Neuroendocrine Physiology in the Transgenic SLOB Rat: A New Obesity Model

A thesis submitted in accordance with the requirements of the University of London for the degree of Doctor of Philosophy

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January 2001

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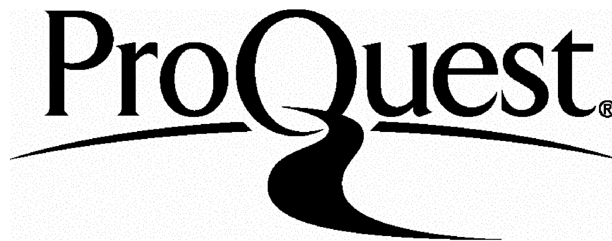

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"Some people have the good genes, some people don't, I think there are some patients especially the very morbidly obese patient, who are going to be pretty much a biological problem. They have a real nasty set of genes. As long as they have enough calories to eat they're going to be fat no matter what environment they're in and despite their best efforts."

Dr David West, excerpt from The Skinny on Fat: Our Obsession with Weight Control by Shawna Vogel (W.H.Freeman, 1999).

ABSTRACT

Previously two lines of transgenic rats with a cosmid containing both vasopressin (VP) and oxytocin (OT) genes, engineered to express human growth hormone (hGH) and bovine neurophysin (bNP) as reporter genes respectively were generated. Unexpectedly, males of one line, bearing multiple copies of the transgene, exhibited an autosomal dominant phenotype of **S**evere **L**ate-onset **OB**esity (SLOB) with selective accretion of visceral fat despite normal intake of a low fat diet. Presented in this thesis are the physiological and some genetic studies carried out to characterise the SLOB phenotype. Studies include analysis of the sex-specific phenotype such as ovariectomy and high fat feeding, both of which result in a rapid development of visceral obesity in young SLOB rats. Lipid analyses revealed reduced lipolytic activity and increased hepatic very low-density lipoprotein (VLDL) production. Despite severe obesity, these animals were not diabetic or insulin resistant. Investigation of hypothalamic feeding neuropeptides showed some changes, including a reduced arcuate neuropeptide Y (NPY) mRNA expression and an increased supraoptic *tub* mRNA expression. The SLOB phenotype could not easily be explained by the known transgene products. Further analysis of the transgene construct revealed a novel gene, which was termed *5'OT-EST*. Studies assessing the possible role of this gene in SLOB obesity indicated it being a good candidate for contributing to the SLOB phenotype. Whatever the underlying genetic mechanism of the SLOB phenotype, this rat is a unique model for late-onset male specific visceral obesity.

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LIST OF ABBREVIATIONS

aa	amino acid
ACTH	adrenocorticotrophic hormone
AgRP	agouti-related peptide
Apo	apolipoprotein
Arc	arcuate
A^{y}	*agouti* lethal yellow
A^{vy}	*agouti* viable yellow
AVP	arginine vasopressin
BMI	body mass index
bNP	bovine neurophysin
bOT	bovine oxytocin
bp	base pair
cAMP	cyclic AMP
CART	cocaine- and amphetamine-regulated transcript
cDNA	complementary DNA
cp	corpulent
CRF	corticotrophin releasing factor
CV	coefficient of variation
db	diabetic
DM	dorsomedial
DMH	dorsomedial hypothalamus
DNA	deoxyribonucleic acid
dw	dwarf
EDTA	ethylenediaminetetracetic acid
ER	oestrogen receptor
EST	expressed sequence tag
FFA	free fatty acids
FPLC	fast protein liquid chromatography
GH	growth hormone

GHR	growth hormone receptor
GHRH	growth hormone-releasing hormone
GRF	growth hormone-releasing factor
GTT	glucose tolerance test
HDL	high density lipoprotein
HDL-C/TG	high density lipoprotein cholesterol/triglyceride
hGH	human growth hormone
HPA	hypothalamic-pituitary-adrenal
HSL	hormone sensitive lipase
i.c.v.	intra-cerebro-ventricular
IDL	intermediate density lipoprotein
IGF-I	insulin like growth factor-I
i.p.	intra-peritoneal
ITT	insulin tolerance test
i.v.	intra-venous
kb	kilobase
LDL	low density lipoprotein
LDL-C/TG	low density lipoprotein cholesterol/triglyceride
Lepr	leptin receptor
LH	lateral hypothalamus
LPL	lipoprotein lipase
MC	melanocortin
MCH	melanin concentrating hormone
MC3/4-R	melanocortin- 3/4 -receptor
mRNA	messenger RNA
α–MSH	α-melanocyte stimulating hormone
NaOH	sodium hydroxide
NIDDM	non insulin dependent diabetes mellitus
NIHDDK	National Institute of Health for Diabetes and Digestive and Kidney Disease
NIMR	National Institute for Medical Research
NP	neurophysin
NPY	neuropeptide Y
nt	nucleotide

ob	obese
OBR	leptin receptor
OBR-EC	OBR-extracellular
OBR-IC	OBR-intracellular
OT	oxytocin
ovx	ovariectomised
PeN	periventricular nucleus
PCR	polymerase chain reaction
POMC	pro-opiomelanocortin
PPAR	peroxisome proliferator-activated receptors
PRL	prolactin
PVN	paraventricular nucleus
PWS	Prader-Willi syndrome
rAVP	rat arginine vasopressin
rGH	rat growth hormone
rhGH	recombinant human growth hormone
RIA	radioimmunoassay
rLH	rat luteinising hormone
RNA	ribonucleic acid
rOT	rat oxytocin
RPA	RNAse protection assay
RT-PCR	reverse transcriptase PCR
SLOB	severe late onset obesity
SOCS3	suppresser of cytokine signalling-3
SON	supraoptic nucleus
SRIF	somatotrophin-release inhibiting factor
SS	somatostatin
SSC	sodium chloride/sodium citrate buffer
STAT	signal transducers and activators of transcription
TAE	tris acetate EDTA
TBS	tris buffered saline
TG	triglyceride
Tgr	transgenic growth retarded rat
TSH	thyroid stimulating hormone

TULPs	tubby like proteins
TZD	thiazolidinediones
UCP	uncoupling protein
VLDL	very low density lipoprotein
VLDL -C/TG	VLDL cholesterol/triglyceride
VMH	ventromedial hypothalamus
VMN	ventromedial nuclei
VP	vasopressin
WAT	white adipose tissue
WHO	World Health Organisation

Chapter 1

Introduction

1.1 Aims of this thesis

The aim of this thesis is to address the nature of the sexually dimorphic phenotype in SLOB rats, characterise SLOB physiology in terms of lipid biochemistry, diabetic status and hypothalamic neuropeptide analysis, and finally to try to explore further the underlying genetic cause of the SLOB phenotype.

The SLOB rat is a model of **S**evere **L**ate-onset **OB**esity. Before I started my PhD, this line of transgenic rats had been generated with the aim of investigating the control and regulation of the hormones released from the posterior pituitary gland. However, unexpectedly males from this line developed severe obesity with age. This obesity was predominantly central in distribution and also males from this line were infertile. Females were fertile and developed obesity at a much later age than male rats. Both sexes also exhibited slightly reduced pituitary rat growth hormone levels. At the time of starting this thesis little more was known about the obesity phenotype of SLOB rats. In this thesis I present my physiological characterisation and some molecular genetic studies of this model.

The following introduction gives a general summary of the existing data in the obesity field. This introduction will cover the role of leptin, lipid metabolism, feeding neuropeptides and rodent obesity models. These will be further investigated and compared in the subsequent chapters of this thesis and should provide a background for my studies on the new rodent obesity model on which this thesis is based.

1.2 Obesity: a world-wide epidemic

1.2.1 The facts and figures

'For the first time in history, the world's total of overweight people equals the total of those who are underfed. Up to 1.2bn people eat more than they need, and as many go hungry each day.'

This statement appeared in The Guardian on the 6th March 2000 and emphasised the increasing problem of obesity in the developed world. Obesity, defined in clinical terms by body mass index (BMI) is calculated by dividing an individuals weight in kilos by their height squared in metres. Ideally, this should fall between 20 and 25; 25-30 is classified as overweight and a BMI of 30 or above as clinically obese. However, this measure does not take into account muscle mass and therefore individuals such as bodybuilders have BMI's of over 30 but are obviously not obese. In the United States 23% of adults are officially obese, 55% are officially overweight, and 20% of children are obese or overweight. In Britain, one in five women and one in six men are officially obese, with 45% of men and 33% of women overweight (Flegal *et al.*, 1998; WHO, 1998; Seidell, 2000). With such a high incidence rate, it is not surprising that research in the obesity field has increased dramatically over the past 10 years.

For reasons that are not fully known, obesity is also associated with an increased risk of hypertension, heart disease, diabetes and cancer (Pi-Sunyer, 1993). As well as the prospect of diminished health, obese individuals are often stigmatised both socially and in the workplace. This makes the obese syndrome a mental as well as physical condition for many people. With all these factors in mind the cost of treating obesity is very large. In the United States alone more than £60bn a year, or more than 10% of the nation's bill for health is used for this condition and a further £20bn spent on diet drugs (Quesenberry *et al.*, 1998; Oster *et al.*, 2000).

The most obvious cause of increased weight gain is thought to be an increase in food intake (Seidell, 2000). The first law of thermodynamics states the amount of stored energy equals the difference between energy intake and work (Stryer, 1981), this can also be applied in terms of bodyweight regulation. The amount of triglyceride in adipose tissue is the cumulative sum over time of the differences between energy (food) intake and energy expenditure (mainly resting metabolism and physical activity). Although homeostatic mechanisms keep this difference very close to zero, small imbalances over a long period can have a large cumulative effect. Thus, it is often misunderstood that in order for late-onset obesity to occur, a large intake of food is not needed, a small increase over a long period of time is enough to cause obesity. For example, the non-obese adult ingests about 900,000 kcal of food per year (Rosenbaum *et al.,* 1997); if intake exceeds by just 2 percent daily for a year, the result is an increase of 18,000 kcal, or approximately 2.3kg (5 lb).

The most obvious treatment of obesity is therefore presumed to be diminishing hyperphagia by caloric restriction (Danforth, 1985; Wadden, 1993). However, treatment directed toward the long-term reduction of bodyweight is largely ineffective, and 90-95% of persons who lose weight by dieting subsequently regain it (Rosenbaum *et al.*, 1988; Wadden *et al.*, 1993). In contrast, despite over-eating some people can maintain a normal body weight, indicating that changes in feeding behaviour do not always correlate with changes in bodyweight. These findings suggest that factors other than food intake may be involved in regulating bodyweight.

As well as food intake, obesity can arise following reduced energy expenditure, for example due to lack of physical exercise. A balance between energy intake and energy expenditure therefore works to retain a normal bodyweight (Bray and York, 1979; Kalra, 1997; Elmquist *et al.,* 1998; Flier and Maratos Flier, 1998; Friedman and Halaas, 1998; Inui, 1999). Animals and humans eat for a variety of reasons, such as time of day, social setting, stress, boredom, palatability/reward, and food availability. Unlike humans, some animals rely on an adaptation in energy expenditure in order to survive. For example, the ability to store substantial amounts of energy as lipid in adipose tissue has been critical for development of the exceptional rates of milk production achieved in the dairy cow (Vernon and Pond, 1997). Energy expenditure in terms of metabolic rate is also important in the survival of hibernating animals.

The hibernating alpine marmot experiences a 95% reduction of metabolic rate facilitating a drop in body temperature, thus energy expenditure is actively downregulated and controlled at a minimum level enabling the animal to survive during the winter (Ortmann and Heldmaier, 2000). Metabolic rate is also altered in the Norwegian and Svalbard deer (Nilssen *et al.,* 1984). These animals have reduced metabolic rate, reduced serum thyroid hormones and reduced food intake during the winter. Similar studies have also been conducted in other species such as the penguin (Robin *et al.,* 1998) and the desert passerine bird (Webster and Weathers, 2000). As well as animals, although humans do not require such adaptive responses to the same extent, some individuals experience changes in energy expenditure. Sufferers of anorexia nervosa have reduced metabolic rate (Luke and Schoeller, 1992) and energy deficits in female athletes have been associated with higher body fat percentage (Deutz *et al.,* 2000).

1.2.2 Does genetics have a role in obesity?

The studies in animals mentioned above show that genes do exist that control the well-regulated process of energy expenditure. In humans, from an evolutionary perspective, given the importance of energy stores to individual survival and reproductive capacity (Frisch, 1990), the ability to conserve energy in the form of adipose tissue in times of excess food for use in times of food deprivation, would at one time have conferred a survival advantage (Neel 1962). Mutations resulting in decreased energy expenditure and also insulin resistance would have meant during periods of limited access to food the chances for survival would be increased. For this reason, humans are thought to be enriched with genes that favour energy intake and storage and diminish energy expenditure. However, in today's western living the combination of easy access to calorically dense foods and a sedentary lifestyle have made the metabolic consequences of these genes maladaptive. These genes have now become disadvantageous by their contribution to fat accumulation, obesity, hypertension and atherosclerosis. As far as 30 years ago, the idea of genetics having a role in obesity was shown with tube-fed animals (Han and Frohman, 1970) or in weanling animals (Frohman *et al.,* 1969) that were not hyperphagic yet still developed obesity. These studies proposed mechanisms other than hyperphagia had to be

involved in the development of obesity and already made discovering the molecular basis of obesity an arduous one.

Studies in twins (Stunkard *et al.,* 1986a, Stunkard *et al.,* 1990; Bouchard *et al.,* 1990), adoptees (Stunkard *et al.,* 1986b), and families (Bouchard *et al.*, 1994) indicate that as much as 80% of the variance in the body-mass index is attributable to genetic factors. Heritability is estimated to be as high as 30-40% for factors such as adipose-tissue distribution, physical activity, resting metabolic rate, changes in energy expenditure in response to overeating, certain aspects of eating behaviour, food preferences, lipoprotein lipase activity, maximal insulin-stimulated glyceride synthesis, and basal rates of lipolysis (Rosenbaum *et al.,* 1988; Bouchard *et al.,* 1994).

Also supporting a role for genetics in obesity are the number of single gene disorders which present obesity as one of their components. Such pleiotropic syndromes include Prader-Willi and Bardet Biedl syndromes. For example in the Prader-Willi syndrome individuals are severely hyperphagic resulting in severe obesity (Prader *et al.,* 1956). In more than 50% of cases, this syndrome is the result of a deletion in the Q12 region of the proximal portion of the long arm of chromosome 15 (Ledbetter *et al.,* 1981).

Among the most important concepts regarding the obese phenotype, as with many other syndromes, is the idea that relevant genes mediate susceptibility to disease in a specific environmental context. Thus, in any effort to elucidate the genetic basis for susceptibility to obesity, the 'environment' in which the obesity is occurring is also a critical factor regarding what sorts of genes are identified. Due to difficulties in obtaining experimentally defined/controlled environments of humans, and because attaining an obese state may actually rectify the metabolic differences predisposing to obesity, animal models of obesity enable researchers to identify clues to the relevant genes in humans. Over the past 45 years, a series of autosomal dominant (*agouti*) and recessive (*obese, diabetes, tubby, fat, fatty*) obesity mutations have been described in mice and rats, however only in the last 10 years have all these been elucidated. All have provided valuable clues to the role of genetics in obesity and will be discussed in section 1.5.

In light of the role of obesity in, evolution, twin studies, pleiotropic syndromes and rodent obesity models, it seems that the chances of susceptibility to obesity having a strong genetic component are high.

1.3 Leptin

Research in the pathophysiology of obesity has greatly intensified over the past 5 years since the discovery of the adipocyte-derived hormone leptin, the lack of which is responsible for severe obesity in *ob/ob* mice (Zhang *et al,* 1994). This result provided the first genetic framework on which the future understanding of body weight regulation and obesity could be built. Leptin, named from the Greek word '*leptos*' meaning 'thin', encodes a 167 amino acid protein whose crystal structure suggests that it belongs to the cytokine family (Zhang *et al.*, 1997; Madej *et al.*, 1998). Leptin is highly conserved in a number of vertebrate species, mouse and human leptin sequences being 84% identical at the amino acid level (Zhang *et al.*, 1994).

Released from adipose tissue, leptin has profound effects on appetite and energy expenditure by interacting with receptors in the hypothalamus to decrease food intake, increase thermogenesis and increase physical activity (Zhang *et al.*, 1994; Frederich *et al.*, 1995; Friedman, 1997a). The product of the leptin *(ob)* gene expressed in white adipose tissue is also found at lower levels, in gastric epithelium and placenta (Friedman *et al.*, 1998). As leptin is released from adipose tissue, levels increase exponentially with increasing fat mass (Lonnqvist *et al.*, 1995; Considine *et al.*, 1996). Leptin production is also thought to be greater in subcutaneous fat than in visceral fat depots (Montague *et al.*, 1997a; Lonnqvist *et al.*, 1997a).

1.3.1 The leptin receptor

Leptin receptors are found in many areas of the brain, including the hypothalamus, cerebellum, cortex, hippocampus, thalamus, choroid plexus and brain capillary endothelium (Steiner, 1996). In the rat hypothalamus, leptin receptor mRNA is densely concentrated in the arcuate nucleus and lower levels are present in the

ventromedial and dorsomedial hypothalamic nuclei and other brain areas known to be involved in energy balance (Schwartz *et al.*, 1996a). Tartaglia *et al.*, (1995) cloned the full-length rat leptin receptor and later Takaya *et al.*, (1996) identified 3 alternatively spliced isoforms, OB-Ra, OB-Rb, and OB-Re. The soluble leptin receptor isoform OB-Re circulates in the serum and functions as a leptin-binding protein (Houseknecht *et al.*, 1996; Sinha *et al.*, 1996; Cumin *et al.*, 1996; Murakami *et al.*, 1997; Muoio *et al.*, 1997; Auwerx and Staels, 1998). OB-Ra (OBR-S for short) and OB-Rb (OBR-L for long) are the two major splice variants and predict single transmembrane proteins (Takaya *et al.*, 1996). Leptin receptors are also expressed in peripheral tissues, including the lung, kidney, liver, pancreas, adrenals, ovaries, hematopoietic stem cells, skeletal muscle and stomach (Fei *et al.*, 1997; Sobhani *et al.*, 2000).

The leptin receptor is a member of the gp 130 family of cytokine receptors which are known to stimulate gene transcription via activation of cytosolic signal transducers and activators of transcription (STAT) proteins (Darnell *et al.*, 1994; Schindler and Darnell, 1995). Studies by Vaisse *et al.*, (1996) show that leptin injection activates Stat3 but no other STAT protein in the hypothalamus of *ob/ob* and wildtype mice but not in *db/db* mice, mutants that lack an isoform of the leptin receptor. This study also showed leptin did not induce STAT activation in other tissues known to express leptin receptors as mentioned earlier, such as the lung, kidney, adrenal and liver. These results indicated that the hypothalamus was a direct target of leptin action and that this activation was critically dependent on the gp-130 like leptin receptor isoform missing in *db/db* mice.

1.3.2 Defects in leptin and leptin receptor in the human population

Injection of leptin into the brain or bloodstream of wildtype rodents results in reductions in food intake and body weight (Pelleymounter *et al.*, 1995; Halaas *et al.*, 1997). These findings raised expectations that human obesity might be due to a leptin-deficient state that could also be treated with exogenous leptin. However, subsequent screening of several obese populations has only identified a handful of subjects with a mutation in leptin or the leptin receptor, as discussed below. The majority of individuals screened had very high plasma leptin levels correlating to

large adipose stores, which raised the suggestion, these individuals perhaps maybe leptin-resistant (Maffei *et al.*, 1995a; Considine *et al.*, 1996). It is most likely that control by leptin is therefore a one way system. It is effective in responding to low fat mass and works to cause an increase in food intake during times of fasting and starvation, but when excess fat mass and subsequent obesity are present, leptin no longer is able to regulate food intake and energy expenditure (Friedman and Halaas, 1998). Thus, excess leptin does not protect individuals from obesity.

