptin (**Section 4.7**). **Section 4.4** described that icv injection of leptin failed to induce reduced food intake and body weights in IL-1RI (-/-) mice that lack type I IL-1 receptors. Conversely, IL-6 deficient mice were hypersensitive to the effects of leptin on these parameters (**Section 4.8**).

4.10 DISCUSSION

The experiments described in this chapter are the first to suggest that the effects of leptin on food intake, body weight and core body temperature are mediated by the cytokine IL-1. Previous investigations have reported only the reverse, i.e. that LPS and proinflammatory cytokines such as IL-1, IL-6 and TNF

stimulate the synthesis and release of leptin from adipose tissue both *in vivo* and *in vitro* (Grunfeld *et al.*, 1996; Sarraf *et al.*, 1997; Faggioni *et al.*, 1998; Finck *et al.*, 1998; Berkowitz *et al.*, 1998). Loffreda *et al.* described how LPS-induced phagocytosis and synthesis of TNF, IL-6, IL-12 is upregulated by leptin in *ex vivo* murine macrophages (Loffreda *et al.*, 1998), although alone, leptin failed to induce a response, thereby implicating leptin in the modulation of an activated immune response. Most studies investigating mediators of leptin actions in the brain have been limited to molecules such as NPY, CRF and POMC products (**Section 1.2.6**). Therefore, induction of cytokines by exogenous leptin is a novel observation.

The observation described in **Section 4.2** that central injection of IL-1ra attenuated (but did not completely abolish) effects of leptin on food intake (**Figure 4.2.1**), indicates that IL-1 is only partially responsible for mediating this response, which may also involve molecules such as CRF, NPY and POMC (Sahu, 1998). Conversely, the increase in core body temperature induced by central injection of leptin was totally blocked by IL-1ra (**Figure 4.2.3**), thus suggesting that IL-1 may be solely responsible for mediating this response. The fact that the leptin-induced suppression of body weight gain was also returned to control values by IL-1ra administration (**Figure 4.2.2**) implies that energy utilised in generating leptin-induced hyperthermia also contributes to the weight loss.

Leptin is synthesised and released into circulation from peripheral adipose tissue (Frederich *et al.*, 1995; Auwerx & Staels, 1998). Therefore the changes in food intake and body temperature observed in **Section 4.3** in response to

peripheral (ip) injection of leptin strongly reinforce the suggestion that the responses to central injection of leptin described here are relevant to pathophysiological conditions. Thus endogenous leptin released from adipose tissue may cross the BBB (Banks *et al.*, 1996; Golden *et al.*, 1997; Bjorbaek *et al.*, 1998), or release some secondary factor, or stimulate afferent nerves (Wang *et al.*, 1997) to increase core body temperature and reduce food intake and body weight. However, the responses to peripheral injection of leptin were not as great as responses to central injection, and were delayed, indicating that BBB transport is saturable (Banks *et al.*, 1996; Caro *et al.*, 1996), and requires time to accumulate enough leptin in brain to stimulate pathways involved in increasing core body temperature. Furthermore, the half-life of leptin in circulation may be shorter than in the brain, accounting for the reduced effects observed in response to peripheral injection of leptin. These results demonstrate that responses to leptin are induced via a time-dependent cascade of responses that involve actions of IL-1.

Like the responses to icv injection of leptin (**Section 4.2**), IL-1ra inhibited the reduced food intake (**Figure 4.3.1**), and completely blocked the increased core body temperatures (**Figure 4.3.3**) induced by ip injection of leptin. However, body weight gains in these animals were not fully restored to control levels (**Figure 4.3.2**). Therefore peripherally administered leptin may have direct catabolic actions on peripheral tissues (Bornstein *et al.*, 1997; Siegrist-Kaiser *et al.*, 1997), which were unaffected by the central injections of IL-1ra, thereby causing attenuated body weight gains to persist. Nevertheless, these results indicate further that central release of IL-1 mediates actions of leptin on food

intake and body temperature. However, these experiments using IL-1ra do not distinguish whether effects of leptin are being mediated by IL-1β and IL-1α.

The finding that hypothalamic levels of IL-6 (**Figure 4.5.2**) and especially IL-1β (**Figure 4.5.1**) were both upregulated in response to icv injection of leptin (**Section 4.5**), also supports this hypothesis, and localises actions of leptin to the hypothalamus. Since levels of IL-1 and IL-6 in circulation were below the detection limit of the assay in both leptin and vehicle-treated rats, the presence of IL-1β in the brain could not have been due to its release from peripheral tissues. However, the ELISA technique used does not distinguish between the pro- and mature forms of IL-1β. Therefore, the increased levels of IL-1β detected may have been intracellular pro-IL-1β, demonstrating increased expression of IL-1β in response to leptin, but not whether the mature IL-1β was released to act extracellularly. Nevertheless, studies described in **Sections 4.2-4.3** suggest that IL-1 mediates actions of leptin, and so probably is released in its mature form.

The suggestion that leptin acts on the hypothalamus to induce actions on food intake and energy balance is complemented by studies such as those demonstrating high concentrations of hypothalamic leptin receptors (Tartaglia *et al.*, 1995; Couce *et al.*, 1997; Hakansson *et al.*, 1998; Elmquist *et al.*, 1998); injection of leptin activates hypothalamic neurones (Elmquist *et al.*, 1997; Elmquist *et al.*, 1998; Huang *et al.*, 1998; Powis *et al.*, 1998); and lesions of the hypothalamus induce obesity (Hetherington & Ranson, 1942; Hervey, 1958; Bray *et al.*, 1982) and increase adipocyte *ob* gene expression (Funahashi *et al.*, 1995). Furthermore, the fact that cytokines such as IL-1 and IL-6 have been

reported extensively to act on the hypothalamus to regulate effects on energy balance such as anorexia (Plata-Salaman, 1998) and fever (Kluger *et al.*, 1995) supports the suggestion that leptin and cytokines are related in mediating effects in these paradigms.

It is interesting that although injection of leptin failed to elicit responses on food intake, body weight gain or core body temperature in obese Zucker rats (**Section 3.12**), leptin still induced significant release of IL-1β (**Figure 4.6.1**), albeit significantly reduced in these animals compared to lean Zucker rats (**Section 4.6**). One might expect that the levels of IL-1β observed in the obese rats in response to leptin were sufficient to induce fever. However, results indicate otherwise. These data support the hypothesis that the leptin receptor in the obese Zucker rat is still capable of limited signal transduction (Yamashita *et al.*, 1997; Yamashita *et al.*, 1998). Indeed Cusin *et al.* reported that injection of leptin at high doses still induces reductions in the food intake and body weights of obese Zucker rats (Cusin *et al.*, 1996). Alternatively, Busbridge *et al.* showed that responses to injection of cytokines in the obese Zucker rat are blunted because of the inhibitory effects high serum levels of corticosteroids, and that adrenalectomy restores effects of cytokine administration (Busbridge *et al.*, 1990). Therefore adrenalectomy in animals injected with leptin may remove the suppression of the febrile response mediated by hypothalamic IL-1β.

Other studies have described mainly neuronal activation (Elmquist *et al.*, 1997; Elmquist *et al.*, 1998; Powis *et al.*, 1998), co-localisation or release of neurochemicals such as galanin, MCH, NT, CRF, POMC and NPY (Hakansson *et al.*, 1996; Hakansson *et al.*, 1998; Sahu, 1998; Huang *et al.*, 1998) in the

brain in response to leptin treatment. Hakansson *et al.* also reported leptin receptor immunoreactivity of cells in the choroid plexus, but mainly neurones in layers II-VI of the cerebral cortex, hippocampus, thalamus, hypothalamic nuclei and brainstem (Hakansson *et al.*, 1998). Conversely, the preliminary immunohistochemical study described in **Section 4.7** failed to show leptin-induced expression of irIL-1β in any neuronal cells. However, staining was observed in choroid plexus cells (**Figure 4.7.2**), hypothalamic microglia (**Figure 4.7.3**) (indicating the source of hypothalamic IL-1β detected in **Section 4.4-5**), and meningeal macrophages (**Figure 4.7.4**). The presence of these irIL-1β-expressing cells is similar to the distribution seen during host defence responses to infection (Van Dam *et al.*, 1995). The IL-1β produced by these cells may serve as a signal for adjacent or more distant targets such as neurones, endothelial or microglial cells, to play a role in the induction of responses such as anorexia and fever. The staining observed at the injection site in both vehicle and leptin-treated animals represents a non-specific response to injection (**Figure 4.7.1**).

IL-1ra blocks all known actions of IL-1, and has no other reported biological action. Nevertheless, further support for the hypothesis that effects of leptin are dependent on endogenous IL-1 is provided by the results of the preliminary investigation described in **Section 4.4**, using IL-1RI (-/-) mice. Central injection of leptin inhibited food intake (**Figure 4.4.1**) and body weight (**Figure 4.4.2**) in normal C57BL/6 mice, but had no effect in mice with the IL-1 type I receptor gene deleted. In contrast, results described in **Section 4.8** suggested hypersensitivity to the effects of leptin on food intake (**Figure 4.8.1**) and body

weight (**Figure 4.8.2**) in IL-6-deficient mice. These results are discussed below. Unfortunately facilities at this establishment were not available for the accurate measurement of core body temperature in mice (Kozak *et al.*, 1994), and so the effect of leptin on this response was omitted. However, in contrast to IL-6 (-/-) mice (**Figure 4.8.3**), it was observed that the pre-injection body weights of the IL-1RI (-/-) mice (**Figure 4.4.3**) were significantly greater than their age-matched controls. This observation is also apparent in aged mice as depicted below.

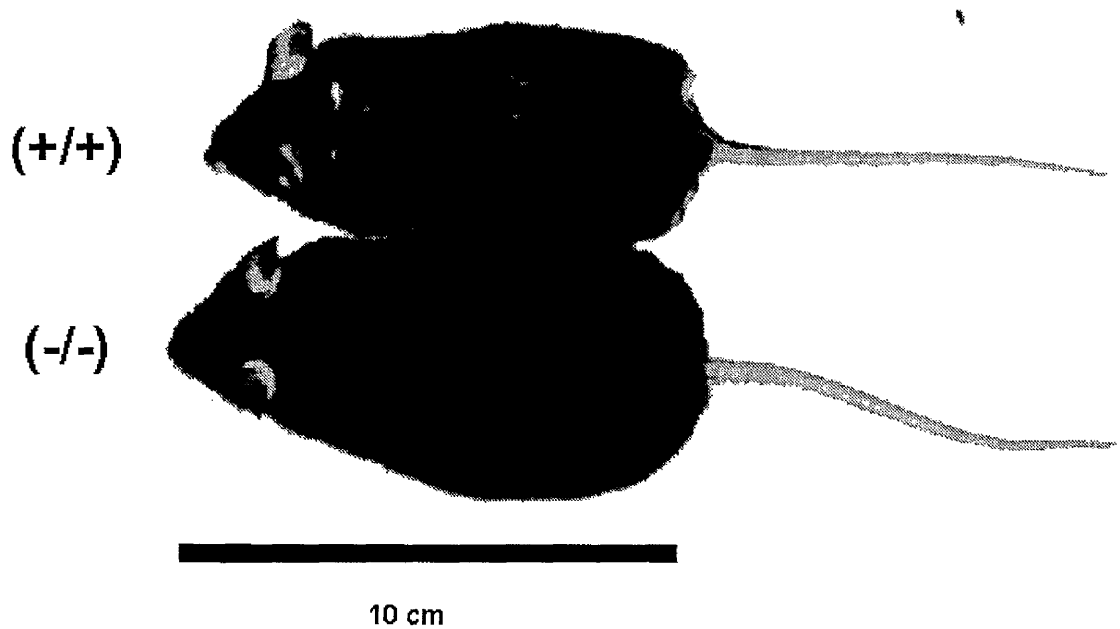


Figure 4.10.1. Photograph of aged (18 month old) IL-1RI (-/-) and C57BL/6 mice

Since IL-6 is a widely accepted mediator of host defence responses such as fever and hypophagia (Akira *et al.*, 1993; Rothwell *et al.*, 1996; Moldawer & Copeland, 1997; Matthys & Billiau, 1997), the data indicating that mice lacking IL-6 exhibit exacerbated hypophagia in response to icv injection of leptin

(**Section 4.8**) is confusing. One possible explanation may be that IL-6 is an endogenous *inhibitor* of leptin actions in the brain. Therefore central injection of leptin would have a greater effect on body weight and food intake in animals lacking the limiting effects of IL-6. This hypothesis is supported by *in vitro* and *in vivo* studies suggesting that IL-6 possesses anti-inflammatory properties (Tilg *et al.*, 1994; Tilg *et al.*, 1997). IL-6 suppresses LPS-induced neutrophil exudate (Ulich *et al.*, 1991) and TNF production (Mizuhara *et al.*, 1994). IL-6 anti-serum completely blocks IL-1ra synthesis in response to bacterial infection (Jordan *et al.*, 1995). Furthermore, IL-6 (-/-) mice produce threefold more TNF-α compared to control animals in response to LPS (Fattori *et al.*, 1994), indicating that IL-6 may exert a protective effect during inflammation. Alternatively, IL-6 (-/-) mice may have upregulation of the IL-6 receptor, including the increased expression of the gp130 protein associated with both IL-6 and leptin receptors, which may result in increased sensitivity to leptin.

The results presented here indicate that leptin causes release of IL-1 and IL-6 in the brain, which may be involved in effects of leptin on food intake and body temperature by stimulatory and inhibitory mechanisms respectively (**Figure 4.10.2**). However, data presented in **Chapter 3** indicate that the actions of leptin on appetite and body temperature appear to depend on separate mechanisms, since only the latter involves release of cyclo-oxygenase products.

Thus, leptin may act as an important mediator of neuroimmune actions, and could serve as a major circulating afferent signal for activation of responses to disease such as fever and loss of appetite. Indeed, there is recent evidence that leptin regulates immune responses (phagocytosis) as well as cytokine

expression (Loffreda *et al.*, 1998). Furthermore, a recent study (Esler *et al.*, 1998) suggests that the brain could also be a major source of circulating leptin, prompting the hypothesis that leptin or leptin-like analogues may also act as neurochemical agents regulating energy balance in the brain.

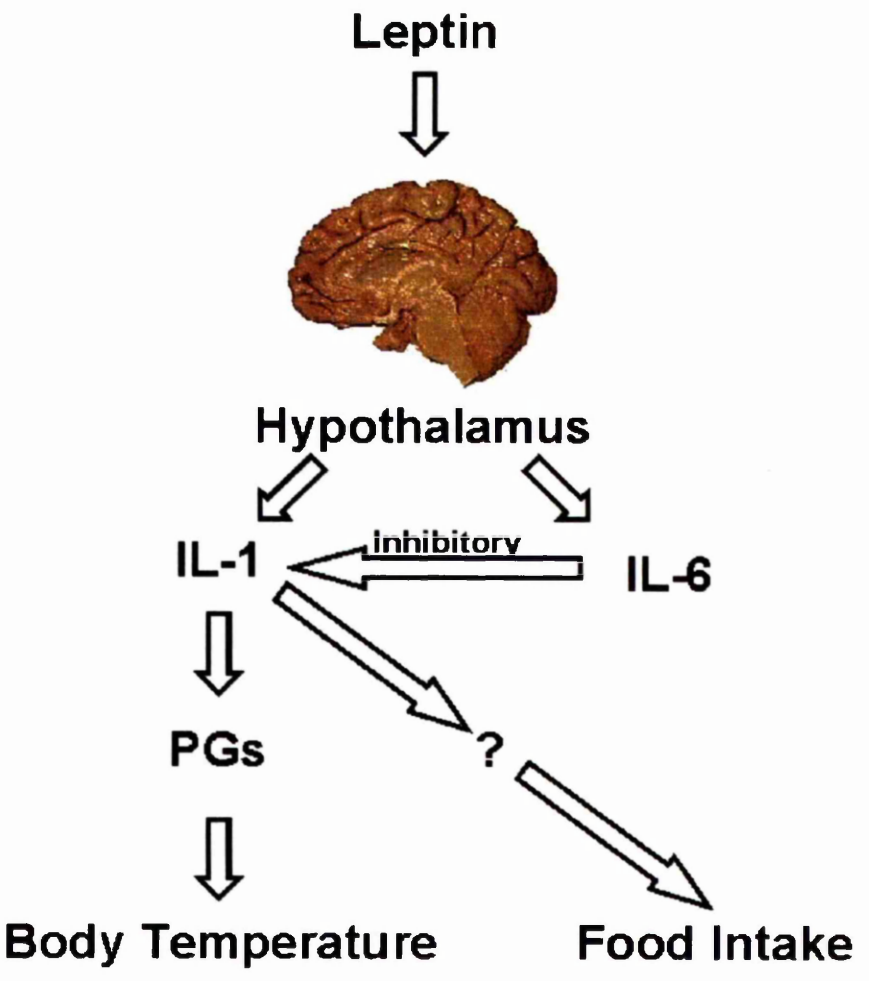


Figure 4.10.2. Schematic diagram showing potential mechanisms of leptin actions on food intake and body temperature via release of IL-1β and IL-6 in the hypothalamus

Chapter 5

Leptin and CRF

5.1 INTRODUCTION

Several mechanisms of action of leptin in the brain have been identified or postulated (Sahu, 1998). One of these includes a possible relationship between leptin and CRF. Neuronal activation (measured by induction of c-fos expression) has been reported in the PVN (the major site of CRF synthesis) in response to an intracerebroventricular (icv) injection of leptin (Elmquist et al., 1998). Leptin also induces expression of CRF mRNA in the same area of the hypothalamus (PVN) (Schwartz et al., 1996), and leptin receptors are present on CRF containing neurones (Hakansson et al., 1998). However, there are few studies describing the relationship between leptin and CRF in physiological and behavioural responses.

CRF, administered icv in the rat, causes marked increases in metabolic rate and core body temperature - responses which are ascribed to the activation of the sympathetic nervous system and subsequent increases in heat production in brown adipose tissue (BAT) (Rothwell, 1990b; Rothwell, 1994). Previous studies have administered CRF over a wide range of doses to elicit various responses in rats. Infusion (icv) of 0.1-5 μg CRF increases sympathetic nerve activity (Katafuchi et al., 1997). CRF (0.03, 0.1, 0.3, and 1 μg, icv) has been reported to induce dose-dependent increases in heart rate, core temperature and activity after only 5 min (Diamant & De Wied, 1991). Anorexia and increased BAT mitochondrial guanosine diphosphate (GDP)-binding (an index of thermogenic activity) was observed after 30 min in response to icv injection of 5 μg CRF (Arase et al., 1988). Morimoto *et al* reported that blood pressure,

heart rate and locomotor activity was increased in response to injection (icv) of 1 or 10 μg CRF (Morimoto et al., 1993). Oxygen consumption and metabolic rate has been reported to be significantly increased by icv injection of 2 nmol (~10 μg) CRF (Rothwell et al., 1991), and by 4-4.7 μg CRF (Rothwell, 1989; Rothwell, 1990a; Strijbos et al., 1992). Furthermore, increases in interscapular BAT temperature and GPD-binding were seen in response to icv injection of 2-5 nmol (~10-25 μg) CRF (LeFeuvre et al., 1987).

Endogenous CRF has also been proposed to mediate pyrogenic responses to cytokines such as IL-1β, IL-6 and IL-8, prostaglandins, and peripheral injury or infection (Rothwell, 1990c; Rothwell & Hopkins, 1995). The effects of these cytokines on oxygen consumption, core temperature and BAT activity in rats are attenuated or blocked by icv administration of CRF receptor antagonists or a neutralising antibody to CRF (Busbridge et al., 1989; Rothwell, 1989; Rothwell, 1990a).

These data suggest an important role for CRF in thermoregulatory responses to disease, though they do not indicate whether CRF acts as a pyrogen, to raise thermoregulatory set point, or modifies effector mechanisms in response to changes in set point. In contrast, some studies suggest that CRF can *inhibit* pyrogenic responses to cytokines (Bernardini et al., 1984; Opp et al., 1989). These apparently conflicting data may be ascribed to species differences between studies, although there are reports of antipyretic or hypothermic actions of CRF (Sausen et al., 1996) or CRF-like neuropeptides in the rat (Broccardo, 1990; Broccardo & Improta, 1994).

Ambient temperature has a major influence on thermoregulation and energy balance (Gwosdow & Besch, 1985). Thermoneutrality is defined as the range of ambient temperatures where resting metabolic rate is minimum and constant. For rats housed individually, this range is between 28 and 32°C (Swift & Forbes, 1939; Herrington, 1940). However, at these ambient temperatures, body temperature is elevated, and rats exhibit behavioural changes associated with heat loss (Hainsworth, 1968; Hellstrom, 1975). The rat has a range of preferred ambient temperatures between 19 and 31°C (Ettenberg & Carlisle, 1985; Gordon, 1987; Gordon, 1990; Gordon et al., 1991). In addition, Poole and Stephenson (Poole & Stephenson, 1977) define a range of 18-28°C, when animals can display 'normal' activity, i.e. perform behavioural thermoregulation. Previously, studies involving actions of CRF have been performed over a range of ambient temperatures (22-26°C), and this may be the cause of some of the discrepancies observed in the literature.

Earlier research into the effects of CRF on core body temperature were conducted using a colonic probe thermocouple on animals housed in groups, or in metabolic chambers for the measurement of oxygen consumption (Lefeuvre et al., 1989; Rothwell, 1990a). Although animals are handled daily before such an experiment in order to minimise the stress associated with insertion of a colonic probe, the level of intervention is such that animals may display stress responses that mask the effects of any injected substances. This problem could be addressed by the use of remote radiotelemetry. This method, introduced in 1985 (Gallaher et al., 1985), allows undisturbed monitoring of free moving animals, and has since become the preferred method over colonic probe

(Clement et al., 1989). Remote radiotelemetry requires that animals be housed individually, since each receiver can detect only the output from a single transmitter. Because rats are social animals, and normally housed in groups, this restriction may affect results.

The objectives of the studies described in this chapter were to investigate the relationship between leptin and CRF by the use of the CRF receptor antagonist D-Phe CRF_{12-4} (Menzaghi et al., 1994). In addition, the discrepancies in the literature concerning responses to CRF were investigated.

5.2 Effects of CRF Receptor Antagonist on Actions of Leptin

Although previous studies have shown activation of CRF neurones, and CRF release in response to injection of leptin (Schwartz et al., 1996; Raber et al., 1997; Hakansson et al., 1998), physiological actions of leptin are yet to be linked causally to CRF. The CRF receptor antagonist D-Phe CRF_{12-41} has been shown to inhibit physiological responses that are dependent on CRF in rats. D-Phe CRF_{12-41} (0.2-5 μg, icv) inhibits defensive behaviour responses to stressful conditions (Rodriguez et al., 1996), reduces CRF-induced locomotor activity (Menzaghi et al., 1994), hypotension, tachycardia, and noradrenaline release (Rivier et al., 1993), and delays CRF-induced seizures (Baram et al., 1996).

Therefore effects of injection of doses of D-Phe CRF_{12-41} similar to those described above in rats were tested in responses to leptin on food intake, body weight and core body temperature.

5.2.1 Experimental Design

Animals were injected (icv) at 18:00 h with vehicle or leptin (4 μg), and with a dose (1, 2.5, or 5 μg, icv) of D-Phe CRF_{12-41} or vehicle. Food intake, body weight and core body temperature were measured over the following 14 h - until the beginning of the subsequent light phase.

5.2.2 Results

Food intake (**Figure 5.2.1**) was significantly reduced by 50% (ANOVA: $p<0.001$) over 14h in response to icv injection of leptin, compared to vehicle-treated animals (28.2±0.8 g). Central injection of all doses of D-Phe CRF_{12-41} alone did

not significantly affect food intake (ANOVA). Co-administration of leptin and D-Phe CRF_{12-41} however, attenuated the suppressed food intake observed in leptin-treated rats in a dose-dependant fashion. The lowest dose (1 μg) of antagonist did not significantly affect leptin hypophagia, but the higher doses (2.5 and 5 μg) of D-Phe CRF_{12-41} significantly attenuated effects of leptin on food intake by 58% (ANOVA: $p<0.001$), such that food intake of these animals was restored to within 80% of the intake of vehicle-treated animals.

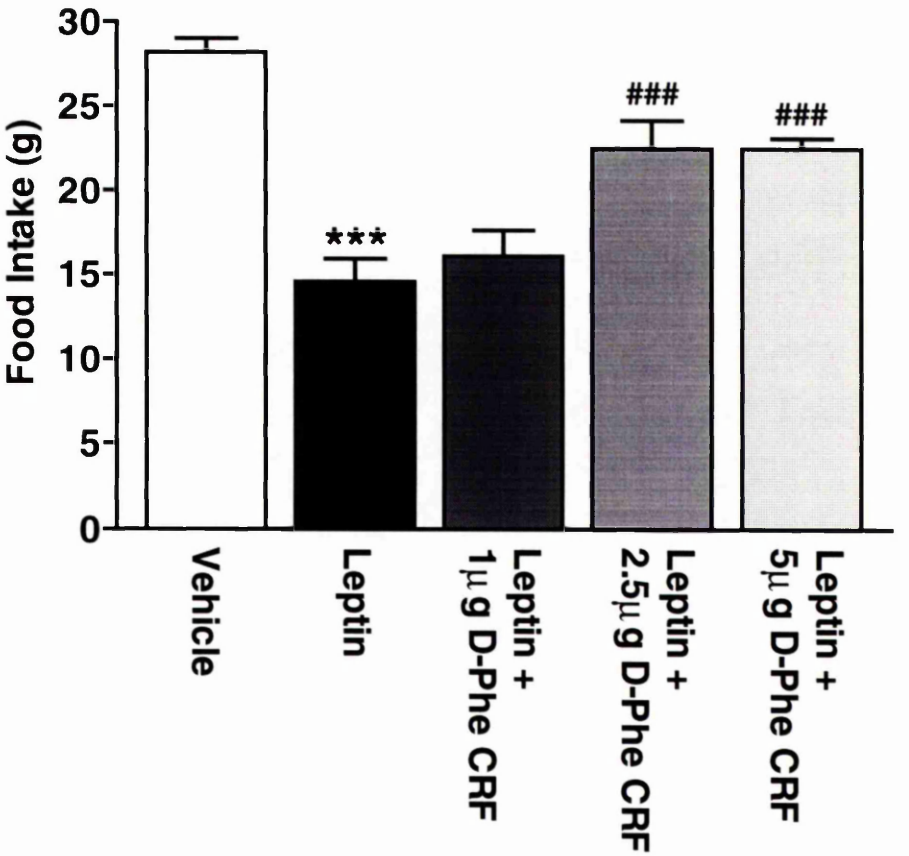


Figure 5.2.1. Food intake over 14 h in response to co-injection (icv) of leptin (4 μg) and D-Phe CRF_{12-41} (1, 2.5 or 5 μg) at 18:00 h

(ANOVA: $^{***}p<0.001$ vs Vehicle; $^{###}p<0.001$ vs Leptin)

In the same animals, body weight (**Figure 5.2.2**) was measured over the 14 h after icv injection of leptin and D-Phe CRF_{12-41}. Leptin significantly reduced body weight (by 12±3 g) in comparison to the weight gain (18±1 g) observed in vehicle-treated rats (ANOVA: $p<0.001$). This weight loss induced by central injection of leptin was significantly inhibited (ANOVA: $p<0.001$) by co-injection of all doses of antagonist. Animals co-injected with the lower doses (1 and 2.5 μg) of D-Phe CRF_{12-41} lost 1±3 g and 0±3 g respectively. The higher dose (5 μg) of antagonist partially reversed leptin-induced weight loss such that animals gained 6±3 g.