Montague *et al.*, (1997b) reported the first case of leptin deficiency in two severely obese children both members of the same highly consanguineous Pakistani pedigree. Both children were normal weight at birth but rapidly gained weight thereafter experiencing severe hyperphagia. Subsequent DNA analysis revealed a homozygous frameshift mutation involving deletion of a single guanine nucleotide in codon 133 of the leptin gene in both children. Since the identification of their mutation, recombinant leptin treatment has resulted in a marked decrease in their food intake and body weight (Farooqi *et al.*, 1998). In 1998, Strobel *et al.*, described a Turkish family in which a homozygous missense mutation in the leptin gene resulted in low plasma leptin and morbid obesity in three affected members. A different family was reported by Clement *et al.*, (1998) describing a large consanguineous family of Kabilian origin in which 3 of 9 siblings had morbid obesity with onset in early childhood. The three affected sisters had normal birth weights, but developed severe obesity in the first few months of life. The sisters were found to be homozygous for a splice site mutation in the leptin receptor. This mutation led to a truncated form of the receptor lacking both transmembrane and intracellular domains thus having no signalling function. All these cases demonstrate the direct role of leptin and the leptin receptor in bodyweight regulation, although the mechanisms that control this have yet to be fully understood.

1.3.3 Gender differences in leptin levels

The individuals described above who are leptin-deficient or have inactivating mutations of the leptin receptor, are morbidly obese and have hypogonadotrophic hypogonadism (Clement *et al.*, 1998). This suggested leptin as having an important role in puberty and reproduction. Further analysis has also revealed a sexual

dimorphism in plasma leptin levels in humans (Maffei *et al.,* 1995a; Considine *et al.,* 1996). Even after adjustment for fat mass, women have higher plasma leptin levels than men (Ostlund *et al.,* 1996; Saad *et al.,* 1997,). The differences between males and females have been identified as early as 1 month of age, when female babies have higher plasma leptin levels compared to male babies (Lönnerdal and Havel, 2000). In normal children, leptin levels increase before puberty as body fat mass increases and reach their peak at the onset of puberty, suggesting that leptin may trigger puberty in humans (Garcia-Mayor *et al.,* 1997; Mantzoros *et al.,* 1997).

Thus, leptin previously hailed as the anti-obesity hormone has since been found to have a role in other endocrine functions and its complete role in metabolic and biochemical pathways has yet to be elucidated. The regulation of leptin and its involvement in various rodent obesity models, including the SLOB rat, will be discussed later.

1.4 Lipid metabolism: an overview

Since the realisation that adipocytes secrete leptin known to play a key role in appetite regulation, a much more complex and dynamic role for white adipose tissue (WAT) has progressively emerged. WAT is the major energy reserve in higher eukaryotes, and storing triglycerides in periods of energy excess and its mobilisation during energy deprivation are its primary purposes (Gregoire *et al.,* 1998). Thus, the regulatory machinery of adipose tissue is of vital importance and a potential contributor to the obese state. An understanding of the biochemistry of this machinery and the genes and factors involved in adipocyte differentiation would aid in understanding the cellular and molecular basis of adipose tissue growth in physiological and pathophysiological obese states. Described in this section are factors involved in adipogenesis and ones that have subsequently been investigated in the SLOB rat.

1.4.1 Triglycerides and Cholesterol

Stored fat is in the form of triglycerides (TG). Just as plasma leptin levels correlate with the amount of adipose tissue, a measure of plasma triglyceride also correlates with the amount of adipose tissue, thus obese individuals generally have both elevated leptin and triglyceride levels (Sirtori and Vega, 1997; Gregoire *et al.,* 1998). Cholesterol is a vital constituent of cell membranes and the precursor of steroid hormones and bile acids. It can be obtained from the diet or synthesised *de novo*, and in mammals the major site of cholesterol synthesis is the liver (Voet and Voet, 1990). In a healthy organism, an intricate balance is maintained between the biosynthesis, utilisation, and transport of cholesterol. Many epidemiological studies have shown a significant positive relationship between blood cholesterol levels and deaths associated with coronary heart disease (Keys, 1970; NIH Consensus Conference, 1985) and measurements of plasma cholesterol levels are commonly used to assess the risk of coronary heart disease. However, some researchers have obtained conflicting data, for example Genest *et al.,* (1991) reported that nearly 50% of patients with ischemic heart disease had plasma cholesterol levels equal to or even lower than those of healthy subjects. In view of these differences, more specific consideration of blood lipid variables were needed in order to assess risk with greater accuracy. Cholesterol is a hydrophobic compound, which together with triglycerides is transported in the blood by lipoproteins (Voet and Voet, 1990). Thus, an assessment of cholesterol-lipoproteins and TG-lipoproteins may offer a greater correlation with heart disease than total cholesterol and TG amount; this is discussed in the next section. **Figure 1.1** shows a model for plasma TG and cholesterol transport.

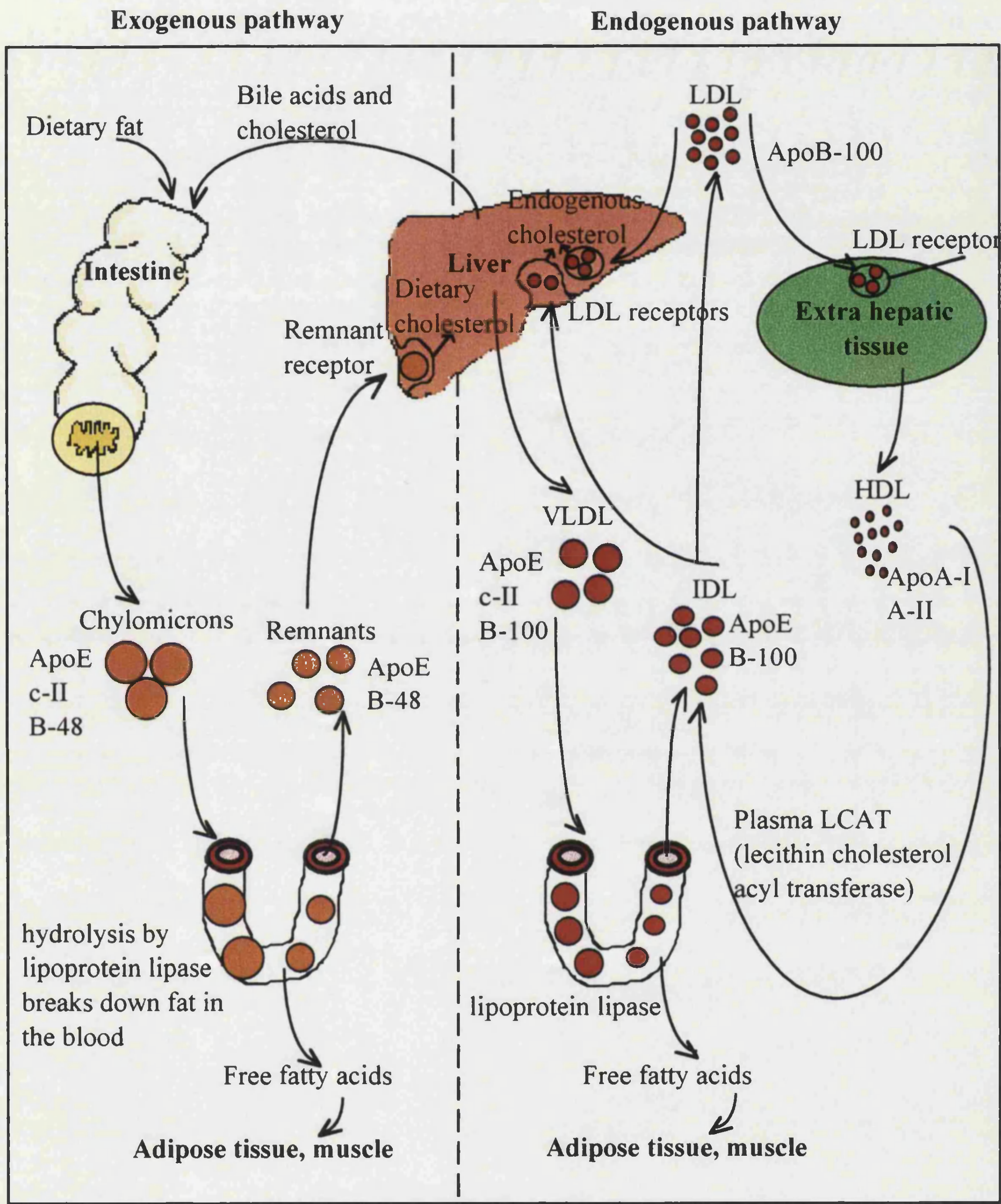


Figure 1. 1 A model for plasma triglyceride and cholesterol transport in humans (from Voet and Voet, 1990).

1.4.2 Lipoprotein metabolism

Lipoproteins vary in size, composition, and density and are thus named accordingly. Four main families can be identified; chylomicrons, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins HDL) (Voet and Voet, 1990). Chylomicrons are large particles found after a meal that are responsible for the transport of alimentary lipids. They are generally absent from the fasting plasma of healthy subjects. Triglyceride and cholesterol molecules of hepatic origin are secreted in VLDL particles, which are converted to LDL following hydrolysis by the enzyme lipoprotein lipase (LPL). During the hydrolysis of chylomicrons and VLDL, excess surface component aggregates to form nascent HDL particles. Additional newly formed and immature HDL particles originate from the intestine and the liver (Voet and Voet, 1990). The protein components of lipoproteins are known as apolipoproteins and are important determinants of lipid metabolism (Siest *et al.,* 1995). Several apolipoproteins have been identified, AI, B, D, E, H and J (Calvert and Abbey, 1985; Franceschini, 1996). LDL receptors located at the surface of hepatocytes bind and clear remnant lipoproteins from an intermediate binding site (Brown and Goldstein, 1986; Herz, 1993). The LDL receptor efficiently removes apoB100-containing LDL, as well as apoB48-containing remnants through interaction with apoE from the bloodstream (Choi and Cooper, 1993; Ishibashi *et al.,* 1993). The ratio of HDL to LDL cholesterol is important in assessing increased risk of coronary heart disease. Of 19 studies reviewed by Austin in 1991, 15 reported that HDL had a protective effect, three noted a tendency, and only one failed to observe a significant association between HDL-cholesterol levels and coronary heart disease. One component of dyslipidemia, secondary to obesity, results from increased hepatic VLDL (Grundy *et al.,* 1979; Kesaniemi *et al.,* 1985; Egusa *et al.,* 1985). Evidence for this contention are partly based on the increased free fatty acid (FFA) flux as obesity is known to supply increased amounts of FFA to the liver which could enhance the secretion of VLDL particles (Lönnqvist *et al.,* 1997). Thus many diabetic and obese humans have high plasma VLDL concentrations (Reaven, 1995; Sirtori and Vega, 1997).

1.4.3 Lipoprotein lipase (LPL)

Lipoprotein lipase (LPL) is located on the capillary endothelium of extrahepatic tissues and is involved in the hydrolysis of plasma triglyceride (Bensadoun, 1991). The ratio of adipose LPL to muscle LPL is thought to be of importance in adipogenesis. Such evidence has arisen from studies in transgenic mice (Weinstock *et al.*, 1995; Levak-Frank *et al.*, 1995) and responses in LPL activity in response to feeding and fasting (Eckel, 1989). These will be discussed in more detail in Chapter 3.

1.4.4 Peroxisome proliferator-activated receptors (PPARs)

The peroxisome proliferator-activated receptors (PPARs) are a group of closely related nuclear hormone receptors that control the expression of a large array of genes involved in adipocyte differentiation, lipid storage and insulin sensitisation (Spiegelman and Flier, 1996). Three distinct PPARs termed α, δ (also called β, NUC1 or FAAR) and γ, each encoded by a separate gene and showing a distinct tissue distribution pattern, have been identified (Kersten *et al.*, 2000). Whereas PPARα operates in the catabolism of fatty acids in the liver, PPARγ influences the storage of fatty acids in the adipose tissue (**Figure 1.2**). Thus, PPARγ is thought to be the key regulator of adipocyte differentiation and energy storage and is the most influential PPAR involved in obesity (Tontonoz *et al.*, 1994; Rocchi and Auwerx, 1999). Interestingly, PPARγ expression is highly enriched in subcutaneous fat in normal-weight subjects, whereas its expression in visceral adipose tissue is significantly higher in obese subjects (Lefebvre *et al.*, 1998). Recent mutations in PPARγ that lead to severe obesity have further validated this factor as having a key role in obesity etiology. A rare Pro115Gln mutation in the NH2-terminal ligand-independent activation domain of PPARγ was found in four very obese subjects (Ristow *et al.*, 1998). Furthermore, a much more common Pro12Ala substitution has been identified (Beamer *et al.*, 1998) and more recently, Valve *et al.*, (1999) reported two polymorphisms in the PPARγ gene which were associated with severe overweight among obese women.

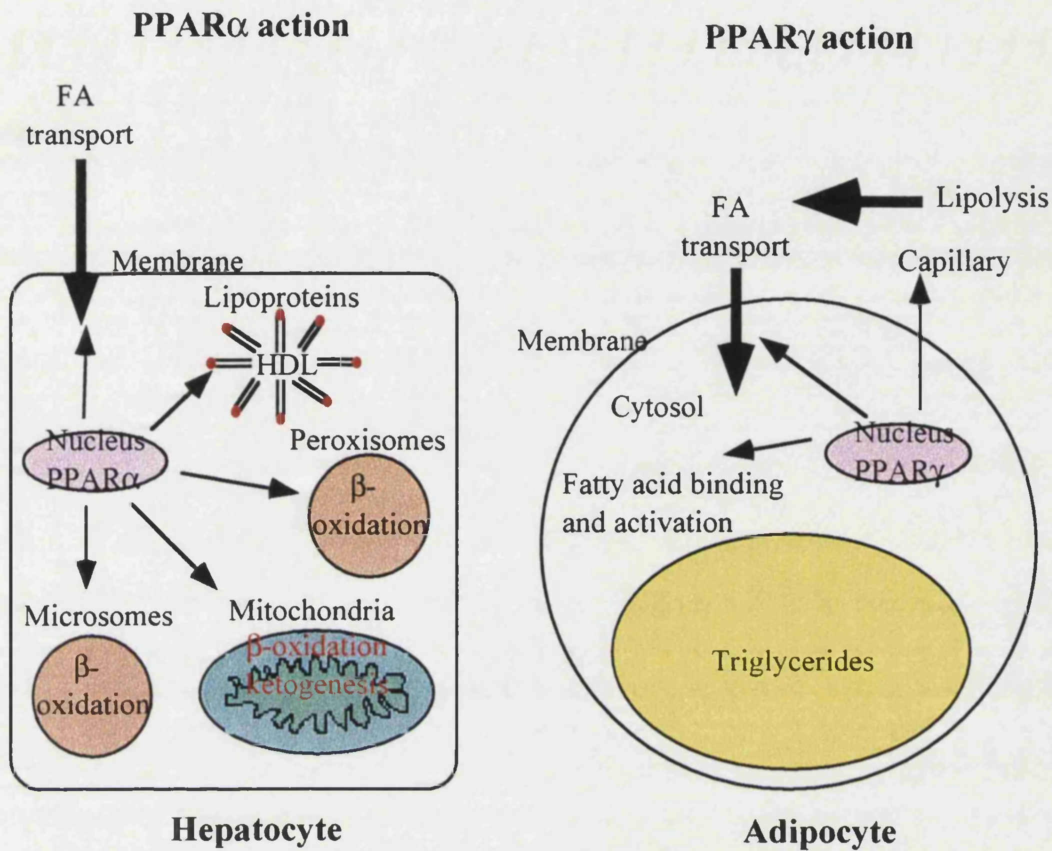


Figure 1.2 Action of PPARα and PPARγ at the cellular level. PPARα stimulates oxidation of fatty acids in various organelles, such as mitochondria, peroxisomes and microsomes. It also stimulates uptake of fatty acids and synthesis of lipoproteins. PPARγ stimulates lipolysis of circulating triglycerides and the subsequent uptake of fatty acids into the adipose cell. It also stimulates binding and activation of fatty acids in the cytosol, events that are required for synthesis of triglycerides. FA=fatty acid; HDL=high density lipoprotein (from Kersten *et al.*, 2000).

Thiazolidinediones (TZD) are a class of insulin sensitising drugs that have recently been used for the pharmacological treatment of insulin resistance and type 2 diabetes. Animal studies have shown that the administration of these drugs reduces plasma glucose levels in insulin resistant diabetic mice and rats (Spiegelman and Flier, 1996; Rocchi and Auwerx, 1999). As well as insulin resistance these drugs have also been shown to have other beneficial effects, such as, reducing plasma triglyceride levels, reducing obesity, increasing HDL, and decreasing blood pressure (reviewed by Olefsky, 2000). The mechanisms for how these drugs work were identified in 1994 by Ibrahimi *et al.,* who showed that TZD could behave as agonists for PPARγ. It is therefore thought that TZDs activate PPARγ receptors leading to the introduction of glucoregulatory molecules and enhanced insulin sensitivity.

1.5 Feeding Neuropeptides

1.5.1 The role of the hypothalamus in feeding behaviour

Hypothalamic obesity as a clinical syndrome has been recognised for 100 years. Two landmark reports by Frohlich in 1900 and by Babinski in 1901 documented the presence of tumours in the hypothalamus that were associated with obesity. These original descriptions were unclear as to whether the problem was of pituitary origin or involved destruction of both the pituitary and hypothalamus. However, later in 1927, Smith distinguished between hypopituitarism and hypothalamic injury by injecting chromic acid selectively into each area. It was the injections into the hypothalamus that were associated with obesity. Later Hetherington and Ranson (1940) showed that obesity could be produced following stereotaxic lesions which injured the ventromedial hypothalamus (VMH) and produced hyperphagia (Brobeck, 1946). In 1951 Anand and Brobeck demonstrated food intake decreased or was totally abolished after injury to the lateral hypothalamus (LH). Later still, small lesions in the paraventricular nucleus (PVN) (Fukushima *et al.,* 1987) and the ventromedial nucleus (VMN) as before (Parkinson and Weingarten, 1990), were also found to produce obesity. In addition, these observations led to the simple yet important conclusion that obesity in animals with such stereotaxic lesions was the result of overeating (Brobeck, 1946). All these findings led to the identification of the 'Dual

Centre' hypothesis: VMH/PVN lesions cause obesity - satiety centre, and LH lesions cause reduced food intake - feeding centre. As well as obesity resulting from hypothalamic lesions, various other endocrine disturbances have also been reported. These include the disruption of oestrus cycles (Chateau *et al.,* 1976; Waloch *et al.,* 1978), abnormalities in temperature control (Elmquist *et al.,* 1999) and a loss of the diurnal rhythm for insulin resistance (Lee *et al.,* 1991).

From all these studies it is obvious that the hypothalamus is an important centre for regulating feeding behaviour and other endocrine functions, the disruption of which has major consequences on maintaining normal homeostasis. Many researchers have since concentrated their efforts to this region and the many neuropeptides found here, in a search to elucidating the etiology of obesity and related disorders.

1.5.2 Feeding peptides – a short history

The field of peptides in the control of food intake and body weight began in 1957 with the report that peripheral injections of glucagon decreased food intake in humans (Schulman *et al.,* 1957). Since then and together with the data on the role of the hypothalamus and the discovery of leptin, numerous peptides, most of which are synthesised in the hypothalamus, have subsequently been identified and found to have a role in feeding behaviour (**Table 1**). The large array of neuropeptides has made it difficult to elucidate which of these are the most important and relevant in terms of feeding behaviour, however all have provided clues into obesity etiology. A particularly important piece of evidence involves the demonstration of degradation in the control of food intake when a specific antagonist, antibody, or antisense oligonucleotide to a peptide is administered. Such research and the direct administration of peptides have elucidated several important neuropeptides and the regulation by leptin on these peptides, which are summarised in **Table 2**. I have focused more on those neuropeptides that were analysed in SLOB rats. These were chosen as they have previously been measured in other rodent obesity models and were readily available as riboprobe or oligonucleotides in the lab. Also some of the neuropeptides when mutated in rodents cause similar phenotypes to that seen in SLOB rats.