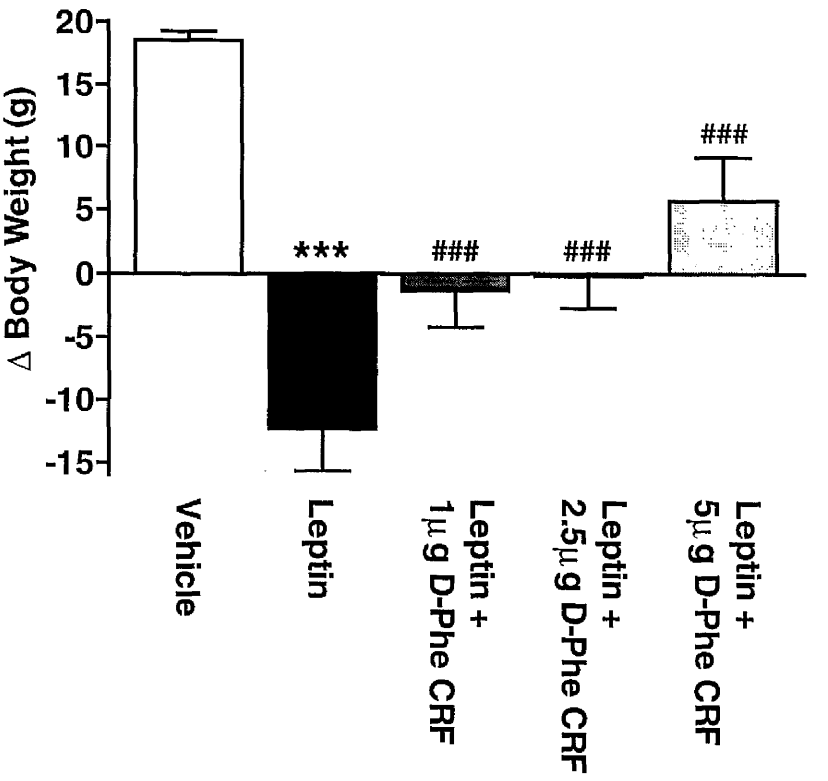


Figure 5.2.2. Changes in body weight in response to injection (icv) of leptin (4 μg) and D-Phe CRF_{12-41} (1, 2.5 or 5 μg) at 18:00 h

(ANOVA: $^{***}p<0.001$ vs Vehicle; $^{###}p<0.001$ vs Leptin)

Increase in core body temperature induced by central injection of leptin was unaffected by the CRF receptor antagonist, which itself did not affect the body temperature of animals (data not shown).

5.3 Dose Response Study on the Effects of CRF on Core Body Temperature

Results described in the above section suggest that in contrast to the role of PGs (**Section 3.13**), CRF is involved in mediating actions of leptin on food intake and body weight, but not core body temperature. Previous reports suggest that CRF is involved in the activation of thermogenic pathways and the development of fever (Rothwell, 1989; Rothwell, 1990b; Rothwell & Cooper, 1992). However, some papers report that CRF inhibits febrile responses (Bernardini et al., 1984; Rothwell, 1989; Opp et al., 1989; Rothwell & Cooper, 1992). Therefore, to clarify the influence of CRF in thermoregulation, the study described here investigated the effects of different doses of icv injection of CRF alone on core body temperature.

5.3.1 Experimental Design

CRF (0.3, 3 or 30 μg) obtained from the Salk Institute for Biological Studies (USA) or vehicle were injected (icv) at 10:00 h (0 h), in animals housed at an ambient temperature of 22°C. Core body temperature was monitored for 6 h after injection.

A 3 μg dose of CRF was also injected ip, sc, or iv, and core body temperature monitored by remote radiotelemetry.

5.3.2 Results

Core body temperatures of rats were not significantly affected by icv injection of 0.3 μg CRF (**Figure 5.3.1**). Injection of 3 μg CRF caused a rapid (within 0.5 h) and reproducible decline in temperature (MANOVA: $p<0.05$ vs Vehicle), to a nadir (1.0°C below control) 1.5 h after injection (ANOVA: $p<0.01$ vs Vehicle and 0.3 μg CRF), which returned to control values after 3 h. Similar results were obtained in response to injection of 1 μg CRF (data not shown). A higher dose of CRF (30 μg) elicited hypothermia of a greater magnitude (2°C below control, 2 h after injection; ANOVA: $p<0.001$ vs Vehicle and 0.3 μg CRF, $p<0.05$ vs 3 μg CRF), which was sustained for 6 h (MANOVA: $p<0.001$ vs Vehicle).

Similar results were obtained using CRF from alternative sources (Sigma, UK; Peninsula Laboratories Inc., USA; data not shown). In all further experiments, 3 μg CRF was used to elicit hypothermia. Furthermore, peripheral injection (ip, sc, or iv) of 3 μg CRF failed to elicit any change in core body temperature.

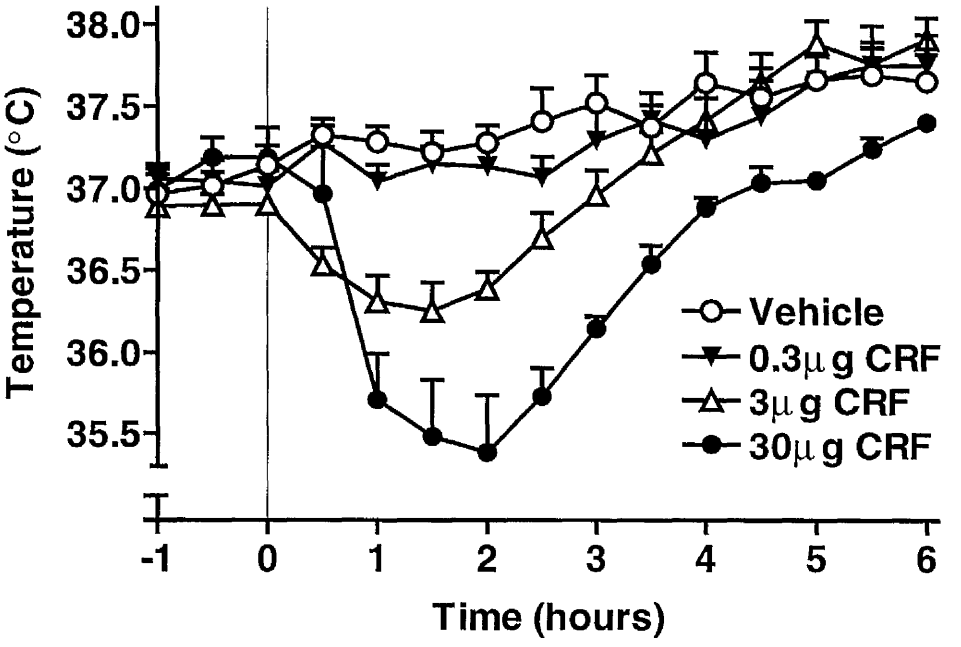


Figure 5.3.1. Dose-response study of CRF (0.3, 3 and 30 μg, icv) on core body temperature

5.4 Influence of Housing and Method of Temperature Measurement on CRF Effects on Core Body Temperature

The results obtained in the previous investigation, describing hypothermic actions of CRF, markedly contradict with the generally accepted role of CRF as a mediator of thermogenesis and fever (Rothwell, 1990b). It was hypothesised therefore that the differing responses observed here may be a result of methodology and housing. Most previous studies were performed using a colonic probe rather than radiotelemetry to measure core body temperature (LeFeuvre et al., 1987; Rothwell, 1989) - a procedure that may elicit a stress response sufficient to affect actions of CRF. Furthermore, both methods of temperature measurement normally require individual housing of the rats, which

are social animals and are usually housed in groups. This may also elicit stressful responses sufficient to affect actions of endogenous CRF. Alternatively, the huddling behaviour exhibited by group housed animals which can maintain raised microclimate temperature may be lost during individual housing, and affect responses to injection of CRF.

Therefore, this study was designed to investigate the effects of altering the method of temperature measurement (colonic probe or remote radiotelemetry), housing conditions (individual or group housed), or ambient temperature (to simulate huddling temperatures) of the animals injected icv with a midrange dose (3 μg) of CRF.

5.4.1 Experimental Design

CRF (3 μg) or vehicle was injected (icv) at 10:00 h (0 h) in rats housed individually or in groups, while core body temperature was monitored by remote radiotelemetry or colonic probe at either 22 or 26°C:

i. Individual housing plus colonic probe

ii. Group housing plus telemetry

iii. Group housing plus colonic probe

iv. Individual housing plus telemetry at 26°C

v. Individual housing plus colonic probe at 26°C

5.4.2 Results

Injection of 3 μg CRF (icv) induced significant hypothermia (MANOVA: $p<0.05$ vs Vehicle) in rats monitored using colonic probe (**Figure 5.4.1A**), similar to that seen in studies described in the previous section (**5.3**) in which body temperature was measured by remote radiotelemetry. Core body temperature declined after 0.5 h, reaching a nadir (1°C below control) at the 1.5 h time point (t-test: $p<0.001$ vs Vehicle), before returning to control values after 3.5 h.

Injection of CRF (3 μg, icv) in rats housed in pairs in small cages, also induced significant hypothermia (MANOVA: $p<0.05$ vs Vehicle) when monitored by remote radiotelemetry (**Figure 5.4.1B**). Core body temperature declined within 0.5 h to a minimum temperature (1°C below control) 1 h after injection (t-test: $p<0.001$ vs Vehicle), before returning to control values after 4 h.

In animals housed in their original home cages (5-6 rats per cage), administration of CRF (3 μg, icv) elicited significant changes in temperature (MANOVA: $p<0.01$ vs Vehicle) (**Figure 5.4.1C**) when measured by colonic probe, which contrasted with those observed previously. As before, core body temperature declined within 0.5 h after injection, to a nadir 0.5°C below control (t-test: $p<0.05$ vs Vehicle) at the 2 h time point. Subsequently, body temperatures of the animals rose dramatically to reach a plateau after 4 h, 1.5°C above control (t-test: $p<0.001$ vs Vehicle).

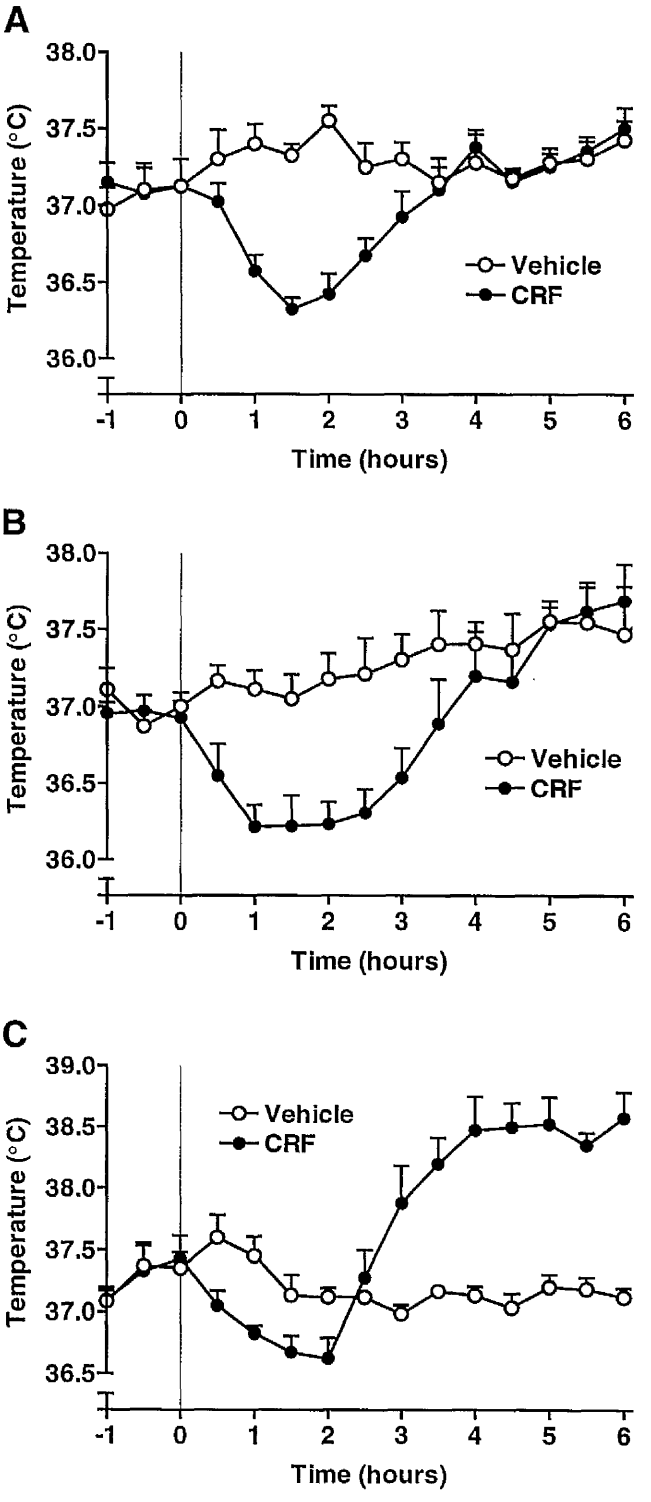


Figure 5.4.1. Core body temperatures of rats injected (icv) with 3 μg CRF and (A) housed individually plus colonic probe, (B) housed in pairs (small cages) plus telemetry, or (C) housed in home cages (5-6/cage) plus colonic probe

In animals housed at an ambient temperature of 26°C, administration of CRF (3 μg, icv) induced changes in core body temperature when monitored by remote radiotelemetry, that were not significantly different between treatment groups when analysed over the whole 6 h time course (MANOVA) (**Figure 5.4.2A**). However, analysis of treatment by time, revealed significant changes (MANOVA: $p<0.001$ vs Vehicle): core body temperature declined within 0.5 h after injection, to a nadir 1°C below control (t-test: $p<0.05$ vs Vehicle) at the 1 h time point, before returning to control values 2 h after injection. MANOVA over this time period revealed significant difference ($p<0.05$ vs Vehicle). Subsequently, body temperatures of animals treated with CRF continued to rise (MANOVA: $p<0.05$ vs Vehicle) to peak (1.5°C above control) at the 6 h time point (t-test: $p<0.001$ vs Vehicle).

In the same experiment, animals injected with 3μg CRF (icv) did not exhibit hypothermia when core body temperature was monitored by colonic probe (**Figure 5.4.2B**). However, CRF induced hyperthermia (MANOVA: $p<0.01$ vs Vehicle) that was maximal 5.5 h after injection (1.5°C above control; t-test: $p<0.001$ vs Vehicle), and was maintained for the remainder of the 6 h experiment.

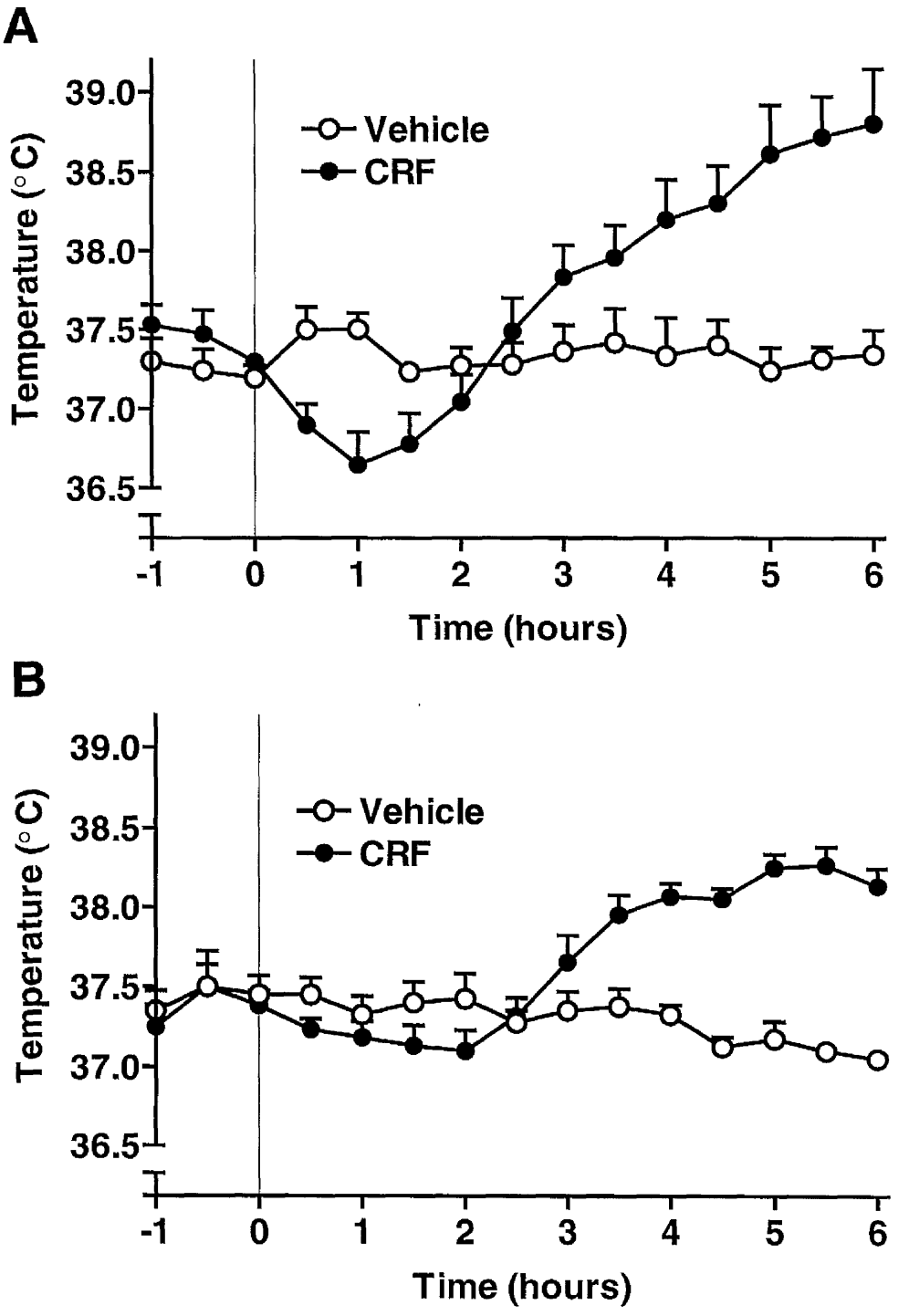


Figure 5.4.2. Core body temperatures measured by (A) telemetry, or (B) colonic probe, in rats housed at 26°C ambient temperature, and injected (icv) with 3 μg CRF

5.5 Influence of Ambient Temperature on Core Body Temperature Responses to Injection of CRF

The studies described in the previous section illustrate the importance of experimental conditions in temperature responses to injection of CRF. Perhaps the most interesting finding is that the core body temperature responses to CRF administration appear to be different at normal room temperature (22°C; **Section 5.3**) and at higher ambient temperatures (26°C; **Section 5.4**) when measured by radiotelemetry or colonic probe. This study investigated further the influence of ambient temperature on effects of CRF on core body temperature.

5.5.1 Experimental Design

In separate experiments, rats were housed individually at ambient temperatures of 22, 24, 26, or 28°C for 24 h before and after injection (icv) of a hypothermic dose (3 μg) of CRF or vehicle at 10:00 h (0 h). Core body temperatures were monitored by remote radiotelemetry.

5.5.2 Results

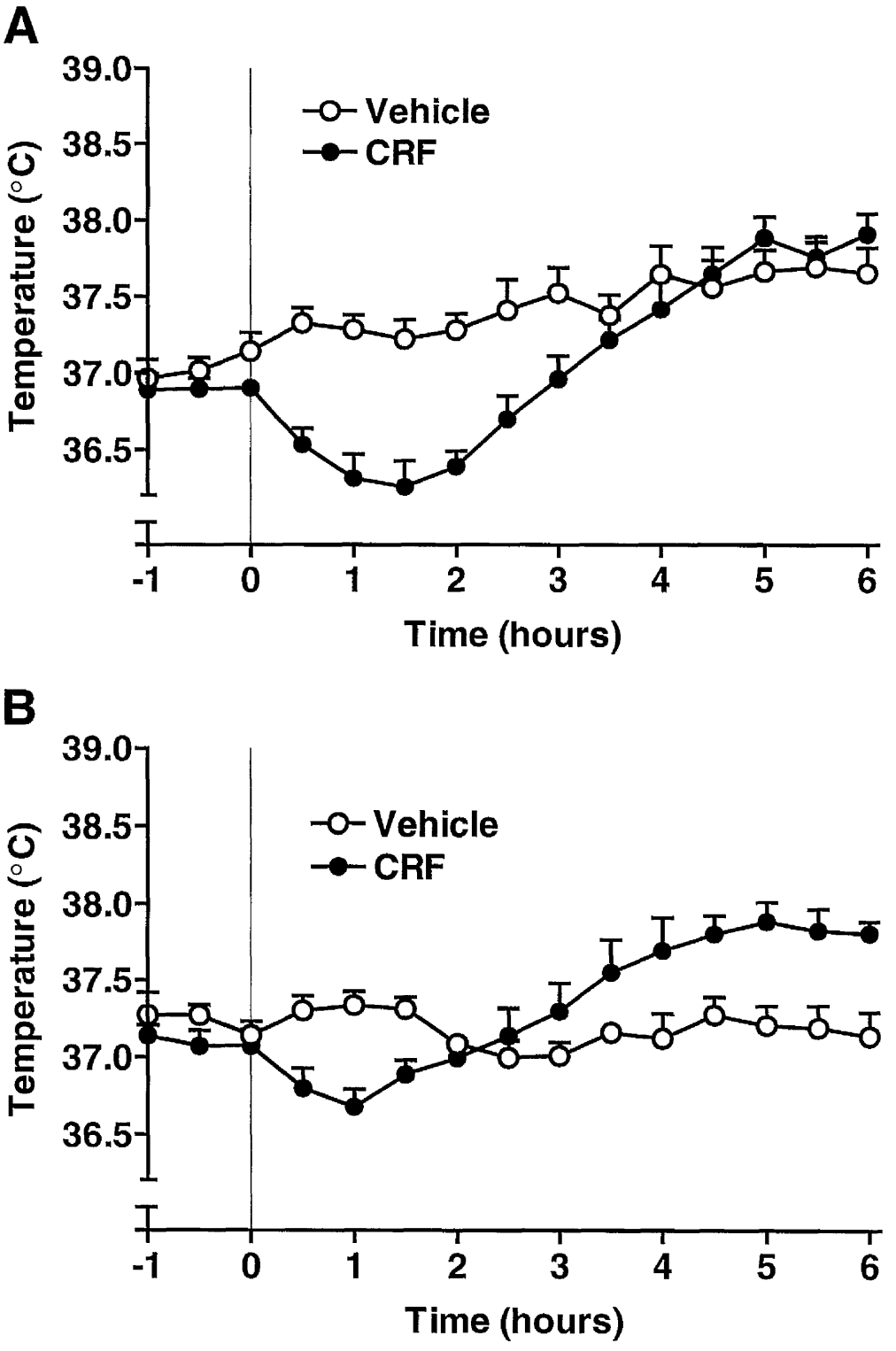
At 22°C, injection of CRF elicited hypothermia (MANOVA: $p<0.05$ vs Vehicle) as seen in the previous section (**5.3**). Body temperatures of rats declined within 0.5 h to a nadir (1.0°C below control) 1.5 h after injection (t-test: $p<0.01$ vs Vehicle), which returned to control values after 4 h (**Figure 5.5.1A**).

CRF administration to rats housed at 24°C induced modest (0.7°C below control; t-test: $p<0.01$ vs Vehicle), transient (2 h), but significant (MANOVA: $p<0.001$ vs Vehicle) hypothermia (**Figure 5.5.1B**). This was followed by an

increase in body temperature above vehicle-treated animals (MANOVA: $p<0.05$), which was maximal (0.7°C above control) at the 5 h time point (t-test: $p<0.01$). A similar response was observed after injection of CRF in rats housed at 26°C (data not shown).

Animals housed at 28°C did not exhibit hypothermia in response to CRF (**Figure 5.5.1C**). In contrast, core body temperature of animals increased 1 h after injection and rose to a maximum value (1.6°C above control; t-test: $p<0.001$ vs Vehicle) 6 h after treatment (MANOVA: $p<0.001$ vs Vehicle).