Table 1.1 – Chronology of peptide effects on food intake after central or peripheral administration (adapted from Smith, 1999).

Peripheral	Central
1957 glucagon	1974 opioids
1973 cholecystokinin	1977 thyrotropin-releasing hormone
1979 bombesin	1979 cholecystokinin, insulin
1980 insulin	1981 bombesin
1981 somatostatin	1982 neurotensin
1983 neurotensin	1983 corticotropin-releasing factor
1984 calcitonin gene-related peptide	1984 calcitonin gene-related peptide, somatostatin
1987 tumour necrosis factor	1985 neuropeptide Y
1988 enterostatin	1986, galanin, α-melanocyte stimulating hormone
1989 interleukin 1-β	1988 interleukin-1 β, tumour necrosis factor α
1991 amylin	1991 amylin, enterostatin
1992 apolipoprotein AIV	1992 tumour necrosis factor β
1998 glucagon-like peptide-1	1993 apolipoprotein AIV, agouti protein
	1994 leptin
	1996 urocortin, glucagon-like peptide 1, melanin concentrating hormone
	1997 agouti related protein
	1998 orexins, cocaine and amphetamine regulated transcript

Table 1.2 Regulation of appetite by neuropeptides (adapted from Lawrence *et al.*, 1999).

Neuropeptide	Effect on feeding *	Regulation by leptin §
NPY	↑	↓
Orexin/Hypocretin	↑	↑↓
MCH	↑	↓
Galanin	↑	↓
AGRP	↑	↓
α-MSH/POMC	↓	↑↓
CRF	↓	↑↓
CART	↓	↑
Neurotensin 1	↓	↑

*icv administration of peptide either increases (↑) or decreases (↓) feeding. § Increased expression (↑) in normal animals treated with leptin (or decreased expression in the absence of a functional leptin pathway such as *ob/ob* or *db/db* mice or *fa/fa* rats); decreased expression in (↓) in normal animals treated with leptin (or increased expression in *ob/ob, db/db, fa/fa* rodents). Where conflicting evidence has been reported, this is denoted by ↑↓.

1.5.3 Neuropeptide Y

Neuropeptide Y is a 36 amino acid peptide that has emerged over the past 16 years as a possible key neurotransmitter candidate for the regulation of energy homeostasis (Gehlert, 1999). Synthesised throughout the brain, it is particularly abundant in the hypothalamus (Pesonen *et al.,* 1992; Billington *et al.,* 1994). Here it is synthesised largely in neurones whose cell bodies lie in the arcuate nucleus and send projections into surrounding hypothalamic structures. These structures include the paraventricular nucleus, where NPY is released from nerve terminals. Injection of NPY into the PVN of rats stimulates food intake and increases lipoprotein lipase activity in white adipose tissue while decreasing thermogenesis in brown adipose tissue (Billington *et al.,* 1994). Administration of human leptin in a dose-dependent manner partially or completely blocks feeding induced by exogenous NPY (Smith *et al.,* 1998) and reduces NPY mRNA arcuate expression (Gehlert and Heiman, 1997). These results were interpreted as indicating that NPY was a central effector of leptin deficiency; as NPY increased food intake and leptin decreased it, the action of leptin on NPY expression fit with the physiology of these peptides

There are 6 NPY receptor subtypes (Y1-Y6) (Gehlert, 1999). Efforts to understand the role of these subtypes in the feeding response are in an early stage. However, based on data from pharmacological, antisense ‘knockdown’ and targeted gene ‘knockout’ approaches, Y1 and Y5 receptors, located in the PVN seem to play an important role in feeding responses (Gerald *et al.,* 1996). Further work with specific receptor antagonists is required to fully understand the role of each receptor subtype in human appetite disorders and obesity.

1.5.4 Corticotropin-releasing factor (CRF)

Corticotropin-releasing factor (CRF) is a 41 amino acid protein and the principal neurohormone of the hypothalamic-pituitary-adrenal axis (Antoni, 1986). CRF is produced in the PVN and its effects on energy balance are catabolic in nature and oppose those of NPY (Heinrichs *et al.*, 1998). This neuropeptide co-ordinates the endocrine, behavioural, and autonomic adaptive responses to stress and is thought to function as a key mediator among the immune, neuronal, and endocrine systems

(Vale *et al.,* 1983; Antoni, 1986; Sapolsky *et al.,* 1987). It is now known that CRF also influences behaviours such as sexual activity, locomotion, grooming, and feeding (Morley and Levine, 1982). It is no surprise that a regulator of stress has a role in feeding behaviour. This is demonstrated by the effect of stress on food intake in humans; binge eating or caloric reduction is often evident during periods of stress. As well as in humans, animals also respond to stress by altering their eating behaviour. Loud sounds, isolation, confinement, electric shock, and tail pinch all lead to changes in feeding in rats (Levine and Billington, 1991). The effects of CRF on food intake are demonstrated by injection of CRF antisense and the functional impairment of hypothalamic CRF neurones with immunotargeted toxins which both increase food intake (Menzaghi *et al.,* 1993). CRF also decreases feeding stimulated by food deprivation or by administration of NPY (Levine *et al.,* 1983).

1.5.5 POMC and the hypothalamic-melanocortin system

Much evidence points to an important role for the hypothalamic-melanocortin (MC) system in the regulation of food intake and body weight. The MC system involves peptides that are processed from the polypeptide precursor, pro-opiomelanocortin (POMC), such peptides include circulating α-melanocyte stimulating hormone (α-MSH), adrenocorticotrophin (ACTH), and β-endorphin (Eipper and Mains, 1980; Cochet *et al.,* 1982). POMC is produced in the neurones of the hypothalamic arcuate nucleus where approximately 30% of the POMC neurones express the mRNA for the leptin receptor long form (Thornton *et al.*, 1997). POMC has also been detected in several peripheral tissues including skin (Slominski *et al.,* 1993), spleen and testis (Mechanick *et al.,* 1992).

(a) Melanocortin receptors

A family of five melanocortin receptor (MC-R) subtypes have been identified (Mountjoy *et al.,* 1992; Cone *et al.,* 1993) of which MC3-R and MC4-R are predominantly expressed in the hypothalamus (Adan and Gispen, 1997). Recent studies have shown that the melanocortin 4 (MC4) receptor and its peptide ligand, α-MSH, are important in the pathogenesis of obesity in the *agouti* mouse (Billington *et*

al., 1996; Spiegelman *et al.*, 1996). A highly selective MC4 receptor antagonist augments feeding in satiated animals and long-term blockade increases food intake and body weight gain leading to obesity (Kask *et al.*, 1998a, 1998b; Skuladottir *et al.*, 1999). In addition, at least 9 cases of mutations in the MC4-R leading to obesity have been identified in humans, indicating this receptor as a strong candidate gene more commonly involved in obesity (Perusse *et al.*, 1999).

(b) Agouti related peptide (AgRP)

AgRP is a 132 amino acid protein that is expressed in the arcuate nucleus and is an endogenous antagonist at the MC3-R and MC4-R (Fong *et al.*, 1997). Overexpression of AgRP in transgenic mice leads to obesity (Ollmann *et al.*, 1997; Graham *et al.*, 1997). The orexigenic actions of AgRP are confirmed by showing that icv injection of an AgRP fragment in rodents increases feeding in a manner similar to that of synthetic MC3/4-R antagonists (Rossi *et al.*, 1998) and that fasting stimulates AgRP in wildtype mice (Wilson *et al.*, 1999). It seems output from the feeding inhibitory MC4 receptor is thus determined by the ratio of agonist (α-MSH) and antagonist (AgRP) at MC4 receptor neurones.

1.6 Rodent Obesity Models

Obese rodents are the most studied experimental models for human genetic obesity. The availability of rodent models of obesity, as well as detailed knowledge of the correspondence between mouse and human genomic maps, has allowed rapid progress in identifying obesity genes (Perusse *et al.*, 1997; Chagnon *et al.*, 1999). Such models have also aided in identifying which of the hypothalamic neuropeptides are of most importance and in the identification and characterisation of leptin and its receptor. Currently the strongest candidates for genes involved in the development of obesity in humans are loci syntenic with mouse genes having defined mutations that result in obesity. The major Mendelian obesity syndromes in mice are summarised in **Table 1.3**, along with their modes of inheritance and chromosomal locations of corresponding human loci (Weigle and Kuijper, 1996). Some genes and specific mutations responsible for obesity at all five of these loci have now been identified by positional cloning and will be discussed here (Spiegelman and Flier, 1996; Weigle

and Kuijper, 1996; Schalling *et al.,* 1999; Pomp, 1999). In addition, a few obesity models have also been characterised in the rat. Most studied are the obese rat models exhibiting a mutation in their leptin receptor, namely the *Zucker* and *Corpulent* rats. Recent descriptions of the genetic defects responsible in these rat and mouse Mendelian obesity syndromes have provided an unprecedented opportunity for understanding both the physiological regulations of body composition and obesity. As with feeding neuropeptides, the pace of progress in this field has been very rapid, therefore an overview of the most studied models are presented here. More detailed results are reported elsewhere in this thesis when making direct comparisons with these models and the SLOB rat model.

Table 1.3 Major Mendelian obesity syndromes in mice (adapted from Weigle and Kuijper, 1996)

Name	Inheritance	Mouse chromosomal location	Human chromosomal location	Mutation responsible for obesity syndrome
Yellow (A^Y)	Dominant	2	20q13	Ubiquitous promoter
Fat (*fat*)	Recessive	8	16q22-24	Missense (Ser202Pro)
Obese (*ob*)	Recessive	6	7q31	Nonsense at Arg105
Tubby (*tub*)	Recessive	7	11p15.1	Unspliced intron
Diabetes (*db*)	Recessive	4	1p31	Splicing error

1.6.1 The *ob/ob* mouse

The *ob* mutation was the first of the recessive obesity mutations to be identified. In 1950 mice homozygous for mutations in the *ob* gene were shown to result in profound obesity and type II diabetes as part of a syndrome that resembled morbid obesity in humans (Ingalls *et al.*, 1950; Friedman *et al.*, 1991). Parabiosis studies suggested that the *ob/ob* mouse lacked a blood-borne factor that could regulate adiposity by modulation of appetite and metabolism (Coleman, 1978). As previously mentioned, in 1994 Zhang *et al.*, isolated the leptin gene, also called the *ob* gene, and it's human homologue by positional cloning. In fact the identification of leptin was a direct result of identifying the mutation responsible for the obese (*ob/ob*) mouse phenotype.

The mutation in these mice results in expression of a truncated inactive form of the leptin protein, thus an absence of this signalling protein results in overeating and mice

become obese, evident from an early age just after weaning. As well as obesity *ob/ob* mice also experience a number of other metabolic and endocrine disorders. Mice are diabetic, have various lipid abnormalities, experience fertility problems and reduced oxygen consumption (Kaplan and Leveille, 1974; Nishina *et al.,* 1994). However, many of these defects are corrected following leptin treatment, supporting the fact *ob/ob* mice are only deficient in leptin itself but leptin signal transduction and function are not impaired. Repeated injections of recombinant leptin reduces excessive bodyweight and normalises serum insulin levels and glucose tolerance in *ob/ob* mice (Pelleymounter *et al.,* 1995; Halaas *et al.,* 1995; Campfield *et al.,* 1995) and treatment with a recombinant adenovirus containing the mouse leptin cDNA also abolishes diabetes (Muzzin *et al.*, 1996). The complications of the *ob/ob* mouse re-emphasise the interplay leptin has with a number of other endocrine pathways as well as providing additional clues for the role of leptin in bodyweight regulation.

1.6.2 The *db/db* mouse

The second recessive model identified was the *db/db* mouse exhibiting an obesity phenotype almost identical to the *ob/ob* mouse (Hummel *et al.,* 1966). These mice were characterised by metabolic disturbances resembling those of maturity-onset diabetes mellitus in man and since its discovery this mouse has been used as an animal model of type 2 diabetes for over 30 years. Parabiosis studies with *ob/ob* and *db/db* mice indicated that *db/db* mice were defective in reception of leptin (Coleman, 1978). When *db/db* mice were linked to lean controls, they did not exhibit weight loss, however when *ob/ob* mice were linked to lean controls they did exhibit weight loss. As *ob/ob* mice were defective in leptin it seemed *db/db* mice were defective in signal reception at the level of the cell surface receptor. Further evidence was provided by the lack of response in *db/db* mice to recombinant leptin therapy, (Campfield *et al.*, 1995; Halaas *et al.*, 1995; Pelleymounter *et al.*, 1995; Stephens *et al.*, 1995). It was in 1996 that the specific mutation in the *db/db* mouse was fully characterised and found to be a G to T point mutation that resulted in the production of an abnormally spliced mRNA for the leptin receptor (Chen *et al.,* 1996). The result of this leads to a mutant protein lacking the cytoplasmic domain and thus defective in signal transduction.

1.6.3 The *agouti* mouse

The first murine obesity syndrome to be fully characterised at the genetic level was the obese yellow mouse (Bultman *et al.,* 1992). The phenotype of *agouti* mice, includes obesity, an all yellow coat colour, hyperinsulinemia, insulin resistance, a form of type II diabetes, and a tendency to develop a variety of spontaneous and induced tumours (Klebig *et al.*, 1995). The wildtype *agouti* gene is only expressed in skin, where it encodes a 131 amino acid protein with a consensus signal peptide (Bultman *et al.,* 1992). Normally, the *agouti* protein acts as a transiently secreted paracrine factor, which antagonises the interaction between α-melanocyte stimulating hormone (α-MSH) and its receptor on the melanocyte (Lu *et al.,* 1994). This regulated *agouti* expression results in black hairs with a subapical band of yellow.

In the mutant mouse, obesity is inherited through any of several dominant mutations at the *agouti* locus, resulting from translocation of DNA causing expression of the *agouti* protein in all cells. The most extensively analysed dominant mutations are the lethal yellow (A^{Y}) and the viable yellow (A^{VY}) (Yen *et al.,* 1994; Michaud *et al.,* 1997). Huszar *et al.,* (1997) and Fan *et al.,* (1997) investigated the primary mechanisms of *agouti* action concluding that the expression of agouti in *hypothalamic* cells as a result of this mutation antagonises the melanocortin-4 receptor (MC4-R) receptor which disrupts food regulation and results in an obese phenotype. Thus, whereas a controlled *agouti* expression in the skin of normal mice leads to black coat colour, an uncontrolled ectopic expression in mutant mice, particularly in the hypothalamus, leads to a yellow coat colour and obesity. The identification and characterisation of the *agouti* mouse also prompted researchers to identify the agouti related peptide (AgRP) as described earlier which causes obesity by mimicking the actions of *agouti* at the MC4-R in the hypothalamus (Fong *et al.,* 1997).

1.6.4 The *fat/fat* mouse

Mice homozygous for the recessive *fat* mutation are markedly hyperinsulinemic from the time of weaning and begin to develop obesity by 8 weeks of age. No sex differences are seen in the rate of weight gain in *fat* mutants in contrast to that seen with normal littermate controls where males are always heavier than females after

weaning (Coleman and Eicher, 1990). It was reported that the *fat* locus maps to within 0.08 centimorgans of the carboxypeptidase E gene on mouse chromosome 8, and that *fat/fat* mice lack carboxypeptidase E activity (Naggert *et al.*, 1995). This lack of enzyme activity results in an inability to remove the C-terminal arginine residues from insulin following C peptide cleavage, and leads to an increase in the ratio of proinsulin-related molecules to active insulin. The discovery of a Ser-to-Pro mutation at position 202 of the carboxypeptidase E gene of *fat/fat* mice, along with the demonstration that introduction of this mutation into wild-type rat carboxypeptidase E results in a loss of enzyme activity in vitro, strongly implicated mutant carboxypeptidase E as the *fat* gene (Naggert *et al.*, 1995).

1.6.5 The *tubby* mouse

Tub was first described in 1990 (Coleman and Eicher) and was the last of the spontaneous, monogenic mouse obesity genes to be cloned (Noben-Trauth *et al.*, 1996). This mutation was shown to segregate as an autosomal recessive with the phenotype characterised by a much slower progression to obesity than in *ob/ob*, *db/db*, or even *fat/fat* mice. In *tubby* mice the onset of obesity is not apparent until 9-12 weeks of age, whereas in *ob/ob* and *db/db* mice obesity is recognised as early as 18 days of age (Coleman and Eicher, 1990). The mild and late-onset weight gain in tubby mice resembles more closely the weight gain in human populations and made this new obesity model more appealing for study in human bodyweight regulation. As well as obesity, *tubby* mice also have insulin resistance but not progressing to overt diabetes, accompanied by retinal degeneration and neurosensory hearing loss (Heckenlively *et al.*, 1995; Ohlemiller *et al.*, 1997). The combination of these phenotypes resembles human syndromes, such as Usher's (retinal and cochlear degeneration) and Bardet-Biedl (obesity and sensory deficits).

The *tub* gene is as yet of unknown function. Northern analysis reveals high-level expression of the *tub* gene transcript in brain, testis and eye (Noben-Trauth *et al.*, 1996). The predicted amino acid sequence of the *tub* gene product is predominantly hydrophilic, suggesting a cytosolic localisation, and the carboxy-terminal portion of the protein demonstrates 62% identity to a putative mouse testis-specific phosphodiesterase (Noben-Trauth *et al.*, 1996). Thus, the expression of defective

tubby protein might be involved in cellular apoptosis since malfunction of phosphodiesterases has been documented to cause cell death (Noben-Trauth *et al.*, 1996). Positional cloning of the *tub* locus identified a 6.3kb gene transcript in wild-type mice and a 6.6kb transcript in mutant mice. The basis of the *tub* mutation was found to be a G-to-T transversion in donor splice site that eliminated splicing of the carboxy-terminal intron and resulted in a longer transcript (Noben-Trauth *et al.*, 1996). Studbal *et al.*, (2000) recently found that targeted deletion of the *tub* gene revealed *tubby* to be a result of a loss of function mutation.

Despite the *tubby* model resembling human obesity in terms of onset when compared to most other murine models characterised so far, no similar mutations have been identified in humans as yet. However, as this model is relatively new, future work may identify such mutations. At the outset of this thesis, rat *tubby* had not yet been cloned.

1.6.6 Rat models of obesity; the *Zucker* and *Corpulent* rats

As well as the *db/db* mouse, leptin receptor mutations have also been identified in two rat models of obesity, the fatty *Zucker* (*fa/fa*) rat and the corpulent (*cp/cp*) rat. The *fa* mutation arose spontaneously in 1961 and showed strong phenotypic similarities to the *db* and *ob* mouse mutations (Zucker and Zucker, 1961). The Zucker rat showed overt obesity, was hyperinsulinemic with normal blood glucose levels (Zucker and Antoniades, 1972) and developed adipocyte hypertrophy and hyperplasia (Johnson *et al.*, 1972). The *fa* locus, was mapped to a region syntenic with the mouse *db* locus (Truett *et al.*, 1991; Streamson *et al.*, 1996), which implied that the *fa* mutation lay within the rat leptin receptor gene. Subsequently, Phillips *et al.*, (1996) identified a missense mutation in the leptin-receptor gene of *Zucker* rats that most likely represented the *fa* mutation.