The results of these experiments are displayed as deviation from mean control temperatures, and are depicted together to allow visual comparison between experiments (**Figure 5.5.1D**).


A
39.0
38.5
38.0
37.5
37.0
36.5
Temperature (°C)
Vehicle
CRF
-1
0
1
2
3
4
5
6
Time (hours)
B
39.0
38.5
38.0
37.5
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Temperature (°C)
Vehicle
CRF
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Time (hours)

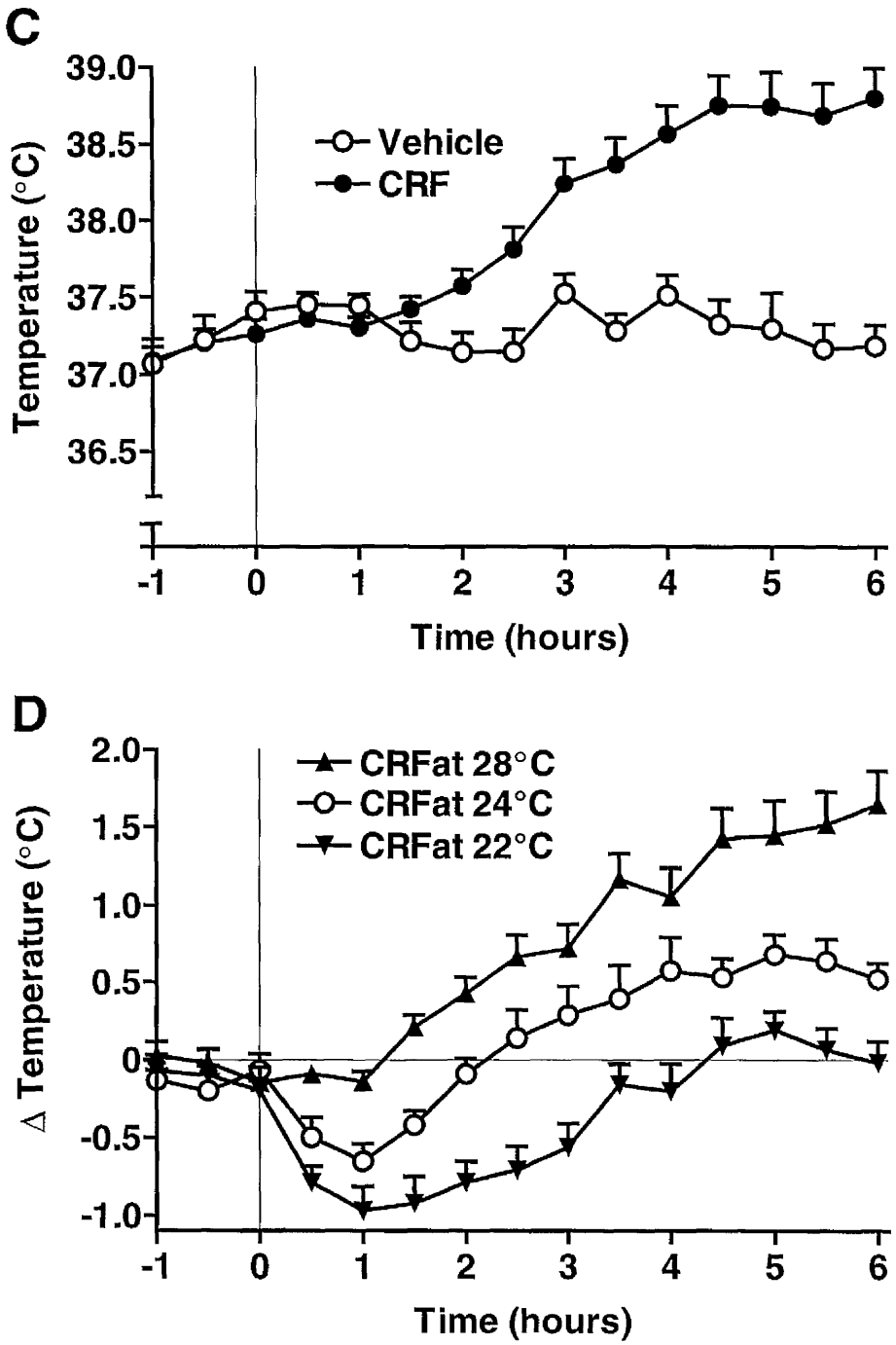

Figure 5.5.1. Core body temperatures of rats housed individually, injected with CRF (3 μg, icv) at ambient temperatures of (A) 22°C, (B) 24°C, or (C) 28°C; deviation of body temperatures from mean control temperature are depicted in (D)

5.6 Effect of CRF Receptor Antagonist on CRF-Induced Changes in Core Body Temperature

Experiments described previously (**Section 5.2**) revealed that the CRF receptor antagonist D-Phe CRF_{12-41} failed to affect leptin-induced changes in core body temperature. Moreover, preliminary studies found injection (5 μg, icv) of this compound ineffective on CRF-induced hypo- or hyperthermia. Therefore in this study, an alternative CRF receptor antagonist, α-helical CRF_{9-41}, injected icv at a higher dose, similar to those administered in previous studies (Rothwell, 1989; Rothwell, 1990a; Richter & Mulvany, 1995), was co-administered with CRF.

5.6.1 Experimental Design

CRF (3 μg) or vehicle was injected (icv) with the CRF receptor antagonist, α-helical CRF_{9-41} (30 μg) or vehicle at 10:00 h (0 h), in animals housed at 22°C or 28°C.

5.6.2 Results

In animals housed at an ambient temperature of 22°C, injection of CRF (3 μg, icv) induced hypothermia (MANOVA: $p<0.05$ vs Vehicle), which was maximal 1.5 h later (0.5°C below control; ANOVA: $p<0.05$) and sustained for 3 h (**Figure 5.6.1A**). Hypothermia over this time period was abolished (MANOVA: $p<0.001$) by concomitant injection (icv) of α-helical CRF_{9-41} (30 μg), so that the response of animals receiving both CRF and α-helical CRF_{9-41} did not differ significantly from vehicle-treated animals (MANOVA). However, injection of the antagonist alone caused marked and sustained (6 h) hyperthermia (MANOVA: $p<0.001$ vs

Vehicle), which peaked at the 3 h time point (1°C above control; ANOVA: $p<0.01$). MANOVA (treatment by time) revealed no significant difference between the shapes of the response to CRF, and CRF plus α-helical CRF_{9-41}.

In contrast (**Figure 5.6.1B**), injection of CRF (3 μg, icv) induced hyperthermia in animals housed at 28°C (as shown previously in **Section 5.5**). Body temperature began to rise 2 h after injection of CRF, was maximal at the 5.5 h time point (1°C above control; ANOVA: $p<0.001$), and was sustained over the 6 h time course (MANOVA: $p<0.05$ vs Vehicle). Injection (icv) of α-helical CRF_{9-41} (30 μg) alone also induced hyperthermia (as seen above, at 22°C) after 0.5 h, reaching a peak value 3 h after injection (1°C above control; ANOVA: $p<0.001$), before declining towards the 6 h time point (MANOVA: $p<0.01$ vs Vehicle). Simultaneous injection of CRF and α-helical CRF_{9-41} induced hyperthermia that showed a similar initial rise to that induced by α-helical CRF alone, but then continued to rise in parallel with the response to CRF (approximately 0.5°C above CRF; MANOVA: $p<0.05$), reaching a plateau after 4.5 h (MANOVA: $p<0.001$ vs Vehicle), 1°C above control (ANOVA: $p<0.001$). MANOVA (treatment by time) revealed that there was no significant difference between the shapes of the response to CRF, and CRF plus α-helical CRF_{9-41}.

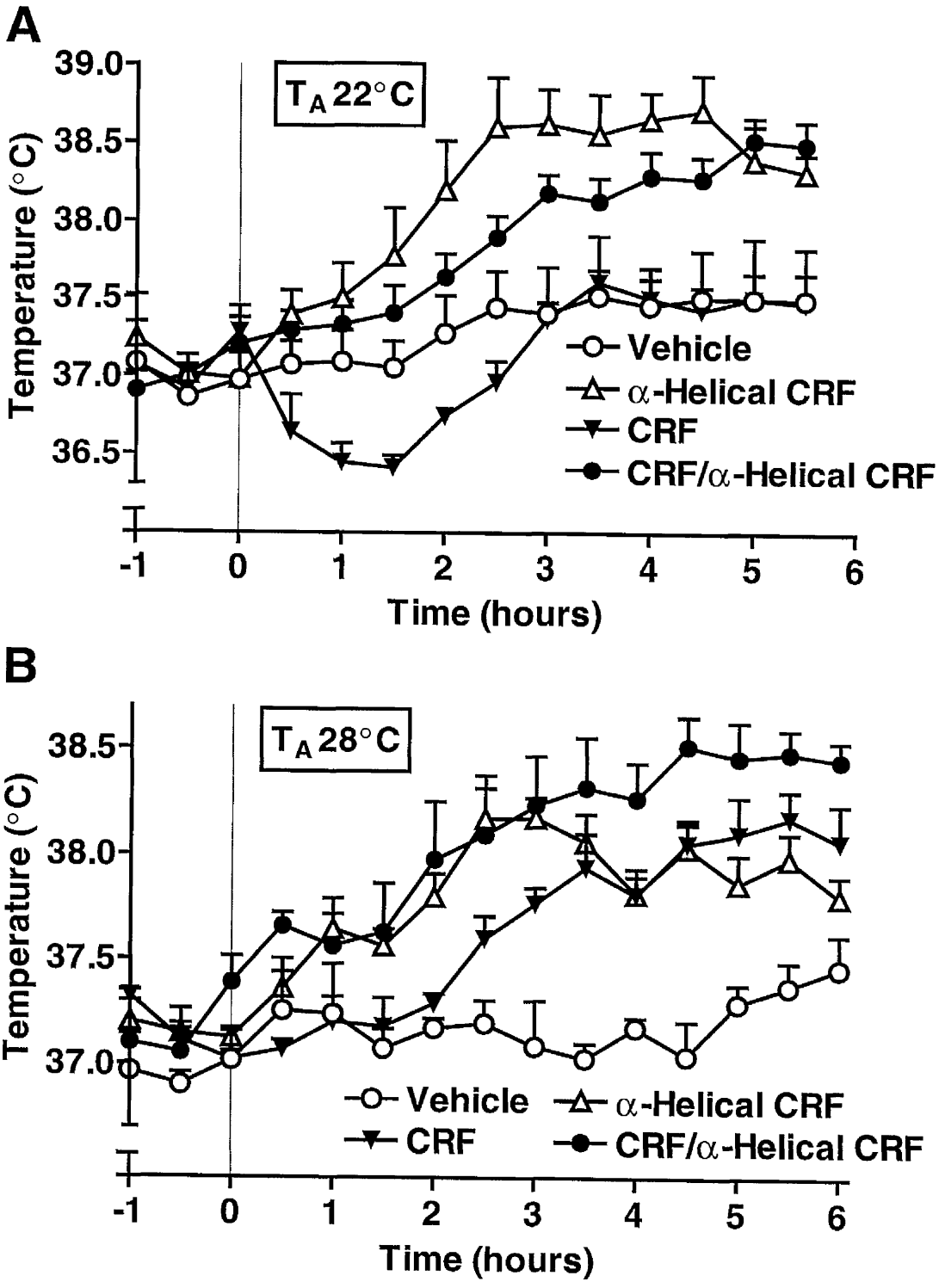

Figure 5.6.1. Core body temperatures of rats co-injected (icv) with CRF (3 μg) and α-helical CRF_{9-41} (30 μg), and housed individually at ambient temperatures of (A) 22°C, or (B) 28°C

5.7 Influence of CRF on Temperature Responses to Leptin

It has been suggested (**Section 5.2**) that CRF mediates actions of leptin on food intake and body weight, but not body temperature. Studies described in this chapter have also suggested paradoxical actions of CRF on thermoregulation (**Section 5.3-5.6**). Therefore, this study investigated whether CRF itself affects increase in body temperature in response to leptin.

5.7.1 Experimental Design

Leptin (4 μg) or vehicle was injected icv at 10:00 h (0 h). Vehicle, or a dose (0.3 μg) of CRF found previously (**Section 5.3**) not to induce changes in core body temperatures (non-hypothermic / subthreshold) was injected (icv) 1.5 h after leptin. Core body temperatures were monitored using remote radiotelemetry for 10 h after injection of leptin, until the end of the light phase.

5.7.2 Results

Administration of leptin (4 μg, icv) induced a significant rise in body temperature (MANOVA: $p<0.001$ vs Vehicle), which began 2 h after injection (**Figure 5.7.1**). This increase was maximal 5 h after injection (1.1°C above control; ANOVA: $p<0.001$), and then body temperature declined steadily towards the end of the time course. A sub-threshold (non-hypothermic) dose (0.3 μg) of CRF administered 1.5 h after injection of leptin (to coincide with the start of leptin hyperthermia), delayed the increase in core body temperature observed in response to injection of leptin (MANOVA: $p<0.05$). Body temperatures of animals that received both leptin and CRF rose after the 3.5 h time point,

peaked 7 h after injection of leptin (1°C above control, ANOVA: $p<0.01$) and then returned to values exhibited by animals injected with leptin alone (MANOVA: $p<0.01$ vs Vehicle).