The fatty *Zucker* rat is a well established and frequently studied model of obesity, less well known is the *corpulent* rat. This model arose spontaneously in Koletsky's hypertensive rat strain (Koletsky, 1973) and the mutation was introgressed into the LA/N and SHR/N strains developed at the National Institutes of Health (Hansen, 1983). The *corpulent* rat was identified as being extremely prone to atherosclerosis

and had lipid abnormalities (Koletsky and Snadjer, 1979). Rats homozygous for the recessive *corpulent* gene are characterised by insulin resistance, hyperphagia, hyperinsulinemia, and hyperlipoproteinemia (Russell *et al.,* 1994; Mathe, 1995; Somanchi *et al.,* 1997). Elwood *et al.,* (1985) reported that the LA/N-*cp* rat, while similar to the *Zucker* rat, appeared to be more insulin resistant.

Based on the phenotypes of *fa/fa*, *cp/cp* and *fa/cp,* animals it was proposed that *fa* and *cp* were mutations in the same gene, the leptin receptor. Complementation analysis and mapping using microsatellite markers revealed that the rat corpulent (*cp*) mutation mapped to the same interval (*Pgm1-Glut1*) on rat chromosome 5 as the fatty *Zucker* (*fa*) mutation (Kahle *et al.,* 1997), implying that *cp* and *fa* were mutations in the same gene. The different phenotypes of *fa/fa* and *cp/cp* animals were thought to be due to the fact that the mutations were segregated on different rat strains. It was then shown that the *cp* mutation was due to a Tyr763Stop mutation in the leptin receptor gene (Wu Peng *et al.,* 1997).

1.6.7 Transgenic and knockout models of bodyweight regulation

With the identification of several genes found to have a role in obesity, for example, genes involved in lipid metabolism and genes for neuropeptides and various receptors, several researchers have generated transgenic and knockout animals to try to elucidate further the genetic basis of the obesity syndrome (Levine and Billington, 1998; York and Hansen, 1998; Inui, 2000). As well as models in which an obese or lean phenotype is expected, several models have also arose in other fields that unexpectedly present obesity or leanness as a component of the phenotype (Johnson *et al.,* 1991; Augustine *et al.,* 1999). A great number of transgenic and knockout models exist, some but not all show obesity as part of their phenotype. These include NPY knockout mice (Erickson *et al.,* 1996), MC4-R knockout mice (Huszar *et al.,* 1997), LPL knockout mice (Weinstock *et al.,* 1995) and overexpressing UCP-3 transgenic mice (Clapham *et al.,* 2000). These models together with others will be discussed elsewhere in this thesis.

1.7 The Transgenic SLOB rat

Presented in this section are a summary of the results from the thesis submitted by Sara Wells (1997) which relate to her findings of obesity in the SLOB rat. For commercial reasons these results remain unpublished except in the patent titled 'Obesity Gene', originally filed in August 1998 (I.C.A.F. Robinson, J.P. Stoye, D.M. Flavell, S.E. Wells, P.R. Le Tissier).

The SLOB rat is a transgenic animal which was initially generated to assess the mutual control and regulation of the hormones released from the posterior pituitary gland namely, vasopressin (VP) and oxytocin (OT). This was carried out by generating a construct for microinjection in which the reporter gene human growth hormone (hGH) was inserted into the vasopressin gene and bovine neurophysin (bNP) inserted into the oxytocin gene. Subsequently, 3 founder rats were generated, one of which died, one gave rise to the SLOB rat line and the other gave rise to a line of rats named JP59.

Wells confirmed that both SLOB and JP59 rats were expressing hGH mRNA and protein in the hypothalamus and posterior pituitary. However, no bNP mRNA or protein was detectable in pituitary, brain or peripheral tissue from either line of transgenic rats. Her further work therefore focussed on hGH expression and manipulation studies. She found that dehydrating SLOB rats increased hypothalamic hGH mRNA expression and concluded that hGH was released in response to stimuli for vasopressin release and thus both vasopressin and hGH were controlled from the vasopressin promoter. She also assessed vasopressin levels in SLOB animals, which were unchanged. Analysis of the transgene in SLOB and JP59 rats revealed that SLOB rats had at least four copies of the transgene whereas JP59 rats had only a single copy. This expression was reflected in hGH levels, where SLOB rats showed a much higher mRNA expression in the hypothalamus compared to JP59 rats.

In her investigation of the general physiology of these animals, Wells found that males from the SLOB rat line developed severe late onset obesity, evident at around 140-200 days of age. This result was completely unexpected. Female SLOB rats also

showed obesity but at a much later age (365 days). Both sexes of the JP59 line of rats showed no changes in overall growth. Before 140 days male SLOB rats were slightly growth retarded and weighed significantly less than normal littermates. Analysis of pituitary rat growth hormone (rGH) levels revealed that both male and female SLOB rats exhibited significantly reduced rGH levels at all ages tested. This growth retardation was also reflected in individual organ weights such as the liver, spleen and kidneys, and body length, which were all reduced in SLOB animals at 140 days, when male SLOB total bodyweights are indistinguishable from normal males. Reduced rGH was thought to be probably due to local hypothalamic release of the hGH protein in these rats that was interacting with the endogenous GH feedback system. JP59 rats did not show growth retardation as in SLOB rats and this was thought to be attributable to a lower hGH production from the reduced copy number of the transgene in these animals. In addition, prolactin levels in SLOB males were reduced. As hGH has lactogenic properties and can bind to the rat PRL receptor (Roupas *et al.*, 1989) this result was thought to be due to a hypothalamic feedback inhibition of hGH on the PRL system as documented in other transgenic rat models expressing a hGH transgene (Steger *et al.*, 1991; Phelphs *et al.*, 1997; Chandrashekar *et al.*, 1997).

Despite GH-deficiency in SLOB males, most bodyweights exceeded those of normal littermates, due to central obesity, as assessed by measurement of central and peripheral fat pads. Wells found that at 140 days of age SLOB males had larger central supra-renal fat pad weights and peripheral testicular fat pad weights compared to normal littermates. At 1 year of age only supra-renal fat pads were significantly greater in SLOB males. Thus SLOB males showed severe central adiposity.

Apart from the original SLOB male founder, subsequent male SLOB rats were infertile. The SLOB line was therefore bred through transgenic females mated with normal Wistar males to produce normal and hemizygous pups. JP59 rats did not show any abnormalities in fertility. Initial analysis of SLOB males could not identify any obvious cause of infertility; testes size and morphology were normal. Finally, Wells also found that SLOB male rats died approximately 6 months earlier than normal males.

It was not possible from the analysis carried out by Wells to determine the cause of obesity and infertility in SLOB males. It was clear however, that this phenotype was an autosomal dominant trait, inherited with the transgene and exhibited by every transgenic hemizygous member of the SLOB line of transgenic rats whilst wild-type littermates were normal. This was therefore the starting point for my work on the SLOB rat, and in this thesis I present my studies on the further physiological and genetic characterisation of this new rodent obesity model.

Chapter 2

Materials and Methods

2.1 Generation of rat and mouse transgenic lines

2.1.1. Generation of JP59 and SLOB rats

The transgenic rats investigated in this thesis had been produced as previously described by Sara Wells and Dave Flavell (Wells, S PhD. Thesis, 1997). Briefly, cosmids containing the rat VP and OT genes were isolated and restriction mapped. Reporter genes for hGH and bNP were inserted into subcloned fragments of the VP and OT genes and assembled into the cosmid cVO14 (**Figure 2.1(a)**). The construct was removed as a 44kb Notl insert and used to generate transgenic rats by pronuclear microinjection into oocytes harvested from superovulated rats and transferred into pseudopregnant adult Wistar recipients. The resulting litter pups were tailed at two weeks of age, DNA prepared using the method by Hogan *et al.,* (1986) and analysed for presence of the transgene using Southern blotting as described in 2.2.2. Three founder rats (two males and one female) were identified by Sara Wells. Unfortunately one of the males died, the remainder rats were used to breed two lines of transgenic animals; the male founder generated the SLOB line and the female founder the JP59 line. On my arrival at the NIMR, SLOB and JP59 stocks were limited; I was responsible for maintaining these lines in terms of setting up breeding pairs and genotyping all subsequent animals. The majority of this thesis is based on my work carried out on SLOB rats.

2.1.2 Generation of SLOB Mice

During the construction of the SLOB transgene, a *Not*I site was present in the construct that needed to be removed to allow excision of the whole construct as a *Not*I fragment for microinjection. This site was therefore removed by Wells and Flavell. Work carried out by Dr Paul Le Tissier at the NIMR later identified a novel gene which we called *5'OT-EST* and which was present in the SLOB transgene (cVO14). Furthermore, during the removal of the *Not*I site by Wells and Flavell, this gene had been truncated in SLOB rats. To investigate the role of this gene in SLOB obesity, Dr Paul Le Tissier generated a

construct containing only the truncated novel gene *5'OT-EST* in order to generate transgenic mice (**Figure 2.1(b)**). The construct was cloned into the vector PCRScript (Stratagene, Cambridge, UK) and excised as a 22.2kb *Sal*I/*Not*I fragment. Transgenic mice were generated using pronuclear microinjection in the same way SLOB rats were produced; this was performed by Dr Kathleen Mathers (NIMR). Details of *5'OT-EST* and its possible role in SLOB obesity will be discussed in Chapter 6.

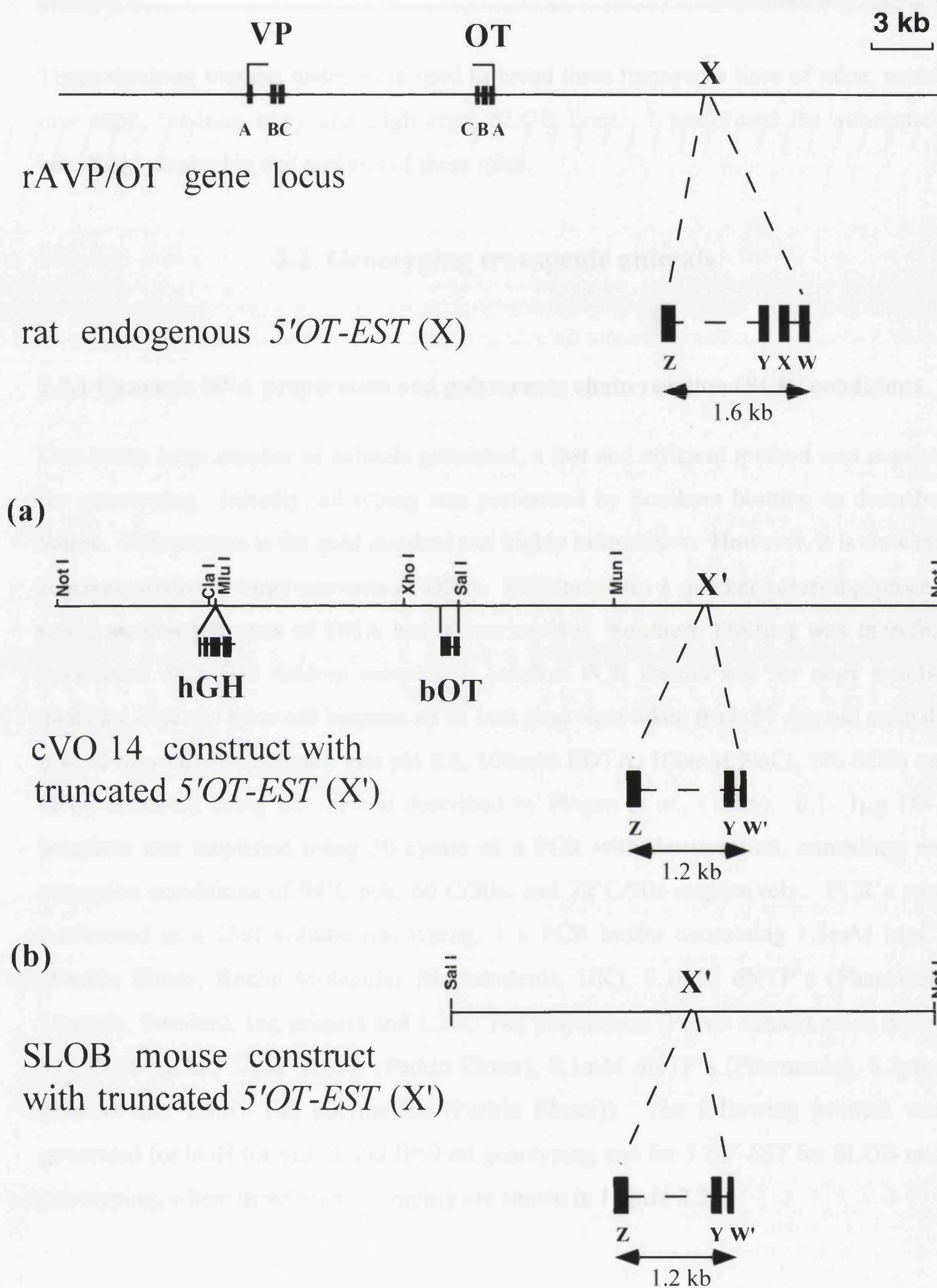


Figure 2.1 SLOB rat and SLOB mouse constructs. (a) cVO 14 was the original construct used to generate transgenic SLOB rats with the reporter genes hGH and bNP inserted into the vasopressin and oxytocin genes respectively. The construct was later found to contain the novel gene *5'OT-EST* (X) in a truncated form (X'). (b) The SLOB mouse construct produced by Dr Le Tissier only contains the truncated novel gene *5'OT-EST* (X').

Three resulting founder males were used to breed three transgenic lines of mice, named low copy, medium copy and high copy SLOB lines. I performed the subsequent breeding, genotyping and analysis of these mice.

2.2. Genotyping transgenic animals

2.2.1 Genomic DNA preparation and polymerase chain reaction (PCR) conditions

Due to the large number of animals generated, a fast and efficient method was required for genotyping. Initially, all typing was performed by Southern blotting as described below. This process is the gold standard and highly informative. However, it is slow and requires relatively large amounts of DNA. PCR provides a quicker screening process using smaller amounts of DNA but is less secure. Southern blotting was therefore performed on a few random samples to confirm PCR results and for copy number analysis. Rat and mice tail biopsies up to 1cm long were taken from 21 day old animals, placed into tail mix (50mM Tris pH 8.0, 100mM EDTA, 100mM NaCl, 1% SDS) and DNA extracted using the method described by Hogan *et al.,* (1986). 0.1- 1µg DNA template was amplified using 30 cycles of a PCR with denaturation, annealing, and extension conditions of 94°C/60s, 60°C/30s, and 72°C/90s respectively. PCR's were performed in a 25µl volume (rat typing; 1 x PCR buffer containing 1.5mM $MgCl_2$ (Perkin Elmer, Roche Molecular Biochemicals, UK), 0.1mM dNTP's (Pharmacia, Uppsula, Sweden), 1ng primers and 1.25U Taq polymerase (Perkin Elmer), mice typing; 1 x PCR buffer, 3mM $MgCl_2$ (Perkin Elmer), 0.1mM dNTP's (Pharmacia), 0.2pmol primers and 1.25U Taq polymerase (Perkin Elmer)). The following primers were generated for hGH for SLOB and JP59 rat genotyping and for *5'OT-EST* for SLOB mice genotyping, where these primers amplify are shown in **Figure 2.2**.

SLOB/JP59 rat primer sequences

hGH forward primer: 5' - AACCACTCAGGGTCCTGTGGACAG

hGH reverse primer: 3' – CCTCTTGAAGCCAGGGCAGGCAGAGCAGGC

SLOB mice primer sequences

5'OT-EST forward primer: 5' - GTCAGGAACTGAGAACAGAG

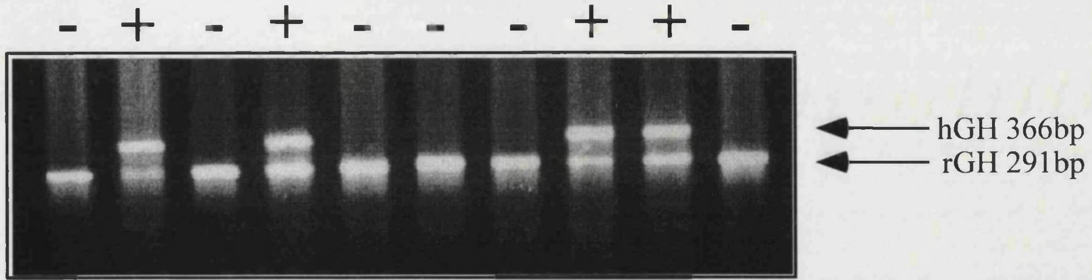
5'OT-EST reverse primer: 3' – GTGATAGGAACTTCCCAGAG

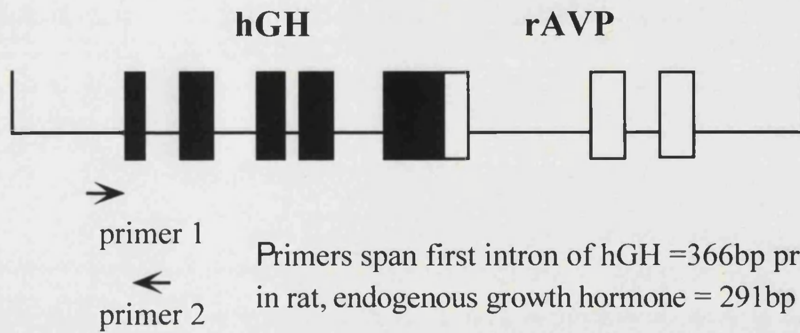
PCR products were separated by electrophoresis in a 2.5% agarose gel for rat genotyping and 3% Nusieve 1% agarose gel for mouse genotyping, both in 1 x TAE buffer containing 5ng/ml ethidium bromide (Sigma, Dorset, UK). Transgenic animals were identified as those having two products, one amplified from the endogenous gene and the second amplified from the transgene. This strategy is important as amplification from the endogenous gene acts as an internal negative control to test for DNA contamination and the presence of DNA in all samples tested. **Figure 2.2(a)** shows PCR results from a typical SLOB or JP59 rat screen. The smaller band (291bp) represents rat endogenous growth hormone, which is also amplified by the hGH primer pair. The larger band (366bp) represents hGH; the appearance of two bands therefore indicates a transgenic animal. **Figure 2.2(b)** shows PCR analysis of SLOB and normal mice using primers to amplify the rat exon Z region of *5'OT-EST*. Amplification of endogenous *5'OT-EST* produces a product which in mice is 19bp larger (234bp) than in rats (215bp). As a rat *5'OT-EST* construct was used, transgenic mice could therefore be identified as having two bands, one representing mouse endogenous *5'OT-EST* exon z and the other the 19bp smaller rat *5'OT-EST* exon Z.

2.2.2 Southern Blotting

Copy number analysis for identifying homozygous animals and confirmation of PCR genotyping, was performed by southern blotting. For rats, 10μg of genomic DNA was digested overnight with *Hind*III (Roche Molecular Biochemicals, East Sussex, UK) which cuts at sites flanking the AVP gene avoiding the hGH sequence. For mice, 10μg

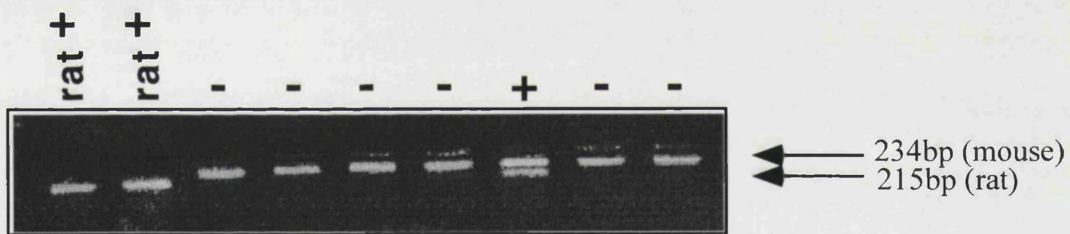
(a) Rat genotyping

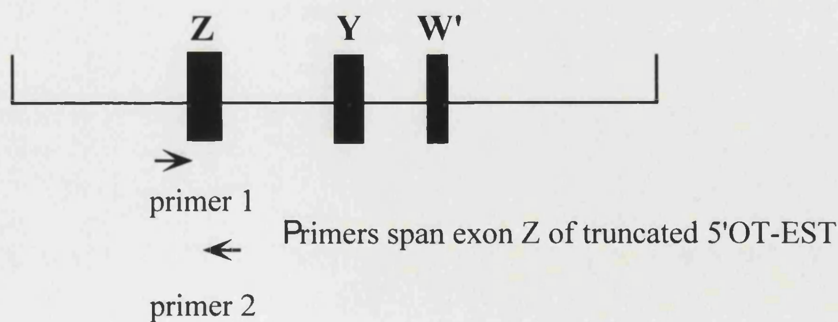




Primers span first intron of hGH =366bp product;
in rat, endogenous growth hormone = 291bp product.

(b) Mice genotyping





Primers span exon Z of truncated 5'OT-EST

<u>Figure 2.2</u> PCR analysis to genotype SLOB rats, JP59 rats and SLOB mice. (a) In rats PCR produces two products, a 291bp band corresponding to rat endogenous GH and a 366bp band corresponding to the hGH transgene. A lane with two bands therefore represents a transgenic animal (+). (b) Transgenic mice (+) are identified as having a second 19bp smaller band (corresponding to rat) when compared to the larger mouse endogenous 5'OT-EST band (234bp). PCR also amplifies the truncated 5'OT-EST as a 215bp band in transgenic rats. The endogenous 5'OT-EST rat band is not visible on this gel as the product is too large.

of genomic DNA was digested overnight with *Sac*I (Roche Molecular Biochemicals) which cuts at sites flanking *5'OT-EST.* Digested DNA was separated by electrophoresis on 0.6% agarose gels with marker DNA of known size fragments and then transferred to Hybond-N+ nylon membranes (Amersham, Bucks, UK) in 0.4M NaOH by capillary transfer method (Southern 1975, Sambrook *et al.,* 1989). Probes (intron 1 of rAVP for rats, and a cDNA clone of exons w-z of *5'OT-EST* for mice) were synthesised using the Prime-a-Gene labelling system (Promega, Southampton, UK) in accordance with manufacturer instructions, using a double labelling method which incorporates both ^{32}P-dATP and ^{32}P-dCTP. The membranes were placed in hybridisation buffer (5 x Denhardts (0.1% BSA, 0.1% Ficoll 400, 0.1% Polyvinylpyrollidine, 5 x SSPE (20xSSPE: 3M NaCl, 0.2M $NaH_2PO_4.H_2O$, 25mM EDTA, pH 7.4), 0.5% SDS, 250µg/ml salmon sperm DNA) and pre-hybridised for 4 hours at 65°C, then hybridised overnight in the same buffer plus denatured probe at 65°C. Blots were washed in 3 x SSC (20 x SSC: 3M NaCl, 0.34M sodium citrate) followed by 0.3 x SSC for 1 hour at 65°C before analysis using autoradiography. Autoradiography reveals two bands in transgenic rats, the smaller (3.4kb) corresponding to the endogenous rAVP gene and the larger band (5.2kb) representing the transgene with its hGH reporter gene insertion. Non-transgenic animals are obviously those with a single 3.4kb band. Since the affinity of the probe for the endogenous fragment should be the same as for the construct, phosphorimaging techniques could be used to compare directly the intensity of the transgene band with the endogenous band. JP59 homozygous animals were identified as having a transgene band with twice the intensity than the endogenous band (**Figure 2.3**). Southern blotting and phosphorimaging techniques also aided in copy number analysis in the three SLOB mice founder males (**Figure 2.4**). Two bands are visible in transgenic animals, the endogenous band as a 5.5kb fragment and the transgene band as a 1.9kb fragment (shorter due to deletion).

For all following procedures described where reagents and kits were used, these were carried out according to manufacturers instructions unless otherwise stated.

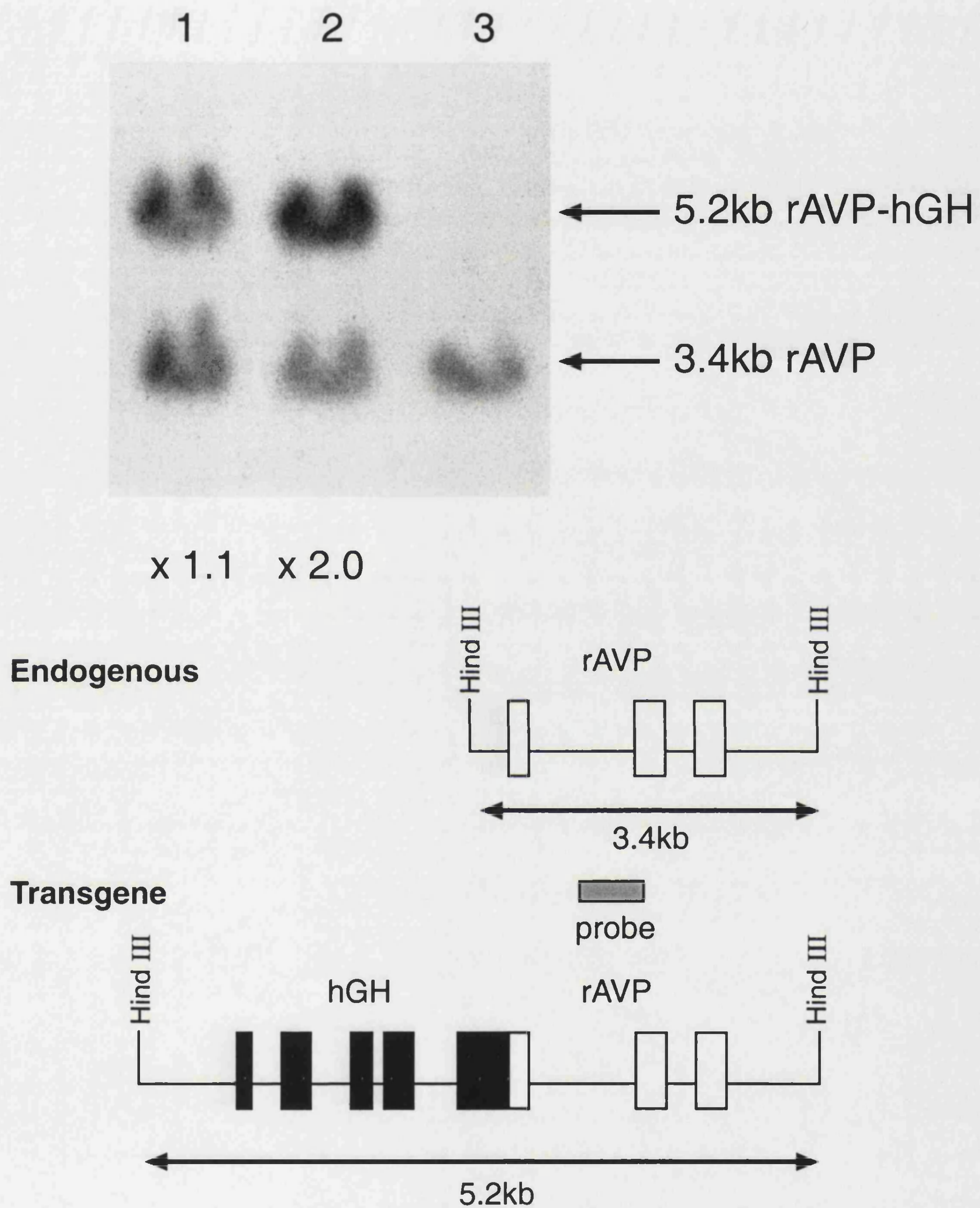
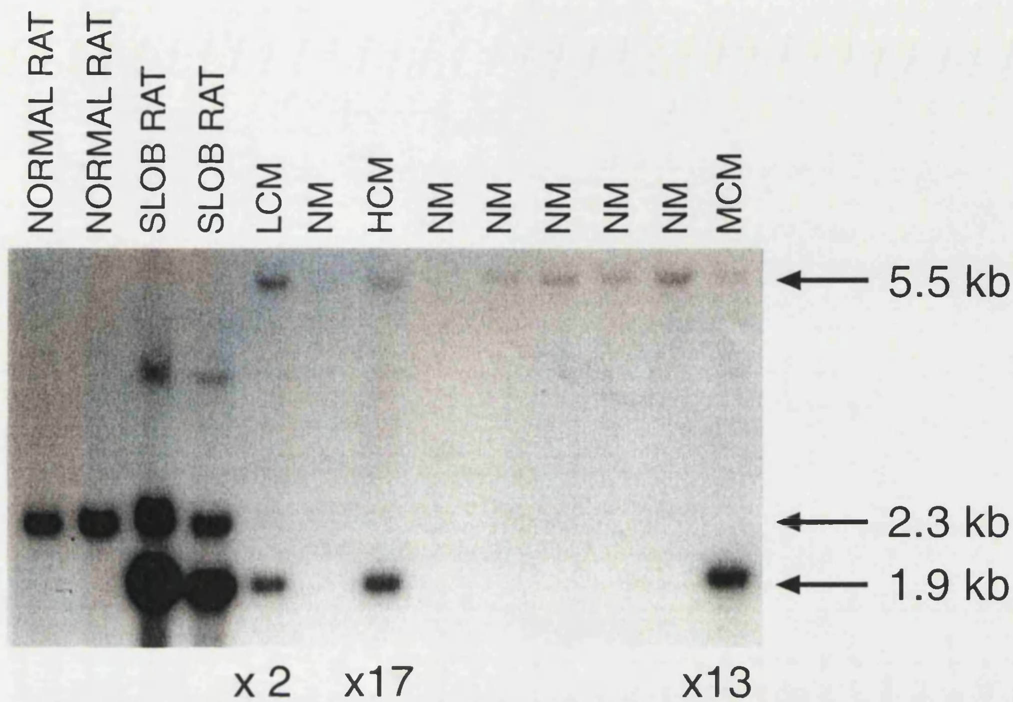


Figure 2.3 Southern blot of JP59 rat genomic DNA probed with a fragment of the rat AVP gene. The larger fragment (5.2kb) corresponds to the rAVP-hGH transgene and the smaller band (3.4kb) to the endogenous rAVP gene. The intensity of the two signals allows copy number analysis (x1.1 and x2.0). Lane 1 shows hemizygous JP59 DNA, lane 2 homozygous JP59 DNA and lane 3 normal rat DNA.



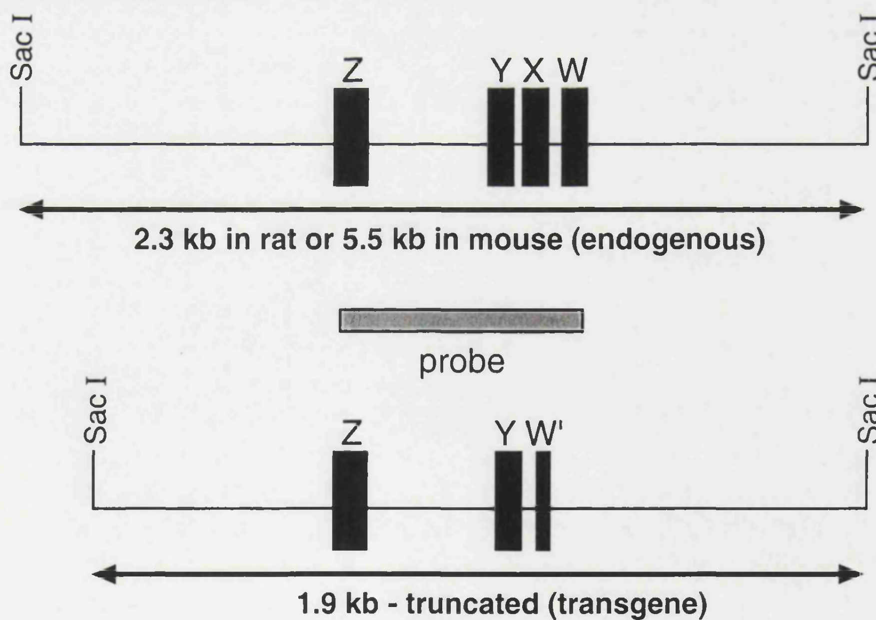


Figure 2.4 Southern blotted SLOB rat and mouse DNA probed with 5'OT-EST (exon w-z). Endogenous mouse 5'OT-EST appears as a 5.5 kb fragment, endogenous rat 5'OT-EST appears as a 2.3kb fragment. The transgene band in both mouse and rat appears as a 1.9kb fragment. The intensity of the two mouse bands allows relative copy number comparisons (LCM = low copy mouse, MCM = medium copy mouse, HCM = high copy mouse, NM = normal mouse).

2.3 RNAse Protection Assay

Tissue RNA was isolated using a Kinematica polytron PT 3000 homogenizer and extracted in TRIzol (Gibco BRL, Paisley, Scotland). RNA for fat was isolated using the single-step method of RNA isolation by acid guanidinium thiocynate-phenol-chloroform extraction described by Chomczynski and Sacchi (1987). Plasmid DNA was linearised using the relevant restriction enzymes, separated on 1% agarose gels and then purified using a Qiaex II gel extraction kit (Qiagen Ltd., West Sussex, UK). Riboprobes were generated by *in vitro* transcription using a SP6/T7 transcription kit (Roche Molecular Biochemicals) and α-^{32}P-UTP. Actin was used as an internal RNA control (RPA III, Ambion, Texas, USA) and a 151nt RNA probe corresponding to exon w (nt 1020-1171 of *5'OT-EST* in the vector pGEM-3Zf(+)). After labelling, probes were DNAse treated and separated on a 5% denaturing, polyacrylamide sequagel (National Diagnostics, Hessle Hull, UK) run for approximately 1.5 hours at 25 watts. The gel still attached to a glass plate was then covered in saran wrap and exposed to autoradiographic film for 2 minutes. Labelled RNA was visualised by autoradiography and the full length labelled probe cut from the gel and eluted by placing in elution buffer (RPA III, Ambion) and incubating for at least 3 hours at 37°C. After 3 hours 1μl of probe was counted in 5ml of scintillation fluid in a Beckman LS 5000CE counter and the relative amounts of probe required for each sample determined. Nuclease protection was performed using an RPA III kit (Ambion). Samples were separated on a 5% denaturing, polyacrylamide Sequagel (National Diagnostics) run for approximately 2 hours at 65 watts. Gels were dried under vacuum at 90°C for 10-15 minutes and then exposed to phosphorimager screens for at least 18-24 hours. The relative quantities of message in various samples were then determined using the computer software Image-Quant (Molecular Dynamics Inc.) and a STORM-860 scanner.

2.4 *In Situ* Hybridisation

2.4.1 Sectioning of brain tissue for *in situ* analysis

Brain samples were thawed slightly at room temperature and the anterior part of each brain in front of the hypothalamus removed. An incision was also made well behind the hypothalamus through the brain stem to provide a flat surface for mounting. Brains were mounted onto chucks in Cryo-M-Bed embedding medium (Bright Instrument Company Ltd., Huntington, UK) ready for positioning in a Bright Model OTF cryostat with chamber temperature held at -16°C. Thereafter, brains were laterally aligned using the anterior commisures as guides and distances between nuclei were mapped from the disappearance of the anterior commisure in the midline. Sections containing the periventricular (PeN), supraoptic (SON) paraventricular (PVN), arcuate (ARC), ventromedial (VMN) and dorsomedial (DM) nuclei were selected. In each case presence of particular nuclei was confirmed visually by briefly staining test sections in 1% toluidine blue (BDH Chemicals Ltd. Poole, UK) at the time of cutting. Coronal brain sections (12μm) were cut through the brain and mounted onto cold (placed in cryostat while cutting) gelatin and chrome alum-coated slides and then placed onto a warm hotplate for a few minutes before storing at -70°C until further use.

2.4.2 Sectioning of other tissues for *in situ* hybridisation

All other tissues apart from adipose tissue were sectioned at -16°C. In each case, tissue was cut until a uniform section representing the majority of the cell types in that tissue was achieved. 12μm sections were mounted onto cold gelatin and chrome alum-coated slides, placed onto a warm hotplate for a few minutes to fix the section onto the slide and then stored at -70°C. Adipose tissue was embedded in Cryo-M-Bed embedding medium onto chucks placed on dry ice. 12μm sections were cut at -30°C and mounted onto warm (room temperature) slides and again stored at -70°C until further use.

2.4.3 Preparation of DNA for labelling

Large scale isolation of plasmid DNA was performed using a Plasmid Maxi kit (Qiagen). 1μg of DNA was linearised using appropriate restriction enzymes. Following the reaction, cut DNA was purified using phenol/chloroform/isoamylalcohol (Sigma) and left to precipitate overnight in 100% ethanol containing 2% 4M NaCl. The following day DNA was centrifuged for 20 minutes at 13500rpm and the resulting pellet resuspended in 4.5μl RNAse-free water. 0.5μl of the DNA solution was mixed with 10μl of Orange-G loading buffer (Sigma, made in 40% sucrose solution) and separated on a 1% agarose gel for 30 minutes at 100 volts. Linear DNA was confirmed when a correctly sized fragment was observed.

2.4.4 Production of ^{35}S UTP-labelled riboprobes

Probes used and details are summarised in **Table 2.1**. cDNA riboprobes were produced using a SP6/T7 in vitro transcription kit (Roche Molecular Biochemicals). Antisense probes were generated and the corresponding sense RNA probe was produced as a negative control. Labelled probes were purified on Sephadex G50 columns (Pharmacia) and eluted using 10mM Tris (pH 7.5) / 0.1% SDS. 6-8 100μl aliquots were taken for each purification from which 1μl was counted in 5ml scintillation fluid in a Beckman LS 5000CE counter.

2.4.5 Production of ^{35}S ATP-labelled oligonucleotide probes

Oligonucleotides were obtained from Oswel DNA Services (Southampton, UK). These were labelled using the Terminal Transferase kit (Roche Molecular Biochemicals) according to manufacturer instructions. Unincorporated label was then removed using phenol/chloroform/isoamylalcohol extraction and the oligonucleotide left to precipitate overnight. The following day oligonucleotides were centrifuged at 13500rpm for 20 minutes, the pellet resuspended and 1μl counted in 5ml scintillation fluid in a Beckman LS 5000CE counter.

Table 2.1 Probes used for *in situ* hybridisation

Clone	Insert size	Vector	Enzymes used to cut/transcribe	Supplied by
hGH	751bp	pGem57f+	*Eco*RI (T3) antisense *Bam*HI (T7) sense	D Flavell, NIMR
OB-IC	316bp	pCR II	*Not*I (Sp6) antisense *Bam*HI (T7) sense	K Lindell, Gôteborg, Sweden
OB-EC	328bp	pCR II	*Not*I (Sp6) antisense *Bam*HI (T7) sense	K Lindell, Gôteborg, Sweden
CRF	770bp	pGem3Z	*Hind*III(T7) antisense *Kpn*I (Sp6) sense	M Grino, Marseille, France
POMC	397bp	pSP64	*Hind*III(Sp6) mRNA *Bam*HI (Sp6) cDNA	M Grino, Marseille, France
NPY	370bp	pGem5Zf+	*Spe*I (T7) antisense *Nco*I (SP6) sense	D Flavell, NIMR
GRF	500bp	PGem7Z	*Sma*I (Sp6) antisense *Bam*HI (T7) sense	D Flavell, NIMR
MC4-R	600bp	pBKS	*Sal*I (T7) antisense *Eco*RI (T3) sense	K Mountjoy, New Zealand
GH-R	2.9kb	pT7T318u	*Xba*I (T7) antisense *Spe*I (T3) sense	G Norstedt, Sweden
TUB	247bp	PCRII-Topo	*Not*I (Sp6) antisense *Bam*HI (T7) sense	P Le Tissier, NIMR
Exon W	145bp	PGem3	*Bam*HI (T7) antisense *Hind*III (SP6) sense	P Le Tissier NIMR
AGRP	oligonucleotide sequence: aggcagtgccaacagcagaacacaactcagcaacattgcagtcagcat			S Dickson, Cambridge
SS	oligonucleotide sequence: gacaccgcccaaagccaggacgatgcagagcgcggccagcgcgcactg			A Levy, Bristol

2.4.6 Pre-hybridisation of sections for riboprobe and oligonucleotide *in situ*

Frozen sections were thawed at room temperature for 10 minutes and then fixed for 5 minutes in freshly prepared 4% paraformaldehyde (Sigma) for riboprobes or 4% formaldehyde (Sigma) for oligonucleotides. Sections were then acetylated in 8.75% triethanolamine (Sigma) and 1.6% acetic anhydride (Sigma) for 10 minutes, then dehydrated through graded ethanol solutions, then delipidated in chloroform for 5 minutes. Finally sections were rinsed in 100% ethanol and allowed to air dry before hybridisation.