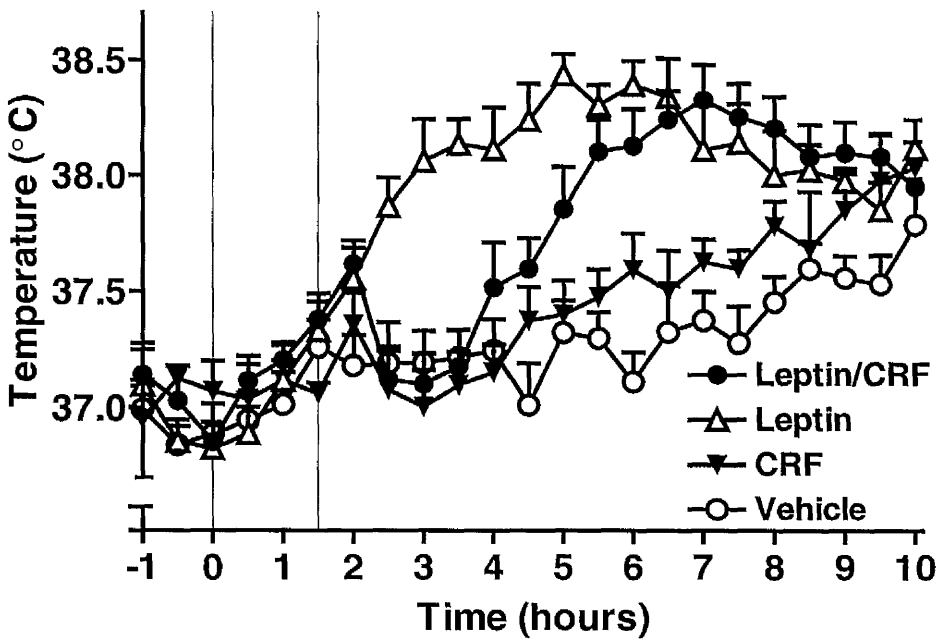

Figure 5.7.1. Effects on core body temperature of a subthreshold dose of CRF (0.3 μg, icv) injected 1.5 h after 4 μg leptin (icv)

5.8 Influence of CRF on IL-1 Fever

The experiment described above suggests that CRF inhibits leptin-induced changes in core body temperature. CRF has been reported to mediate various actions of cytokines (e.g. IL-1) including fever (Rothwell, 1989; Rothwell, 1990b; Rothwell & Cooper, 1992), and data presented in **Chapter 4** suggests that IL-1 mediates body temperature responses to leptin. Therefore this study investigated whether CRF inhibited IL-1 as well as leptin fever.

5.8.1 Experimental Design

A subthreshold (non-hypothermic) dose (0.3 μg) of CRF or vehicle (as in **Section 5.7**) was injected (icv) 0.5 h (to coincide with onset of fever) after vehicle, IL-1β (5 ng, icv) or IL-1α (50 ng, icv) at 10:00 h (0 h). Injections were performed in rats housed at an ambient temperature of 22°C.

To investigate whether responses were dependent on ambient temperature, animals were acclimatised to an ambient temperature of 28°C for 24 h before administration of IL-1β (5 ng, icv) or vehicle at 10:00 h (0 h). These injections were followed (0.5 h later) by icv injection of a subthreshold dose (0.3 μg) of CRF or vehicle.

5.8.2 Results

At an ambient temperature of 22°C, administration of IL-1β (5 ng, icv) induced a rise in body temperature which began 0.5 h after injection and was maximal at the 2.5 h time point (1.5°C above control; ANOVA: $p<0.001$), and sustained beyond 6 h (MANOVA: $p<0.001$ vs Vehicle) (**Figure 5.8.1A**). A sub-threshold (non-hypothermic) dose (0.3 μg) of CRF administered after 0.5 h (to coincide with the initiation of fever), attenuated the febrile response to IL-1β until 5 h after IL-1β injection (MANOVA: $p<0.05$).

In contrast, fever induced by injection of IL-1α (50 ng, icv) that rose after the 1 h time point, peaked 2 h after injection (1.9°C above vehicle, ANOVA: $p<0.001$) and remained elevated for the remainder of the experiment (MANOVA: $p<0.001$), was unaffected by a subthreshold dose of CRF (**Figure 5.8.1B**).

Injection (icv) of IL-1β (5 ng) at an ambient temperature of 28°C, induced fever 0.5 h after injection, that peaked at the 2 h time point (2°C above vehicle; ANOVA: $p<0.001$), and was sustained for 6 h (MANOVA: $p<0.001$ vs Vehicle; **Figure 5.8.1C**). A sub-threshold dose (0.3 µg) of CRF administered after 0.5 h (as above), attenuated the febrile response to IL-1β over the first 4 h after treatment (MANOVA: $p<0.01$).

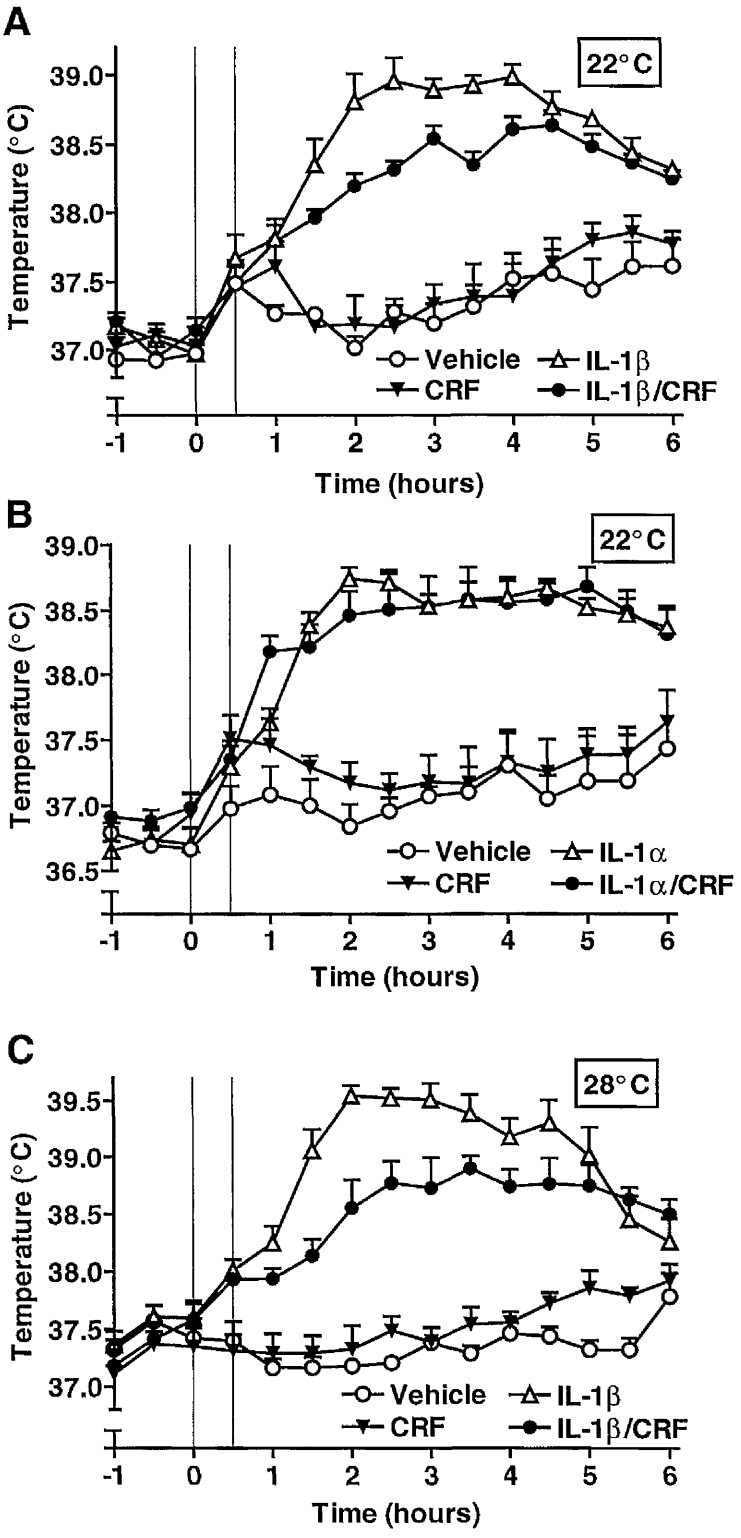

Figure 5.8.1. Effects on core body temperature of a subthreshold dose of CRF (0.3 μg, icv) injected 0.5 h after (A) IL-1β (5 ng, icv), or (B) IL-1α (50 ng, icv) at 22°C, or (C) IL-1β (5 ng, icv) at 28°C

5.9 SUMMARY

Studies described here indicate that leptin-induced reduction in food intake and body weight, but not its effects to increase core body temperatures, are mediated by CRF (**Section 5.2**). However, increased core body temperatures observed in response to injection of leptin were attenuated by administration of a subthreshold dose of CRF (**Section 5.7**), indicating a paradoxical relationship between leptin and CRF in regulating energy balance.

The results of these experiments also indicate that CRF can markedly influence body temperature in the rat, but that the duration, magnitude and direction of the response is dependent on the method of temperature measurement, housing conditions, and ambient temperature. Thus, at an ambient temperature of 22°C, CRF caused dose-dependent hypothermia (**Section 5.3**) in individual animals whose core body temperature was measured by remote radiotelemetry or colonic probe (**Section 5.4**). However, in group-housed rats, in which body temperature was measured using colonic probe, CRF caused transient hypothermia followed by hyperthermia (**Section 5.4**). This response was also observed in animals injected with CRF, and housed at raised ambient temperatures (**Section 5.4-5.5**).

CRF-induced hypothermia was inhibited by α-helical CRF_{9-41} at 22°C (**Section 5.6**). In contrast, at an ambient temperature of 28°C, the receptor antagonist exacerbated CRF-induced hyperthermia (**Section 5.6**). However, the antagonist alone induced hyperthermia at both ambient temperatures (**Section 5.6**). Furthermore, a subthreshold dose of CRF attenuated fever induced by leptin (**Section 5.7**) and IL-1β, but not IL-1α (**Section 5.8**).

5.10 Discussion

These studies provide the first direct evidence that effects of leptin on food intake (**Figure 5.2.1**) and body weight (**Figure 5.2.2**) are dependent on the action of CRF (**Section 5.2**). These results were published in the first volume of *Nature Neuroscience* (Gardner et al., 1998), and preceded a similar publication by Uehara *et al* some weeks later (Uehara et al., 1998). This later study reported that morning injection (into the third ventricle) of 3 μg leptin, inhibited food intake after 2 h by 33% in rats that were deprived of food for the previous 18 h. Simultaneous icv administration of 5 μg α-helical CRF_{9-41} attenuated this anorexic effect of leptin. It was also reported that injection of leptin increased hypothalamic CRF content at this 2 h time point. Attention may be drawn to the use of food-deprived animals in this study, which in addition to exhibiting increased appetite responses, may also have activated HPA axes and increased levels of CRF due to stress. Nevertheless, these studies (Uehara et al., 1998; Gardner et al., 1998), together with those reporting activation of CRF-containing neurones by injection of leptin (Schwartz et al., 1996; Elmquist et al., 1998), and the presence of leptin receptors on these hypothalamic neurones (Hakansson et al., 1998), provide strong evidence that CRF mediates central actions of leptin on food intake and body weight in rats.

The CRF receptor antagonists (α-helical CRF_{9-41} and D-Phe-CRF_{12-41}) injected icv in these studies (Uehara *et al.*, 1998; Gardner *et al.*, 1998) do not distinguish between the different CRF receptors. Therefore actions of leptin affected by these antagonists cannot be localised to specific brain regions or CRF receptor subtype. Indeed, the effects of leptin on food intake and body weight, which are

mediated by these CRF receptor antagonists may be due to the inhibition of actions of urocortin on CRF receptors. Neither do these studies elucidate the involvement of potential pathways subsequent to the actions of CRF, such as POMC and MSH in stimulating these responses to leptin.

Considering that the effects of leptin on food intake and body weight were attenuated by antagonism of the CRF receptor, and that CRF has been reported previously to induce thermogenesis (Rothwell, 1989; Rothwell, 1990b; Rothwell & Cooper, 1992), the observation that CRF reduced core body temperature in rats is a paradox. Nevertheless, data presented here reveal that CRF induces dose-dependent hypothermia (**Figure 5.3.1**), measured by remote radiotelemetry in individually housed rats, at 22°C ambient temperature (**Section 5.3**). One potential explanation for these effects of CRF on body temperature may be that CRF injected into the brain is 'leaking' out across the blood-brain barrier to exert systemic effects on the circulation and peripheral tissues. Previous studies have reported that CRF has differential effects on cardiovascular functions when acting in the brain or periphery. Peripheral administration of CRF induces hypotension and bradycardia, whereas central administration has been reported to increase blood pressure and heart rate (Richter & Mulvany, 1995). However, peripheral sites of action are unlikely to explain the results obtained here because additional experiments showed that intravenous, intraperitoneal or subcutaneous injection of the same dose of CRF (3 µg) produced no change in core body temperature, indicating that the effects observed here result from the direct action of CRF in the brain.

The doses of CRF used in this study are high compared with some studies (0.1 μg) that demonstrate physiological responses of CRF (Holt & York, 1989; Diamant & De Wied, 1991; Behan et al., 1995). However similar doses to those used here have been applied to investigate thermoregulation and energy balance in previous studies in this laboratory (LeFeuvre et al., 1987; Rothwell, 1989; Rothwell et al., 1991) and by other groups (Britton et al., 1984; Eaves et al., 1985; Negri et al., 1985; Arase et al., 1988).

In addition to studies demonstrating that CRF can *inhibit* pyrogenic responses to cytokines (Bernardini et al., 1984; Opp et al., 1989), there is one previous report that describes hypothermia in response to icv injection of CRF, similar to the effects on body temperature observed here (Sausen et al., 1996). This study described how core body temperature (measured using radiotelemetry) and oxygen consumption were recorded for 30 min before and for 90 min after icv injection of saline, 0.3 or 3 μg CRF in Long-Evans rats. Core temperature fell significantly at 90 min in response to 0.3 μg CRF, and at 60 and 90 min in rats injected with 3 μg CRF. Oxygen consumption also fell over the course of the post-injection period for saline and 0.3 μg CRF, but not in response to injection of 3 μg CRF. These data suggest a complex relationship between metabolic and thermoregulatory responses to CRF, and strongly support the data presented here.

The dose-dependent hypothermia described here in response to icv injection of CRF (**Section 5.3**), contrasts markedly with previously published reports from this laboratory, which suggest that CRF increases core temperature and metabolic rate (Rothwell, 1989; Rothwell, 1990b; Rothwell, 1994). The studies

from which this latter conclusion was drawn, were conducted at ambient temperatures of 24°C, using the colonic probe method for measuring core body temperature, while animals were confined to small metabolic cages for the measurement of oxygen consumption (Rothwell, 1989; Rothwell, 1990a; Rothwell, 1990b; Rothwell et al., 1991; Strijbos et al., 1992). Therefore, these differing conditions may have contributed to the contrasting responses observed. This hypothesis is supported by the data presented here, which reveal that responses to injection of CRF were dependent on housing conditions, method of temperature measurement, but predominantly by ambient temperature (**Section 5.4-5.5**).

Injection of 3 μg CRF elicited similar hypothermic responses in rats exposed to the following conditions (**Section 5.3-5.4**):

i. Individual housing plus telemetry (**Figure 5.3.1**)

ii. Individual housing plus colonic probe (**Figure 5.4.1A**)

iii. Group housing plus telemetry (**Figure 5.4.1B**).

However, the combination of group housing (home cages) plus the colonic probe method of temperature measurement resulted in modest hypothermia, followed by hyperthermia (**Figure 5.4.1C**). These results may be explained by considering that although rats were familiarised to temperature measurement by colonic probe for a week before experiment, prolonged exposure to this procedure (every 30 min for 6 h) and the disturbance caused by monitoring core temperatures of littermates, may have induced stress in the animals. The associated increase in stress hormones (including CRF) therefore may have

contributed to the increased core temperatures seen in response to injection of CRF. Moreover, heat conservation and increased microclimate temperature induced by huddling behaviour is likely to have played a role in this paradigm where animals were housed in groups of 5-6 in their home cages, thereby facilitating any increases in core body temperature.

Indeed, the influence of ambient temperature in responses to injection of CRF was found to be of primary importance (**Section 5.4-5.5**), as demonstrated by the results of increasing ambient temperature from 22°C to 26°C to simulate thermal protection provided by huddling behaviour. Individually-housed animals, which at 22°C to exhibit hypothermia only, exhibited both hypo- and hyperthermia with the use of radiotelemetry (**Figure 5.4.2A**). This effect was exacerbated in animals, in which temperature was measured by colonic probe, such that at 26°C hyperthermia only was observed (**Figure 5.4.2B**). These studies further support the proposal that a combination of environmental factors (i.e. stress from colonic probe method and increased ambient temperature) alters effects of exogenous CRF on core body temperature.

Further studies however, found that altering ambient temperature alone was sufficient to influence temperature responses to icv injection of CRF (**Section 5.5**). These studies were performed using radiotelemetry, allowing continuous, undisturbed temperature-measurement, and therefore limiting stress that may otherwise be exhibited by animals monitored using colonic probe. It is possible that animals were exposed to stress and/or infection during peritoneal surgery required for implantation of the radiotransmitter. However, all animals appeared to recover from the operation at the end of the one week post-operative period.

Experiments show that CRF, rather than inducing hypothermia, results in hyperthermia at higher ambient temperatures (22-28°C) (**Figure 5.5.1A-D**).

Sauvagine, a CRF-related peptide isolated from the skin of a South American frog (*Phyllomedusa sauvagei*), with similar biological activity to CRF (Turnbull & Rivier, 1997; Vale et al., 1997) has been demonstrated to induce hypothermia via a dopaminergic mechanism (Broccardo, 1990; Broccardo & Improta, 1994). Therefore, CRF hypothermia may be mediated by similar ambient temperature-dependent dopaminergic pathways, whereas CRF-induced hyperthermia may be mediated by activation of sympathetic mechanisms (Brown et al., 1982; Rothwell, 1990b; Rothwell, 1994).

Perhaps the most likely mechanism of these ambient temperature-dependent effects is that CRF influences both heat production and heat loss. It has been demonstrated that CRF induces thermogenesis, which at higher ambient temperatures (as described here) could increase core body temperature. However, CRF (like sauvagine) may also increase heat loss (Broccardo & Improta, 1994). This action is unlikely to have significant effects on body temperature in warm environments where the potential for heat loss is minimal, but may induce hypothermia at lower ambient temperatures, when body heat may be lost more readily.

Thus, at low ambient temperatures (22°C) CRF-induced heat production mechanisms may not be sufficient to balance heat loss, and so the latter predominates, resulting in hypothermia. Conversely, at higher ambient temperatures, the potential for heat loss is reduced, thereby allowing heat

production mechanisms to predominate. This rather complex hypothesis is depicted in the following schematic diagram (**Figure 5.10.1**).

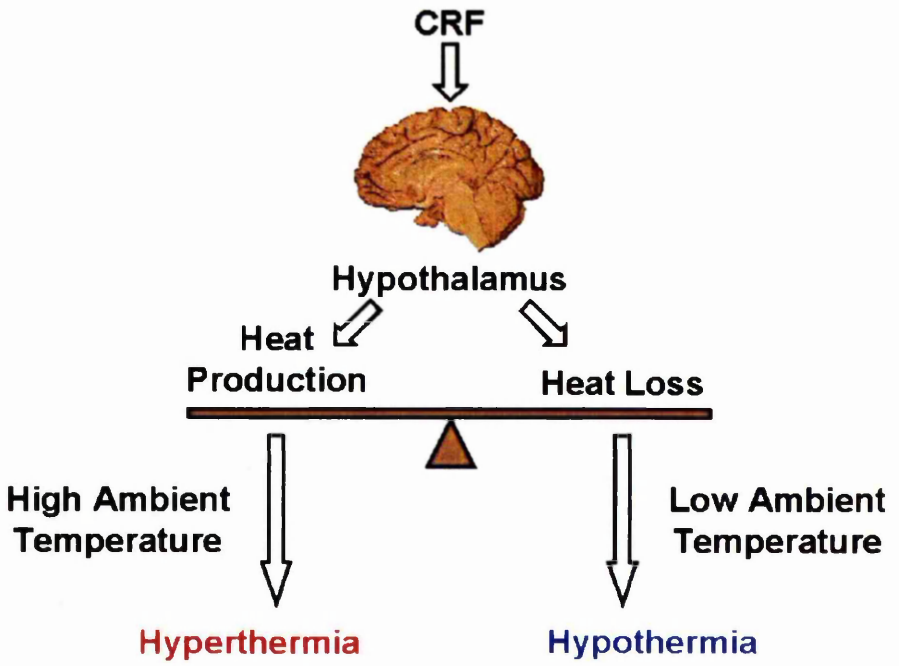

Figure 5.10.1. Schematic representation of proposed mechanisms of effects exogenous CRF on body temperature at different ambient temperatures

These mechanisms appear to be mediated centrally since central injection of α-helical CRF_{9-41} inhibited CRF-induced hypothermia at 22°C ambient temperature (**Section 5.6**). However, these data are difficult to interpret because injection of the antagonist exacerbated CRF-induced hyperthermia at 28°C, and alone induced hyperthermia at both 22 and 28°C (**Figure 5.6.1A-B**), suggesting that CRF may have a role in the regulation of normal body temperature. Effects on core body temperature induced by α-helical CRF_{9-41}

were not induced by endotoxin contaminants, since the results of a LAL assay on samples of the CRF receptor antagonist were negative. These responses may however result from complex interactions of α-helical CRF_{9-41} with other CRF-like neuropeptides, CRF-binding protein, or from partial agonist effects (Behan et al., 1996; Turnbull & Rivier, 1997; Vale et al., 1997). This antagonist, at doses of 5 and 25 μg (icv), has been shown to dose-dependently induce tachycardia and behavioural activation, suggesting partial CRF agonist activity (Diamant & De Wied, 1991). However, the hyperthermic response induced at 22°C ambient temperature is the reverse of that induced by CRF (which elicited hypothermia). It is also perhaps misleading to attribute an exacerbation of CRF-induced hyperthermia to this antagonist, since the response to injection of CRF plus α-helical CRF_{9-41} was less than the sum of the individual responses.

These results, although confusing, allow discussion of the study on the effects of CRF on leptin-induced hyperthermia (**Section 5.7**). The data described in this section show that a subthreshold dose of CRF attenuated the increase in core body temperature induced by leptin (**Figure 5.7.1**). Because CRF has also been implicated in mediating effects of cytokines such as IL-1β, but not IL-1α (Busbridge et al., 1989), this experiment was repeated to observe the effects of a subthreshold dose of CRF on fever induced by these cytokines (**Section 5.7**). Interestingly, a similar inhibitory effect of CRF on leptin hyperthermia was exerted on IL-1β, but not IL-1α fever (**Figure 5.8.1A-B**). Furthermore, this response was apparent at both 22°C and 28°C ambient temperatures (**Figure 5.8.1A and C**).

These data may be explained by considering the level of involvement of CRF in each pathway. If CRF-mediated thermogenesis is maximal during leptin and IL-1β-induced fever, injection of exogenous CRF may affect only the heat loss mechanisms active during CRF-mediated hypothermia, resulting in an attenuation of the febrile response. This may also explain why IL-1β fever is still attenuated by administration of CRF at 28°C, rather than exacerbated as might be predicted from the hyperthermia observed in response to injection of CRF at 28°C. The failure of CRF to affect IL-1α fever may be explained by considering that the CRF pathway is not activated during this response (Busbridge et al., 1989). Therefore a dose of CRF that alone does not affect core body temperature, will have neither stimulatory nor inhibitory effects on IL-1α fever.

The results presented in this chapter support the suggestion that leptin effects on food intake and body weight are mediated by CRF in the brain. Furthermore, the data indicate that central administration of CRF can exert either hyper- or hypothermia. The magnitude and duration of which depends on a combination of environmental factors such as the housing conditions, method of temperature measurement, ambient temperature, and/or the balance between heat loss (via dopaminergic pathways) and heat production (via sympathetic pathways). In addition, CRF can modulate fever involving the activation of CRF-mediated pathways. These effects of CRF on core body temperature are likely to be due to actions on effector mechanisms, rather than an influence on the body temperature set point. This suggestion could be investigated further by additional experiments to measure the metabolic rate and preferred ambient temperature of animals in response to injection of CRF. These data

demonstrate complex actions of CRF, and underline the importance of experimental conditions when investigating the role of CRF in thermoregulation. Nevertheless, the results described in this chapter involving injection of CRF, although fascinating, pose more questions than they answer.

Chapter 6

General Discussion

6.1 Thesis Aims

The general aims of the studies described in this thesis were to investigate the actions of leptin and its interactions with cytokines and other neuroimmune mediators. Experiments were designed to elucidate whether actions of leptin resembled, (**Chapter 3**) and were mediated by proinflammatory cytokines (**Chapter 4**), and to investigate the involvement of CRF in normal untreated animals, or those injected with leptin or cytokines (**Chapter 5**).

6.2 Summary of Results

The results presented in **Chapter 3** described how injection of leptin induced cytokine-like actions, not only on food intake and body weight, but also on core body temperature, and that the latter response only was mediated by release of PGs. However, work described in **Chapter 4** showed that all three responses, but particularly core body temperature, were mediated by release of IL-1β in the brain. Furthermore, leptin-induced release of IL-6 in the brain may be involved in suppressing such responses. **Chapter 5** demonstrated that in contrast to the involvement of PGs, CRF mediates food intake, but not temperature responses to injection of leptin. Furthermore, responses to injection of CRF in normal and febrile animals varied by altering housing conditions, method of measuring core body temperature and ambient temperature, underlining the importance of experimental conditions when investigating the role of CRF in thermogenesis.