2.4.7 Hybridisation and post-hybridisation of sections for riboprobe *in situ*

Sections were hybridised overnight at 45°C, in buffer containing 1 x 10^6 cpm of denatured probe in hybridisation buffer (50% formamide, 0.025M Tris pH 7.5, 0.001M EDTA pH 8.0, 0.4M NaCl, 1 x Denhardts solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% BSA) and 10% dextran sulphate (MW 500,000), 10μg sheared single stranded salmon sperm DNA/ml and 5μg yeast tRNA/ml). Nescofilm (Bando Chemical Industries Ltd., Kobe, Japan) was cut into small squares and used as coverslips. Following overnight incubation, sections were washed in 2 x SSC three times at room temperature, twice in 2 x SSC/50% formamide at 45°C each for 15 minutes, rinsed briefly in 2 x SSC at 37°C, incubated in 2 x SSC containing 20μg RNase A/ml at 37°C for 30 minutes, rinsed in 2 x SSC, washed three times in 2 x SSC/50% formamide at 45°C for 15 minutes each, twice in 2 x SSC at room temperature for 5 minutes each, dipped in water and then 100% ethanol and air dried. Slides were exposed to autoradiographic film (Biomax MR, Kodak, NY, USA) for up to 14 days before quantifying integrated densities. The total integrated densities of hybridisation signal were determined by computerised densitometric scanning (NIMH Image Analysis, Bethesda, Md., USA).

2.4.8 Hybridisation and post-hybridisation of sections for oligonucleotide *in situ*

Sections were hybridised overnight at 37°C in 45µl of oligonucleotide hybridisation buffer (50% formamide, 4 x SSC, 1 x Denhardts solution, 10% dextran sulphate, 0.5mg/ml salmon sperm DNA, 0.25mg/ml yeast transfer RNA) containing 1 x 10^6cpm of probe per slide. Following incubation Nescofilm coverslips were gently floated off in 1 x SSC and sections washed in three changes of 1 x SSC. Sections were then washed in 4 changes of 1 x SSC at 55°C in a shaking waterbath, each wash lasting for 15 minutes, followed by two incubations for 30 minutes each at room temperature in 1 x SSC. Finally, sections were dipped in water and then 100% ethanol before air-drying and exposing to film for 5-7 days.

2.5 Hormone Analysis

2.5.1 Preparation of pituitaries for hormone analysis

Pituitaries were separated into anterior and posterior parts on removal from the animal and stored in 1.5ml microfuge tubes at –70°C until further use. Homogenisation was performed in a 1ml glass homogenizer in 1ml of PBS buffer (50mM NaH_2PO_4, 100mM NaCl, 0.6mM Thimerosal, pH 7.4).

2.5.2 Pituitary rGH, rPRL, rLH, rACTH, rTSH radioimmunoassays (RIA)

These were performed using reagents supplied by the National Institute of Health for Diabetes and Digestive and Kidney Disease (NIHDDK) in methods previously described by Robinson 1980, Horn *et al.,* 1985, and Fairhall *et al.,* 1992. I was responsible for making tracers for rat growth hormone (rGH) and rat prolactin (rPRL). Keith Fairhall in the lab kindly generated rat adrenocorticotrophin hormone (rACTH) tracer and Danielle Carmignac generated rat luteinising hormone (rLH) and rat thyroid stimulating hormone (rTSH) tracers. I subsequently performed all assays.

Table 2.2 NIHDDK antibodies and standards used for radioimmunoassays

ASSAY	ANTIBODY	FINAL ANTIBODY CONC.	STANDARD	STANDARD RANGE
rGH	rabbit anti-rGH NIHDDK	1:30,000	GH-RP-2, NIHDDK	16ng-1.6pg
rPRL	rabbit anti-rPRL NIHDDK	1:45,000	GH-RP-3, NIHDDK	16ng-1.6pg
rLH	rabbit anti-rLH NIHDDK	1:750,000	LH-RP-3 NIHDDK	10ng-20pg
rACTH	rabbit anti-rACTH NIHDDK	1:60,000	ACTH-RP-3, NIHDDK	10ng-20pg
rTSH	rabbit anti-rTSH NIHDDK	1:1.500.000	TSH-RP-3, NIHDDK	10ng-20pg

2.5.3 Plasma leptin and insulin assays

Plasma leptin and insulin concentrations were determined using commercial RIA kits (Linco Research, Inc., St. Charles, MO). The limits of sensitivity for the leptin and insulin assays were 0.5 ng/ml and 0.1 ng/ml respectively. To maximise use of these kits and to reduce the amount of plasma required, all reagent and sample volumes were halved throughout the procedure.

2.5.4 Plasma testosterone assay

Plasma testosterone concentrations were determined using a Coat-A-Count Total Testosterone kit from Diagnostic Products Corporation (Los Angeles, USA) with a detection limit of ~ 4ng/dL.

2.5.5 Plasma IGF-I assay

Plasma IGF-1 concentrations were determined by a hydrochloride acid-ethanol extraction RIA using recombinant rat IGF-I for labelling (Diagnostic Systems Laboratories, USA).

2.5.6 Plasma corticosterone assay

Plasma corticosterone concentrations were determined by a double antibody RIA kit available from ICN Biomedicals Inc. (Costa Mesa, USA). Again, reagent volumes were halved to obtain double the sample number per assay and reduce the amount of sample plasma required.

2.6 Lipid Analysis

The following work was carried out in collaboration with Professor Staffan Edén at the University of Gôteborg in Sweden. I was responsible for collecting and preparing plasma and tissues for dispatch to Sweden, where Fredrik Frick (PhD. student at the University of Gôteborg) carried out analysis of serum lipoproteins, lipoprotein lipase, apoB and apoE measurements. Lipolysis and VLDL secretion studies were carried out by myself together with Fredrik Frick whilst he was on a 3 month visit to the NIMR. I carried out all initial plasma analysis of triglyceride and cholesterol and fat cell size and number. Triglyceride and cholesterol levels were confirmed by Fredrik Frick in Sweden and fat cell diameters confirmed in live cells by him during his work at the NIMR.

2.6.1 Fat cell size and number

Adipose tissue was removed from various adipose depots and placed into Bouins solution (75% saturated aqueous picric acid, 20% formalin, 5% acetic acid). Following routine histological procedures samples were embedded in wax, sections cut using a microtome, and then stained with haemotoxylin and eosin dye. Cell size and number were then determined using computer software NIH Image and counting random frames of known area. Approximately 8-10 frames were counted and 30-40 cell diameters recorded per

tissue section. In addition, fat cell diameters were also determined in live fat cells according to the method of Smith *et al.*, (1972) using a haemocytometer, eyepiece graticule and light microscope.

2.6.2 Serum/plasma analysis

Plasma cholesterol and triglyceride concentrations were determined by enzymatic colorimetric assays in kits according to manufacturers instructions (Sigma). The intra-assay coefficient of variation (CV) was 4.6% for the triglyceride assay and 1.3% for the cholesterol assay. Serum apolipoprotein B (apoB) and apolipoprotein E (apoE) concentrations were determined in samples sent to Sweden, using electroimmunoassay as previously described (Sjöberg 1994). The inter-assay CV of the apoB determinations was 8.6% and intra-assay CV = 5.3%. Serum free fatty acid concentrations were measured by an enzymatic colorimetric method according to manufacturer instructions (NEFA C, Wako Chemicals GmbH, Neuss, Germany).

2.6.3 Total lipid content of liver

This procedure was again carried out by Fredrik Frick on samples sent to Sweden. Approximately 200mg of frozen liver was homogenised in ice-cold distilled water (6ml/g tissue). Total lipid was then extracted from the homogenate as previously described by Bligh and Dyer (1959). The extracted lipids were then re-dissolved in isopropanol (400µl) before total triglyceride and cholesterol concentrations were determined as above.

2.6.4 Size distribution of serum lipoproteins

Determination of size distribution of lipoproteins was performed by Fredrik Frick in Sweden by gel filtration using fast protein liquid chromatography (FPLC) (Pharmacia). Fractions containing lipoproteins were identified with a 280nm UV-detector, and stored at -20°C until determining triglyceride and cholesterol amounts as before.

2.6.5 Western blotting analysis

Western blotting for serum apoB and apoE were performed using enhanced chemiluminescence (ECL) by Fredrik Frick in Sweden.

2.6.6 Lipoprotein lipase (LPL) activity assay

LPL-activity in heart, soleus muscle and adipose tissue was determined according to the method by Peterson *et al.,* (1985) by Fredrik Frick in Sweden. The intra-assay CV for determinations in adipose tissue was 6%, and in heart was 5%. Activity was expressed as mU/g tissue (1 mU=1 nmol free fatty acid (FFA) released per minute).

2.6.7 Preparation of isolated adipocytes

Isolated fat cells were obtained in a method similar to that previously described by Rodbell (1964) with the modifications of Gause *et al.,* (1985). Immediately after an animal was culled, fat was removed from supra-renal and testicular adipose depots. This was then minced into smaller pieces with scissors and transferred to plastic vials containing the relevant buffer containing collagenase A (Sigma). For lipolysis experiments, the buffer used was Dulbecco's medium containing 4% bovine serum albumin (fraction V, Sigma). Vials containing fat were incubated for 1 hour at 37°C in a shaking waterbath. After digestion, liberated adipocytes were collected by filtration through a nylon mesh filter (250 μm) and washed three times with medium before using cells for lipolysis. Some cells were taken at this point for quantification of cell diameter, determined as previously described (Smith *et al.,* 1972), each observation being the mean of 100 cell diameters.

2.6.8 β-adrenergic stimulated lipolysis

Cell suspensions from 2.6.7 were diluted 1:2 with Dulbecco's medium. For each incubation, 100μl of the 50/50-cell suspension was added to plastic vials containing 2ml incubation medium (Dulbecco's), in the absence (basal lipolysis) or presence of

isoproterenol (Sigma), 10^{-7} M, $10^{-6.5}$ M, 10^{-6} M, $10^{-5.5}$ M and 10^{-5} M. In parallel, 100µl 50/50 cell-suspension was also added to Dole's solution for subsequent extraction of lipids (Dole and Meinertz, 1960). After incubation for 2 hours at 37°C, cells and medium were separated by centrifugation (2400 rpm, 3 min) through silicon oil (Kebo Lab, Spånga, Sweden). The glycerol content in the medium was then determined using an enzymatic colorimetric assay (Randox Laboratories, UK) and taken as an index of lipolysis. Lipolysis was expressed as nmol glycerol released per mg triglyceride.

2.7 Animal physiology experiments

2.7.1 Animal housing and general experimental procedures

All animals were housed in environment controlled conditions (12 hour light, 12 hour dark cycle) in designated animal houses. Unless otherwise stated animals were allowed free access to a standard chow diet (3.4% fat, 18.8% protein, 3.7% fibre, 3.8% ash and 60.3% carbohydrate – gross calorie content 15.6 MJ/kg (Special Diet Services, Witham UK) and tap water to drink. Animals were kept in plastic cages in-groups of 2-4; older animals were sometimes housed on their own depending on their bodyweight. On completion of an experiment, animals were anaesthetised using fluothane, weighed, and then decapitated to collect truncal blood. Blood was collected in tubes containing 20µl of heparin (10,000units/ml, Leo Laboratories Ltd., Bucks, UK), centrifuged at 3000rpm for 15 minutes and plasma supernatant aliquoted into 0.5ml microfuge tubes and stored at –20°C until further use. Organs were also collected, weighed if relevant, frozen on dry ice and stored at –70°C until further use. For all studies, control animals referred to as 'Normal' in this thesis are non-transgenic littermates from the same strain colonies.

2.7.2 Diet variation

High fat feeding and food restriction procedures were both carried out in individual metabolic cages (Tecniplast, Kettering, UK) to allow food intakes to be recorded. In both case's animals were placed in the cages for 3-4 days to acclimatise to the change in

housing conditions with free access to food and water. Once the experiment had started all animals were weighed at the same time each day and food intake's recorded and hoppers topped up at the same time. For fat feeding a diet consisting of ~30 % fat was used. This was achieved by purchasing a 60% fat diet (60% fat, 20.1% protein, 1.5% ash and 13.4% carbohydrate – gross calorie content 28.2 MJ/kg (Special Diet Services, Witham)) which was mixed with an equal proportion of chow diet to give a diet of approximately 30% fat (31.7% fat, 19.5% protein, 1.9% fibre, 2.7% ash and 36.9% carbohydrate – gross calorie content 21.9 MJ/kg).

2.7.3 Gonadectomy, ovariectomy and oestrogen treatment

Animals were gonadectomised or sham operated under fluothane anaesthesia at 10 weeks of age, housed in-groups of 2-3, and fed *ad libitum* on chow pellets and tap water. Body weight was recorded weekly or every 5 days. Animals receiving oestrogen were given 30μg in sesame oil every 5 days.

2.7.4 GH treatment

Osmotic minipumps (Alzet, Palo Alto, CA) were inserted subcutaneous under fluothane anaesthesia into 20-22 week old males. Pumps delivered a daily dose of 200μg of recombinant human growth hormone (Genotropin, Kabi Pharmacia AB, Milton Keynes, UK). 2 week-minipumps were inserted and replaced with new minipumps after two weeks. Treatment was therefore performed for a total of 4 weeks during which body weight was registered daily.

2.7.5 Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

Food was removed from animals at 9am on each procedure day, rats were later cannulated and procedure started at 2pm. Rats were anaesthetised with Sagatal (Rhône Mérieux Limited, Harlow, Essex) at a dose of 1μl/g bodyweight and then fitted with indwelling cannulae into the left jugular vein. Cannulae consisted of 20cm Esco silicone tubing with an internal diameter of 0.5mm (Bibby Sterilin Ltd., Stone, UK). For glucose

tolerance test tail veins were also cannulated using polythene tubing (Portex) attached to a 0.6 x 0.25mm sterile needle (Sherwood Medical, West Sussex.UK). For an intravenous insulin tolerance test, animals received 0.4U/kg bodyweight of insulin (Human Actrapid, Novo Nordisk) which was administered via the jugular vein after taking an initial basal blood sample. For an intravenous glucose tolerance test, animals received 0.5g/kg bodyweight of D-glucose injected into the tail vein after taking a basal blood sample from the jugular cannulation. Samples were then taken from all animals from the jugular vein at 5, 10, 15, 30, 45, 60 and 90 minutes after administration of glucose or insulin. 250μl of blood was collected in tubes containing heparin and immediately centrifuged to separate plasma, which was then analysed for glucose using a Beckman Glucose analyser. Plasma insulin levels were analysed by RIA as previously mentioned in 2.5.3.

2.7.6 In vivo hepatic VLDL-TG production using Triton WR1339

Food was removed from animals at 8am on the day of the procedure. Animals were then anaesthetised using an i.p. injection of sagatal (1μl/g bodyweight). Once animals were anaesthetised, a scalpel blade abraded the tail tip to allow bleeding. Approximately 600μl of blood was collected into microfuge tubes by milking the tail. The tail vein was then cannulated using polythene tubing (Portex) and a saline filled 1ml syringe and 0.6 x 0.25mm sterile needle placed at the end of each cannulae. Each animal was injected with Triton (WR1339 500mg/kg bodyweight made up as a 0.2g/dl solution in 0.9% NaCl), followed by approximately 200μl of saline. The time of injection was recorded and then 100μl of blood was collected every hour for a further 4 hours. Upon each collection, blood was centrifuged and the serum supernatant removed and aliquoted. Triglyceride was measured in serum samples using a kit available from Roche Molecular Biochemicals (TG-GPO-PAP). This kit was preferred over the previously used Sigma kit as many samples contained haemolysis which the Roche kit was not as sensitive to as the Sigma kit. This occurrence was probably due to the sampling technique and was the first and only time this procedure was carried out in SLOB and normal rats during my PhD. A more reliable method of multiple blood sampling is cannulating the jugular vein as was carried out for glucose and insulin tolerance tests. However, I was unable to do this for

this experiment as anti-clotting factors such as heparin and EDTA interact with lipoprotein lipase activity, which would interfere with VLDL-TG production.

2.7.8 Automatic sampling to obtain 24 hour profile of corticosterone measurements

This surgical procedure was carried out by Keith Fairhall. I assisted in this experiment and was responsible for preparing samples for analysis and the subsequent assaying of plasma corticosterone. Animals were anaesthetised with fluothane, and indwelling cannulae introduced into the left jugular vein, as previously described (Clark *et al.*, 1985). Blood samples were obtained by an automatic microsampling method (Clark *et al.,* 1986), and collected as 33% blood diluted in heparinised (20U/ml) saline. 10-12 animals were sampled simultaneously and 120-150μl of diluted blood collected every 30 minutes for 24 hours. 48 samples were collected from each animal, centrifuged to remove red blood cells, and the resulting plasma/saline supernatants frozen for subsequent assay of corticosterone as previously mentioned in 2.5.6.

2.7.9 Sperm number and motility

This procedure was kindly performed by Dr Kathleen Mathers at the NIMR. The canda epididymides and vas deferentia were dissected out from 4 male wildtype and 2 transgenic male rats into individual petri dishes. Fat and blood vessels were trimmed away and 5ml of phosphate-buffered-saline (PBS) buffer added to the remaining tissue. Sperm was collected by mincing the epididymes and squeezing the vas deferentia to allow the sperm to swim out into the surrounding buffer. The sperm were then incubated for 10 minutes at 37°C. Total sperm count (in 5ml) and percentage sperm motility was calculated from 6 counts/sample using a Makler sperm counting chamber.

2.8 Statistical Analysis

All data were analysed by the Student's t-test if the standard deviations of the two populations being compared were not statistically different. Where the standard deviations were different, the non-parametric Mann-Whitney U test was used. When

comparisons across a number of groups were made, data was analysed using Analysis of Variance (ANOVA) followed by a Student-Newmann-Keuls test. Numerical results and histogram data show mean values ± the standard error of the mean (SEM) unless otherwise stated. Significance levels are shown as $p < 0.05$ = *, $p < 0.01$ = ** and $p < 0.001$ = ***.

Chapter 3

Characterisation of the SLOB Rat Phenotype

3.1 Introduction

When I started my PhD., it was already known that SLOB rats showed a late-onset male-specific visceral obesity. SLOB animals were also growth hormone deficient and apart from the original founder male, subsequent males were infertile. Up until now little more was known about this model; presented in this chapter are my investigations on the SLOB rat that give an overall characterisation of the SLOB phenotype.

3.1.1 Defining a general obese phenotype in SLOB rats

In the thesis by Sara Wells, she concluded that male SLOB rats showed a slight but significant reduction in body weight up to 120 days of age ($p<0.01$). From 200 days onwards however SLOB animals began to weigh more than controls and abdominal obesity was apparent. The nature of the initial reduced bodyweight is discussed later in section 3.4, but is basically attributable to growth hormone deficiency in SLOB rats. Since these findings many more animals have been studied at a range of ages, and my results suggest that this bodyweight data may be an oversimplification as not all transgenic animals weigh more than their normal littermates (**Figure 3.1**). However, by 200 days of age all are obese in terms of their body composition, but bodyweight is offset because of a reduction in lean body mass in some rats. Since the majority of SLOB rats do weigh more, the mean bodyweight changes are significant by 400 days of age in male rats (**Figure 3.2**).

Measuring various adipose tissue depots gives an indication of where the primary obesity lies in SLOB rats. Supra-renal fat pads and testicular fat pads are taken as indicators of central and peripheral adiposity respectively. These depots were routinely chosen as measures of adiposity in male animals at the termination of each experimental study. The extent of obesity in terms of fat pad weight in 1 year old SLOB males is shown in **Figure 3.3**. There was no difference in testicular fat pad weight compared to age-matched normal rats but there was a significant increase in supra-renal fat pad weight, demonstrating the extent of the abdominal obesity.

(a)

(b)

Figure 3.1 SLOB and normal littermates. Age matched male littermates (2 yrs of age), in both photos the normal animal is shown above the SLOB animal. (a) Littermates with different bodyweights and different body compositions. (b) Littermates with similar bodyweights but still different body compositions.

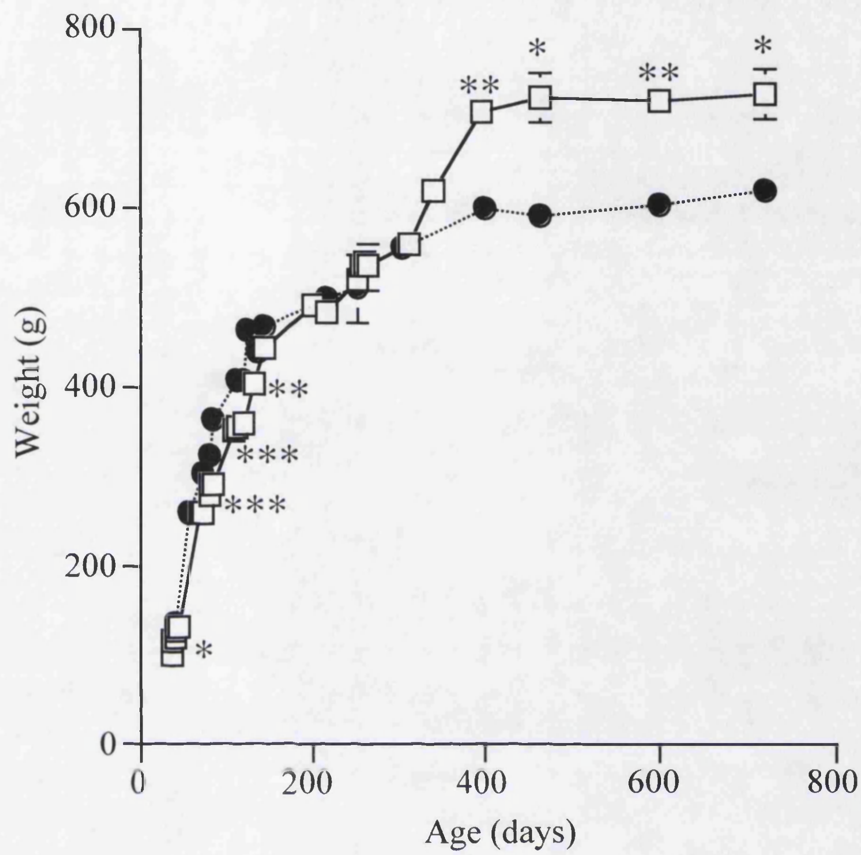
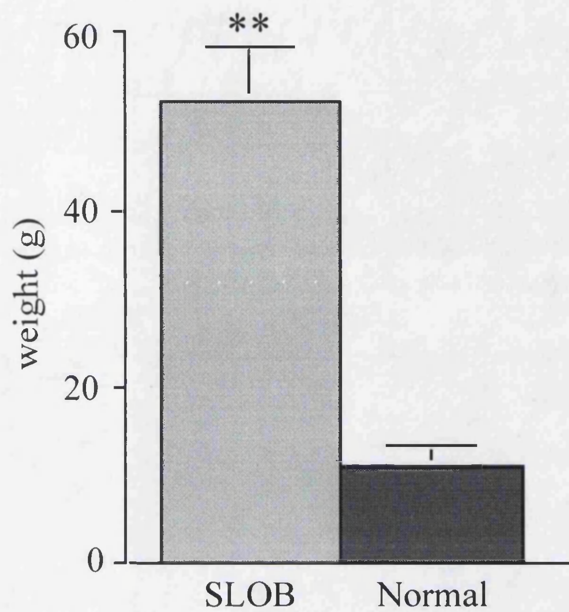


Figure 3.2 Body weight in male SLOB and normal rats. (*p<0.05, **p<0.01, ***p<0.001). —□— SLOB ····●···· Normal

(a)



(b)

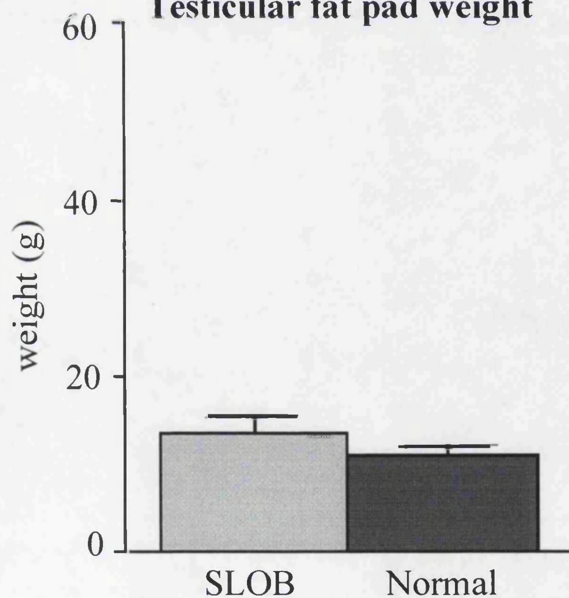


Figure 3.3 Fat pad weights in SLOB and normal rats. (a) Supra-renal fat pad weight in male SLOB and normal animals at 1 year of age (**$p<0.01$ n=6). (b) Testicular fat pad weight in male SLOB and normal animals at 1 year of age (n=6).

Mesenteric (omental) adipose tissue is also a good indicator of abdominal obesity, although this fat pad was not routinely taken following experimental studies since it is more difficult to dissect. Nevertheless, it was measured on a few occasions. At 1 year of age SLOB rats have greater mesenteric fat mass compared to normal rats, however the difference was not as marked as seen in the supra-renal fat pad mass of the same animals (SLOB mesenteric 9.34±1.0g vs normal mesenteric 6.7±0.5g $p<0.05$ compared to SLOB supra 13.7±1.3g vs normal supra 3.26±0.4g $p<0.0001$). A simple marker of fat cell mass is a measure of plasma leptin levels. Assuming there are no abnormalities in leptin production or elimination, the amount of leptin is proportional to the amount of adipose tissue (Lonnqvist *et al.,* 1995; Considine *et al.,* 1996). In 1 year old SLOB males plasma leptins are significantly higher than normal animals (**Figure 3.4 (a)**).

So far, I have discussed the SLOB phenotype in male rats. Although the phenotype is by far most predominant in males, females do show some obesity at a later age. This is in concordance with females in the human population who show visceral obesity after the menopause (Colombel and Charbonnel, 1997). In 1 year old SLOB females, leptin levels were also significantly greater than in normal littermates (**Figure 3.4 (b)**). However, this increase was not reflected in supra-renal fat pad weight or ovarian fat pad weight (**Figure 3.5**), neither of which showed a significant difference compared to normal female fat pad weights. The overall difference in SLOB phenotype is depicted in **Figure 3.6** which shows weight gain in SLOB and normal, male and female rats. The extent of the obesity is much greater in males compared to females confirming the observations of Sara Wells. Although my data shows female SLOB rats do not weigh significantly more than normal females until about 500 days of age, by 365 days they begin to show an obesity phenotype in terms of overall body composition.

3.1.2 Some simple metabolic analyses

One of the most obvious parameters to assess when a new obesity model is discovered is to measure food intake and determine whether obese animals are hyperphagic. Food intake measured before and after severe obesity is evident shows

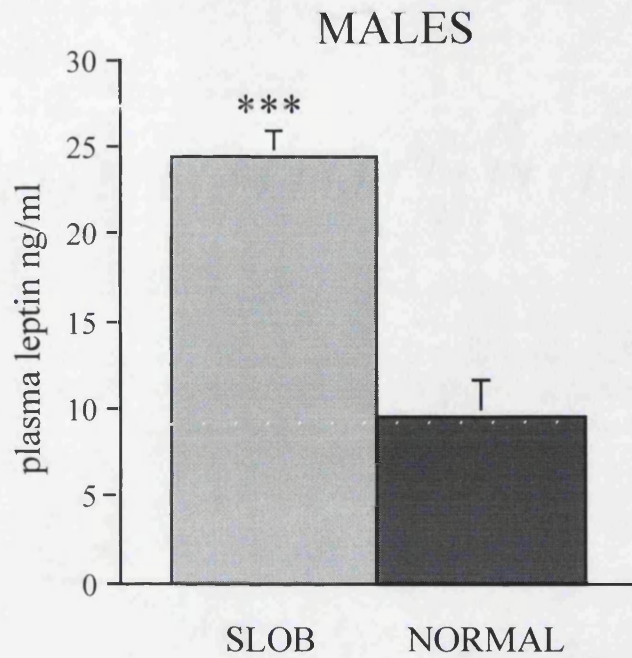


Figure 3.4 (a) Plasma leptin levels in male SLOB and normal rats. Measured in animals at 1 year of age (***=$p<0.001$, n=6).

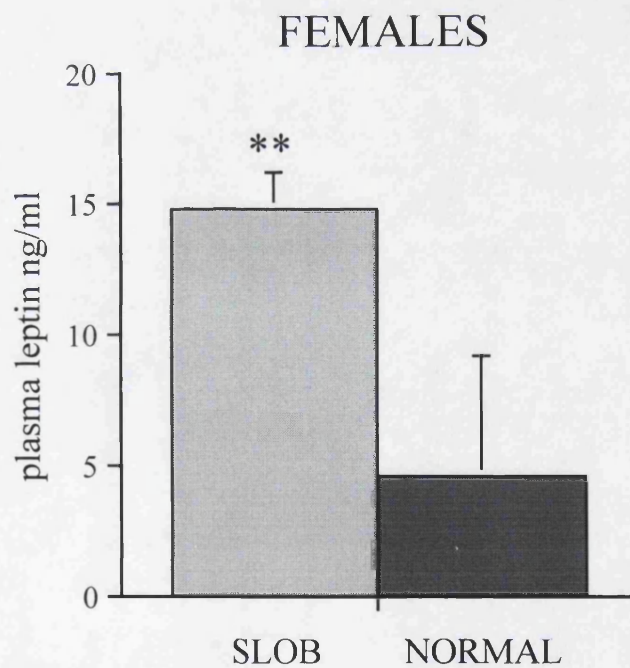
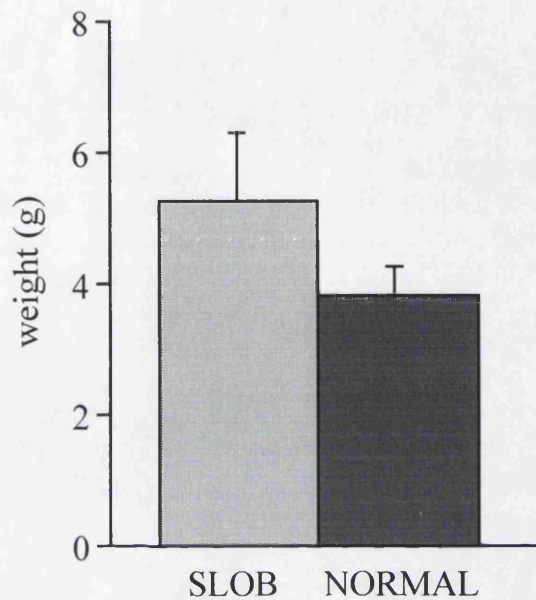


Figure 3.4 (b) Plasma leptin levels in female SLOB and normal rats. Measured in animals at 1 year of age (**=$p<0.01$, n=6).

(a)



(b)

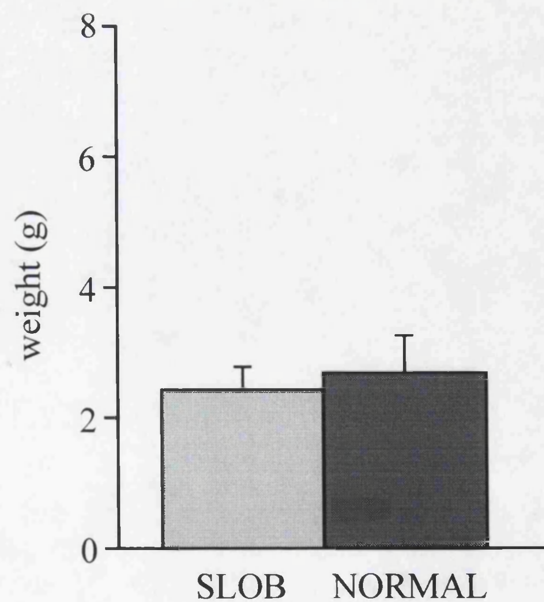
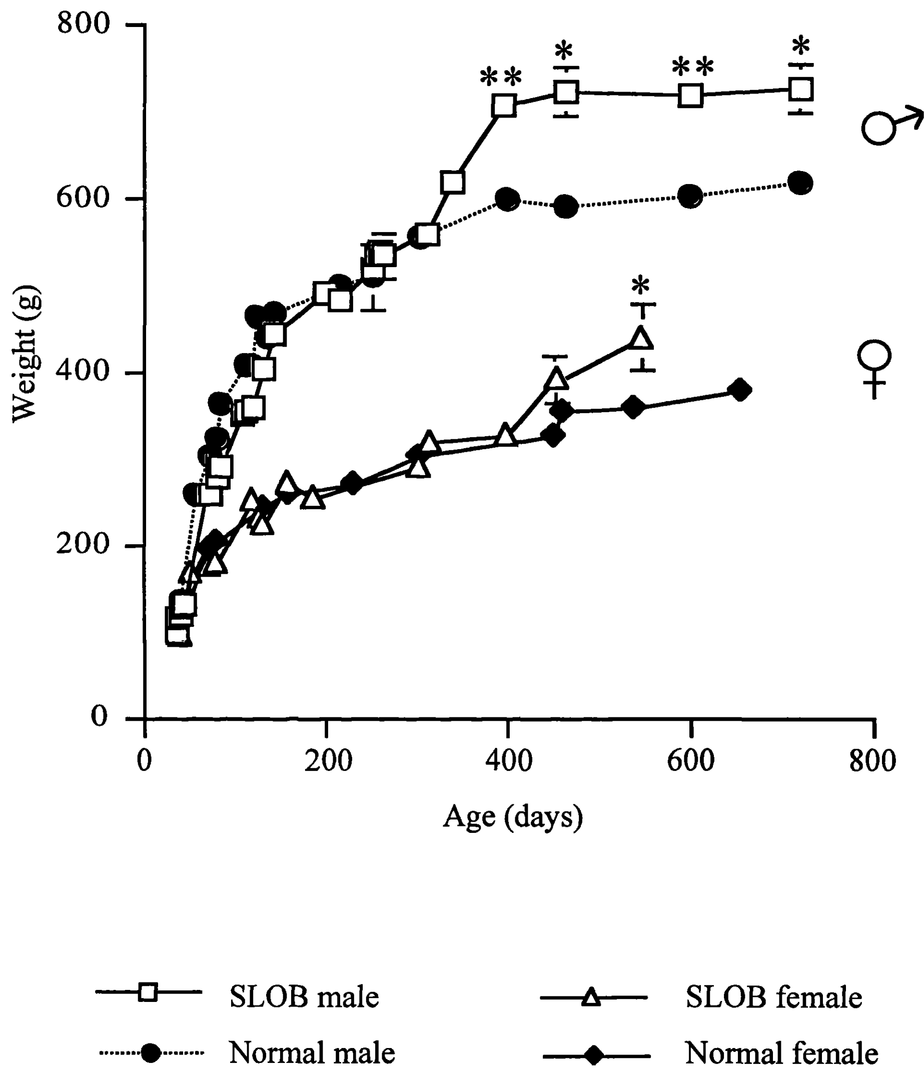


Figure 3.5 Fat pad weights in SLOB and normal female rats. (a) Supra-renal fat pad weight in animals at 1 year of age (p=0.06, n=6). (b) Ovarian fat pad weight in animals at 1 year of age (n=6).



<u>Figure 3.6</u> Body weight in male and female SLOB and normal rats. (*p<0.05, **p<0.01, ***p<0.001).

SLOB males are not hyperphagic (**Figure 3.7(a)**). However, when calculated per 100g bodyweight, and as SLOB rats weigh less than normal littermates at a younger age, food intake was significantly greater than normal animals at 100 days of age (**Figure 3.7(b)**). Thus SLOBs are hyperphagic at a young age before gross obesity is apparent, but not at an old age when severe obesity is evident. As energy input overall is the same in severely obese transgenic and non-transgenic animals, it therefore suggested there must be a difference in energy expenditure. I have not yet attempted to assess this directly but some simple indicators of metabolic state would be to assess the thyroid axis and to measure body temperature. Pituitary thyroid stimulating hormone (TSH) levels were normal in 1 year old male and female rats (male SLOB 4.7±0.47μg/pit vs. normal 5.1±0.61μg/pit; female SLOB 3.1±0.39μg/pit vs. normal 2.6±0.88μg/pit). In male 1 year old rats, (a) conscious rectal temperatures, (b) anaesthetised rectal temperatures and (c) anaesthetised core temperatures were all measured separately. All three measurements showed no difference between SLOB and normal rats (SLOB vs normal (a) 35.5±0.6°C vs 35.8±0.4°C; (b) 36.3±0.2°C vs 36.0±0.5°C; (c) 37.0±0.1°C vs 37.2±0.3°C). These preliminary investigations showed no statistically significant differences.