These data have important implications in understanding the mechanisms involved in energy balance in host defence responses to disease. A summary of these responses is presented in the following schematic diagram (**Figure 6.2.1**).

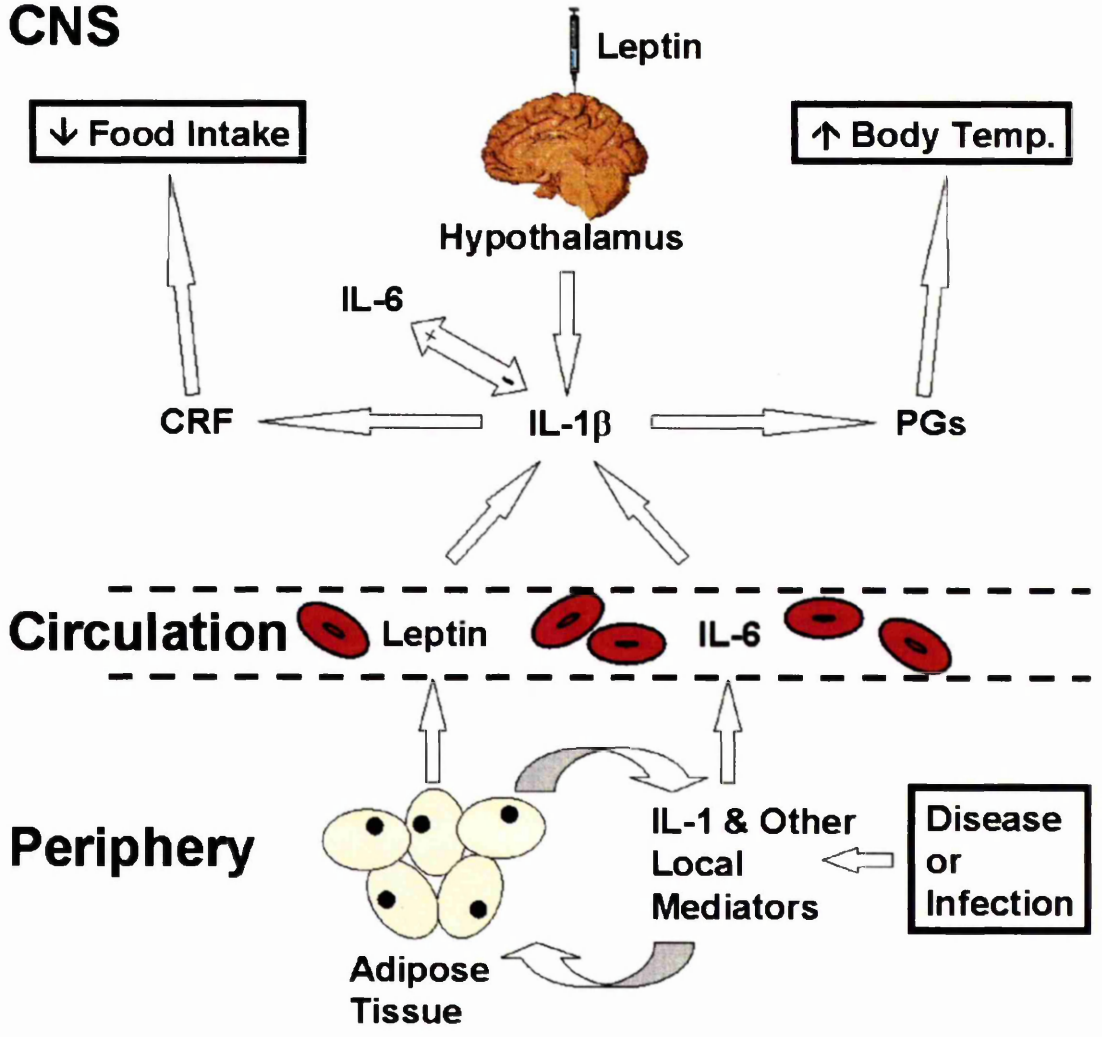

Figure 6.2.1. Schematic representation of potential mechanisms involved in mediating actions on leptin in experimental and disease responses

6.3 Relevance of Experimental Approaches

There are three major points of discussion about the experimental procedures performed in studies described in this thesis:

6.3.1 Administration of substances into the brain

Although similar acute doses of LPS, IL-1β and leptin as used in this study, have been administered to rodents for the observation and characterisation of responses in previous reports (Strijbos *et al.*, 1992; Schwartz *et al.*, 1996; Luheshi *et al.*, 1996), there remains doubt whether such doses are relevant to actual pathophysiological conditions. Osmotic minipumps have been used effectively for the chronic administration of substances in concentrations that purport to resemble physiological concentrations (Plata-Salaman *et al.*, 1996; Gayle *et al.*, 1997). Even so, such studies requiring chronic treatment are not without their disadvantages, such as the extended experimental time course before responses may be observed, and the development of tolerance to the substances administered (Plata-Salaman *et al.*, 1996). Pathophysiological concentrations of IL-1β in human brain CSF have been reported to extend from 0.5 to 1.5 pg/μl (Lopez-Cortes *et al.*, 1993). Caution must be applied when comparing these data with concentrations of substances injected into brains of rodents, since substances present in patient's CSF is 'spill-over' from brain tissue, whereas in contrast, substances injected icv have to diffuse into those tissues from the CSF. Therefore concentration gradients in each case are likely to be in opposition. Nevertheless, assuming that a rat's normal CSF volume is 400 μl (Plata-Salaman, 1994), after initial diffusion, a 5 ng dose of IL-1 (and

assuming no loss) could lead to a concentration of 12.5 pg/μl, which is tenfold higher than for CSF measured in human patients. Assuming a turnover of approximately 1% total CSF per minute (Lai *et al.*, 1983) (and excluding any active clearance of IL-1β), this concentration would be halved every hour, thereby reaching acceptable concentrations within 3 h after injection (at the febrile peak). Thenceforth IL-1β concentrations would be within pathophysiological limits for the remainder of the experimental time course. It may be argued therefore, that the observed rise in core body temperature of animals injected with IL-1β was a result of excessive concentrations. However, febrile body temperatures persisted beyond the 3 h time point, indicating that acute administration can induce pathophysiological responses. Similarly, previous investigations using rats have reported induction of physiological responses after chronic infusion (icv) of leptin (12 μg/day for 4 days) (Cusin *et al.*, 1998). Therefore an acute 4 μg dose of leptin as used in this study would elicit similar acceptable CSF concentrations within 3 h after injection.

It may also be questioned whether icv administration of substances is a physiologically useful approach, since icv injection may influence brain regions that are not normally exposed to the endogenous ligand. Moreover, the fact that leptin is produced in peripheral adipose tissue (Zhang *et al.*, 1994; MacDougald *et al.*, 1995; Guerre-Millo, 1997) implies that systemic administration of leptin is the more physiologically relevant route of administration for the observation of responses *in vivo*. However, leptin has been suggested to have direct actions on the brain where it is more potent and so requires lower concentrations to elicit responses (Seeley *et al.*, 1996; Schwartz *et al.*, 1996). Therefore

performing icv injections prevented the high costs of injecting large doses of leptin peripherally.

6.3.2 IL-1RI (-/-) Mice

There are several flaws the preliminary experiment described in **Section 4.4**, not least the use of mice that are not exact controls for the IL-1RI (-/-) mice. The knockout mice appear to exhibit a degree of heterogeneity. This is most apparent by the fact that some (most) animals exhibit the black coat colouring of C57BL/6s, while the others possess the faun colouring of the 129 strain. There also appears to be variation in body weights between these black and faun mice, and between different litters.

Solutions:

1. A comprehensive breeding programme should be established such that original IL-1RI (-/-) mice are back-crossed with either C57BL6s or 129s so that C57BL6 or 129 mice may be used as a control.
2. Alternatively, knockout mice should be crossed with a C57BL/6 or a 129, to obtain heterozygotes (+/-). Inbreeding these heterozygotes would then yield an mixed 1:2:1 (+/+):(+/-):(-/-) F1 litter, whose weights should be devoid of variation other than that elicited by differences in genotype.

6.3.3 Telemetry Versus Colonic Probe

Experiments presented in **Chapter 5** indicated that although animals exhibited similar responses when core body temperature was measured using either remote radiotelemetry or colonic probe, additional environmental influences

caused resulting responses to deviate. These findings reinforce the argument that remote radiotelemetry, which allows continuous undisturbed monitoring of core body temperature has a distinct advantage over the colonic probe method in obtaining physiologically accurate data (Gallaher *et al.*, 1985; Clement *et al.*, 1989; Matthew, 1997). The only negative aspect of this method is the 1 week period necessary to facilitate recovery from implantation surgery. However, animals implanted icv with a guide cannula were required to undergo this treatment anyway.

6.4 Relationship to Other Work

These studies are the first to describe that injection of leptin induces increases in core body temperatures of normal rats (**Chapter 3**). Others have demonstrated that the suppressed body temperatures of *ob/ob* mice are normalised in response to leptin administration (Pelleymounter *et al.*, 1995; Harris *et al.*, 1998). Earlier experiments performed on rats have shown modest increases in body temperatures, although these effects were induced in response to injection of leptin peptide fragments, not the complete molecule (Fruhbeck *et al.*, 1998).

These studies are also the first to demonstrate induction of cytokines in response to leptin administration (**Chapter 4**). Whereas others have shown that leptin upregulates LPS-induced cytokines (Loffreda *et al.*, 1998), and that conversely, proinflammatory mediators induce increased leptin expression

(Grunfeld *et al.*, 1996; Sarraf *et al.*, 1997; Janik *et al.*, 1997; Bornstein *et al.*, 1998; Faggioni *et al.*, 1998).

Furthermore, work presented in **Chapter 5** demonstrating that CRF mediates actions of leptin on food intake resulted in the first publication of such effects (Gardner *et al.*, 1998). Uehara *et al.* later reported similar findings, although these were observed in food-restricted rats (Uehara *et al.*, 1998).

Studies described in **Chapter 5** agree with a study performed by Sausen *et al.* showing that icv injection of CRF induces hypothermia in rats (Sausen *et al.*, 1996). These effects were also similar to those observed in response to injection of the CRF-like molecule Sauvagine (Broccardo, 1990; Broccardo & Improta, 1994), indicating the possibility that these molecules are stimulating shared receptor-mediated pathways.

6.5 Conclusions Relating to Human Conditions

It is difficult to relate the data obtained in this thesis using rodents to the actions of leptin in the human condition of obesity, due to its heterogeneous nature (Leibel, 1997; Comuzzie & Allison, 1998). Although humans may exhibit genetic predispositions to the development of obesity, environmental factors such as poor diet and inactivity are likely to be of primary aetiological importance in determining body fat content (Hill & Peters, 1998). There has only been one recent study, which has demonstrated congenital leptin deficiency in man (Farooqi *et al.*, 1998). Most studies in humans have suggested that deficiencies in the leptin receptor pathways are linked to the aetiology of obesity (Clement *et*

al., 1998; Comuzzie & Allison, 1998). However, others have detected leptin receptor polymorphisms in obese individuals, but these have not been significantly linked with the obese phenotype (Rolland *et al.*, 1998). Therefore leptin-related effects resulting in obesity are proposed to be present in the downstream signal transduction mechanisms regulating energy balance, rendering direct pharmacological targeting of the leptin receptor obsolete in prospective treatments of obesity. Nevertheless, the studies described here indicate that leptin may play an important cytokine-like role in pathophysiological responses such as food intake and body temperature. Indeed, it is possible that leptin, rather than IL-6, is the 'elusive' circulating neuroimmune mediator involved in inducing CNS-mediated responses to disease such as fever. Leptin is synthesised in the periphery, released into circulation, from where it is able to cross the BBB to stimulate areas of the brain known to be involved in regulating energy balance. Therefore leptin appears to satisfy many of the criteria that such a molecule should possess.

Results presented in this thesis have important implications in host defence responses to disease and infection. By targeting the leptin pathway, it may be possible to inhibit cytokine actions in the brain that contribute to cachexia and other disorders (Moldawer *et al.*, 1992; Tisdale, 1997; Moldawer & Copeland, 1997; Matthys & Billiau, 1997). Furthermore, the finding that leptin induces febrile 'side-effects' mediated by IL-1 also has serious implications for the use of leptin, or leptin-derived molecules in the future treatment of obesity.

6.6 Questions Arising from Work Presented in this Thesis

There are several questions and points of further study arising from the data presented in this thesis:

- What is the role of leptin in IL-1RI (-/-) and IL-6 (-/-) mice?
- What are the sites and mechanisms of interaction between leptin, IL-1, CRF and PGs?
- How does CRF modify body temperature?
- Does CRF affect the hypothalamic thermoregulatory set point?
- Is CRF pyrogenic or cryogenic?
- Is leptin the circulating afferent mediator of fever?
- Are IL-1 and CRF involved in the normal regulation of energy balance and body temperature?

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Publications arising from this Thesis

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