3.2 How early is the phenotype detected?

Although it was previously reported that the SLOB phenotype is not evident until around 200 days, this was based on little data and only as overt phenotype. I therefore sacrificed groups of animals at a younger age to evaluate this issue. 80 day old male and female SLOB and normal animals were sacrificed and various measurements taken (**Figure 3.8**). Both testicular and supra-renal fat pad weights and plasma leptin levels were significantly greater than normals in male SLOB rats. At this age both adipose depots are greater in weight compared to normal animals, however, at a later age only the supra-renal fat pad weight in SLOB males is significantly greater (**Figure 3.3**). Therefore at a young age SLOB obesity is apparent in both abdominal and peripheral fat pads but at an old age is expressed predominantly as visceral obesity. This could possibly be due to a developing GH-deficiency, which may favour fat deposition in abdominal rather than peripheral

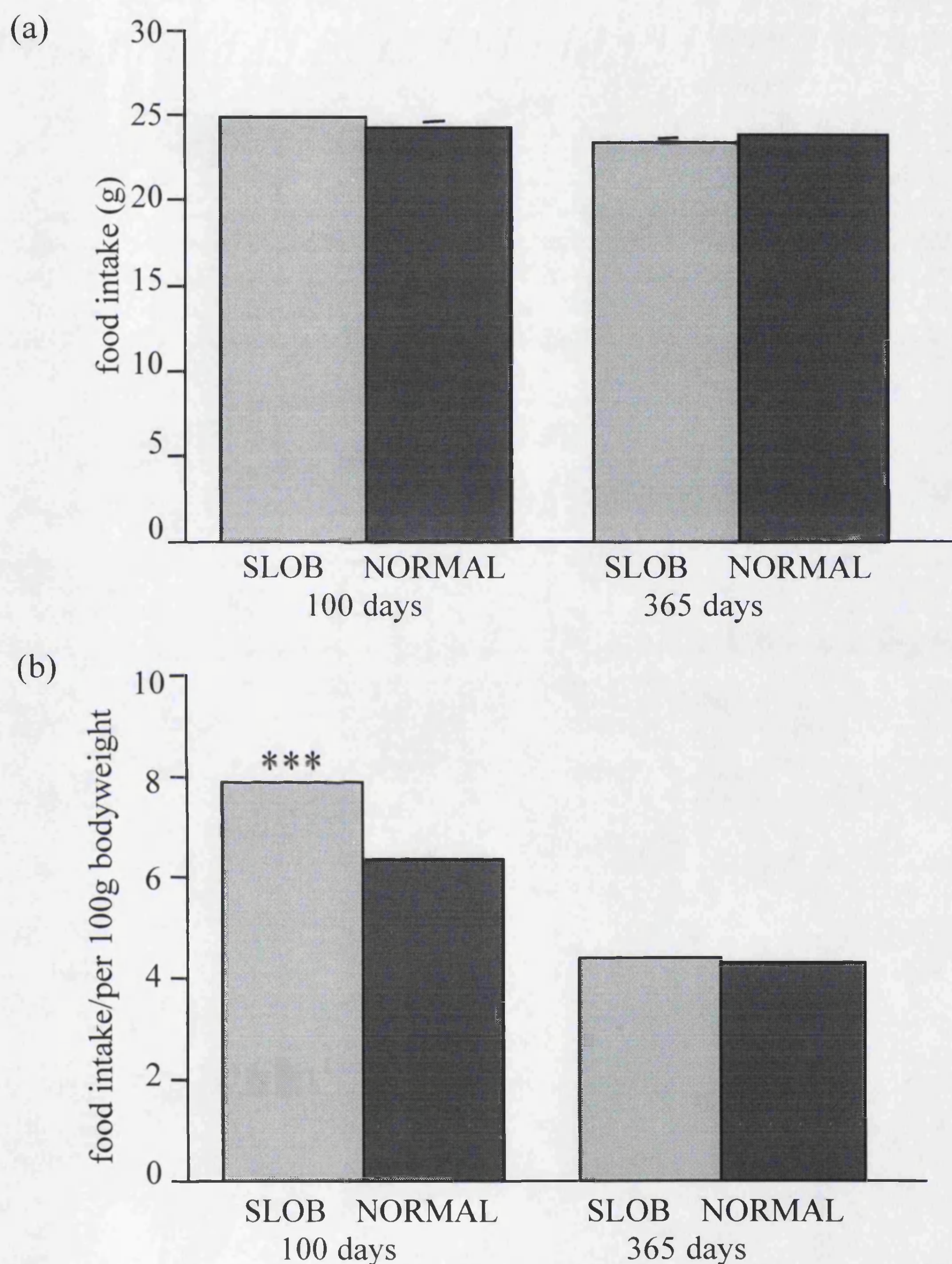
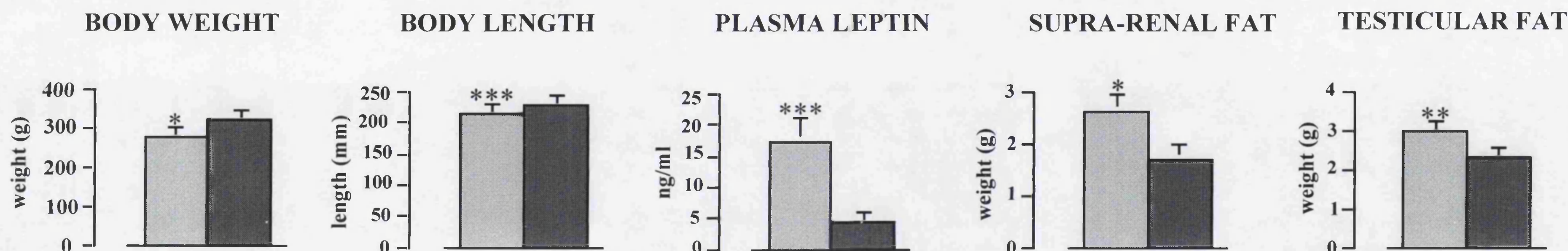


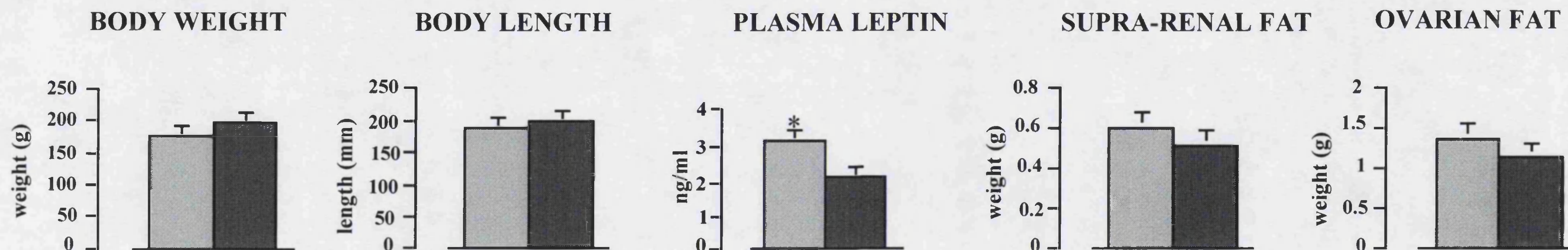
Figure 3.7 Food intake in young and old adult SLOB and normal rats. Animals were placed in metabolic cages and food intake recorded daily after acclimatisation. Means were calculated over 4 days, mean food intake per animal, per day. (a) Food intake, (b) corrected for per 100g bodyweight (n=6-7).

Figure 3.8 Measurements in 'Young' adult SLOB and normal, male and female rats. 80 day old male and female rats were sacrificed, organs weighed, and blood taken for leptin analysis (n=7, *p<0.05, **p<0.01, ***p<0.001). ▢ SLOB ■ NORMAL

MALES



FEMALES



stores. In female SLOBs only leptin levels were significantly greater. Body length and body weight were found to be significantly reduced in both males and females which is explained by the reduced endogenous growth hormone levels in these animals. I therefore believe that SLOB rats do in fact develop an incipient obesity phenotype at an early age, but one that does not become evident in gross morphology until middle age.

3.3 Sexual dimorphism and SLOB rat fertility

The sexual dimorphism of the SLOB phenotype remains unclear. Not only is the obesity phenotype more severe in SLOB males, but males also have a lowered fertility. The original SLOB founder was male and eventually bred after about 6 months of crossing with normal females. Subsequently, the SLOB rat line was bred through transgenic females crossed with normal Wistar males to yield non-transgenic and hemizygous transgenic pups.

3.3.1 Sperm number and motility

In order to try to elucidate the nature of the SLOB male infertility I decided to check sperm count and motility although gross morphology of testes appeared normal from the outset. Sperm analysis was carried out by Dr Kathleen Mathers from the division of Biological Services at the NIMR. Analysis revealed average sperm count in an adult rat as 1.21×10^8 in normal males with 71% motility and 7.38×10^7 in SLOB males with 70% motility, neither of these results were significantly different. Thus, SLOB males have normal sperm count and motility. In addition, plasma levels of the obvious hormone related to male fertility, testosterone, was normal (SLOB 92.2±6.1 ng/ml vs normal 81.7±13.2 ng/ml). I also measured pituitary luteinising hormone in both males and females. Levels in both sexes were insignificant when compared to normal animals (male SLOB 3.92±0.71 ng/pit vs normal 2.75±0.41 ng/pit; female SLOB 3.57±0.96 ng/pit vs normal 4.11±1.7 ng/pit). Therefore these studies did not reveal any obvious indicators of where the source of SLOB male infertility originated.

3.3.2 Breeding behaviour

Extensive studies on breeding behaviour have yet to be carried out, however short term observations reveal mating behaviour in both male and female SLOB rats to be normal. In order to maximise mating behaviour I decided to make use of the Whitten effect (Ross, 1956). This relies on the fact that the regular oestrous cycle in the rat is 3-4 days in length, the female therefore comes into heat every 3rd or 4th night. A specially designed wire cage is used for this experiment consisting of two compartments. A male rat is confined to the smaller compartment and female rats are placed into the larger of the two compartments. When the male is released into the female compartment two days later, peak mating takes place on the first night after the males release. The oestrous cycle of the females is synchronised by the introduction of the male, so that a peak mating occurs on the third night. Use of the Whitten cage on several occasions did not produce pregnant females from SLOB males, although some of the females had been plugged by the males. Several pregnancies and resulting litters were produced from SLOB and normal females in control cages containing normal Wistar males.

As *fat* and *tubby* mice are able to breed if bred at an early age before obesity develops (Coleman and Eicher, 1990), I decided to try to breed from young transgenic SLOB males. Male SLOBs from different litters were set up just after tailing at 3 weeks of age with non-transgenic female littermates. This process proved successful and I was subsequently able to produce a few litters from SLOB males that contained transgenic male and female pups. I further crossed these males with SLOB females to yield potentially homozygous SLOB rats. When the same SLOB males that had produced a litter were left to breed with further females, at an older age they were no longer able to produce pregnant females; this is also true for *fat* and *tubby* mice (Coleman and Eicher, 1990). At present only a few litters have been produced but it may now be possible to produce homozygous SLOB rats. This will require further study, as establishing a homozygous line may be difficult. As the SLOB rat line had now bred through many generations, the SLOB transgene was thought stable. However, it is always possible that in individual animals the original transgene construct may be further mutated resulting in a change in the SLOB phenotype in terms of fertility. To check that the recent male SLOB breeders were definitely transgenic and carried the

same insertion construct (cVO14) as SLOB males from first or second generation rats, a Southern blot was performed. DNA samples were digested with a number of restriction enzymes in an attempt to identify any alterations that may have occurred in the transgene and where it had originally inserted in the rat genome. Results showed DNA fragments of exactly the same size in old original male SLOB rats and new male SLOB breeders (data not shown). I therefore concluded that the male SLOB rats that bred carried the same transgene as old SLOB males and that there was a brief early 'fertility window' in these rats as in other rodent obesity models.

3.4 The role of growth hormone deficiency in the obesity phenotype

As GH promotes lipolysis low levels of GH have been suggested to be of importance for the maintenance of the obese condition (Richelsen, 1997). Diminished growth hormone levels have subsequently been shown to contribute to abnormal body composition, energy expenditure, linear growth, muscle strength, pulmonary function, and carbohydrate and lipid metabolism (Bray *et al.*, 1983; Costeff *et al.*, 1990; Lee *et al.*, 1995). These effects have specifically been observed in the human Prader-Willi syndrome (PWS), caused by functional deletion of the paternal allele of chromosome 15 (Prader *et al.*, 1956; Holm *et al.*, 1993). The condition is characterised by obesity, short stature, hypogonadism, and behavioural abnormalities (Ledbetter *et al.*, 1981; Nicholls *et al.*, 1989). Treatment of children with PWS for 2 years with growth hormone has shown to sustain increases in lean body mass, decreases in percent body fat, improvements in physical strength and agility, and increased fat oxidation (Myers *et al.*, 2000). In particular, blunted GH secretion and corresponding low serum insulin-like growth factor-I (IGF-I) concentrations are associated with abdominal obesity (Hartman *et al.*, 1993; Mårin *et al.*, 1993). GH-treatment of abdominally obese men has been shown to reduce abdominal fat mass, improve glucose and lipoprotein metabolism, and reduces diastolic blood pressure (Johannsson *et al.*, 1997). In addition, a line of heterozygous transgenic rats carrying a chimeric gene comprising a regulatory portion of murine whey acidic protein and a structural portion of the hGH gene, have suppressed endogenous GH levels and develop severe obesity with age (Ikeda *et al.*, 1998). As SLOB rats present abdominal obesity and exhibit hGH as part of their transgene, it would be interesting to assess rat GH and IGF-I

levels in this model and to try and elucidate if any differences in these have a role in the SLOB obesity phenotype.

3.4.1 Pituitary rGH and plasma IGF-I levels

The pituitary content of rGH in both sexes of SLOB rats is reduced at all ages that have been investigated. **Figure 3.9 (a)** shows pituitary levels in rats at 1 year of age. This reduction can be explained by products of the transgene, as was reported by Sara Wells (1997). As hGH was inserted under the control of the vasopressin promoter it is synthesised in the magnocellular neurones of the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) and released peripherally. A local supply of hGH in the hypothalamus therefore causes a negative feedback affecting growth hormone-releasing hormone (GHRH) and thus altering the amount of endogenous rGH released from the pituitary. Together with negligible amounts of hGH in SLOB plasma (Wells, 1997), this explains the initial growth retardation observed in SLOB rats both in overall bodyweight and in individual organ weights such as the kidneys, heart and liver, which all weigh significantly less than in normal rats (Wells, 1997). This growth retardation persists throughout adulthood in SLOB rats, however is masked by the development of the obese phenotype and thus overall bodyweight is usually greater. Nevertheless, individual organ weights such as the kidneys, liver and spleen, at an old age (1 year and above) still weigh less in SLOB rats when compared to normal rats.

Growth hormone promotes growth by stimulating insulin like growth factors (IGF-I) (Vander *et al.,* 1994). As rGH levels are reduced I also expected a reduction in IGF-I levels. **Figure 3.9(b)** shows IGF-I levels are significantly reduced in both SLOB male and female rats.

3.4.2 Does the *dwarf* rat, a spontaneous model of GH deficiency, develop obesity?

The *dwarf* rat, which has resulted from a spontaneous mutation within a gene involved in GH regulation has profoundly reduced pituitary growth hormone levels (Charlton *et al.,* 1988). This growth hormone deficiency and a corresponding reduced IGF-I

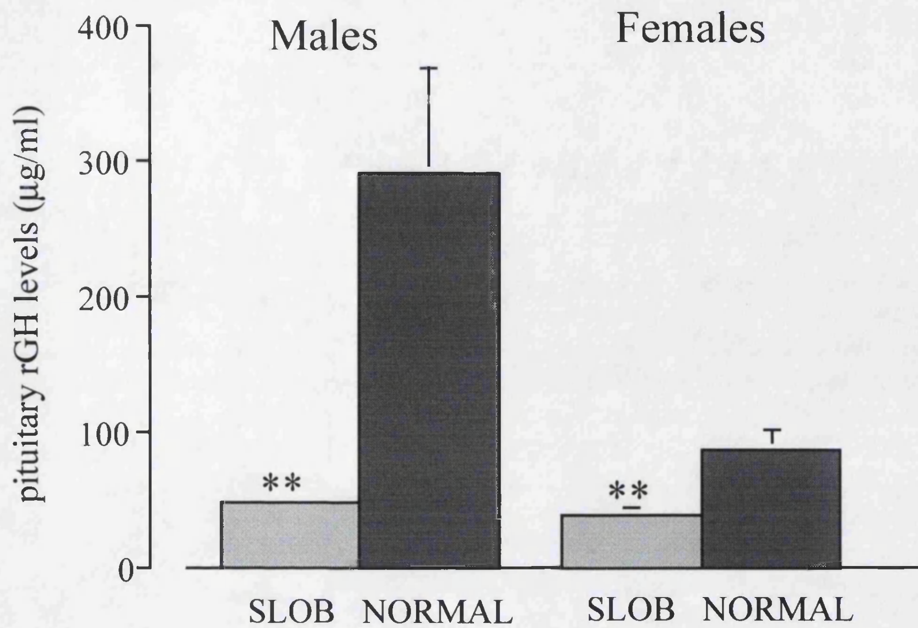


Figure 3.9(a) Pituitary rGH levels in male and female, SLOB and normal rats. Measured in animals at 1 year of age (** = $p<0.01$, n=6).

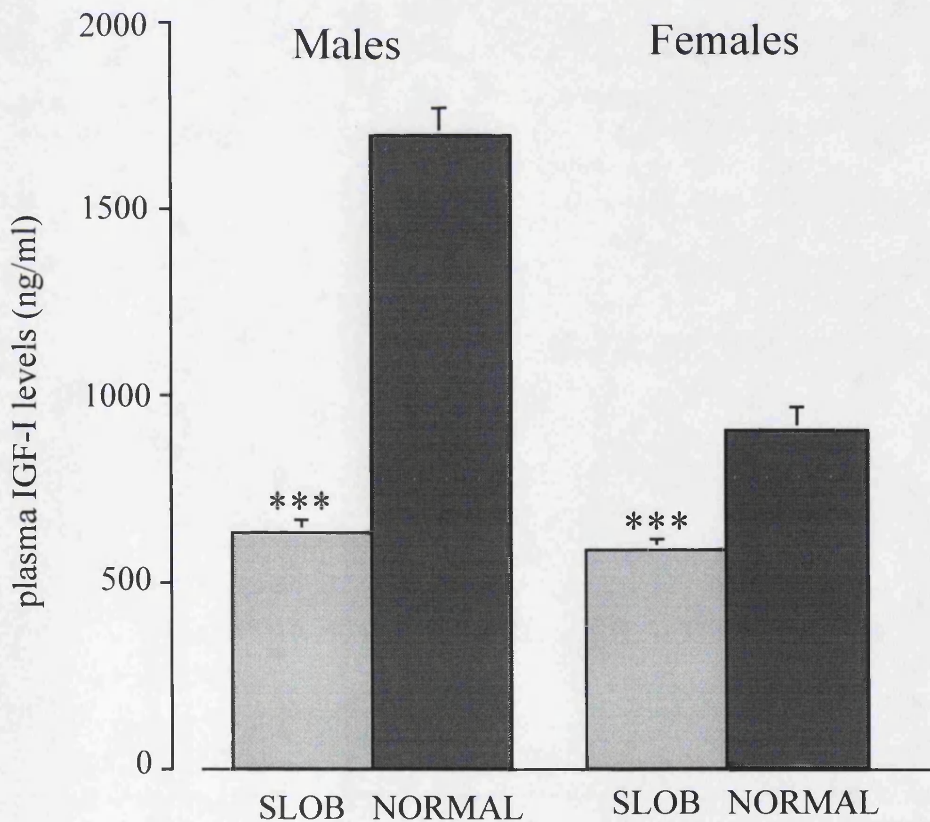


Figure 3.9(b) Plasma IGF-I levels in male and female, SLOB and normal rats. Measured in the same animals as those used for rGH analysis, at 1 year of age (***= $p<0.001$, n=6).

concentration is more severe than that seen in SLOB rats. When female *dwarf* rats are fed a high fat diet they become obese and insulin-resistant compared with chow-fed controls (Clark *et al.*, 1996). To assess obesity in chow-fed male *dwarf* rats these animals were kept to 1 year of age. Thereafter, growth hormone, leptin levels and fat pads were measured and compared to measurements in age-matched SLOB and normal males (**Figure 3.10**). Data are presented per 100g bodyweight to account for the large difference in bodyweight between *dwarf*, normal and SLOB groups. Despite very low rGH levels *dwarfs* did not develop the SLOB obesity phenotype in old age. Even when corrected for bodyweight, supra-renal fat pad weights were still significantly lower in *dwarf* rats when compared to normal rats. Plasma leptin levels also remained low. Whereas in SLOB males, plasma leptin levels and supra-renal fat pad weight were elevated as previously found. I therefore concluded that isolated GH deficiency per se does not account for SLOB obesity, as a more profoundly specific GH deficient model did not develop the SLOB phenotype. In order to strengthen this conclusion, I also treated SLOB males with GH and analysed the effects on the obese phenotype. The results of this experiment are presented in Chapter 5.

3.5 Analysis of the hypothalamic-pituitary-adrenal axis

In order to investigate the HPA axis I decided to measure some hormones involved in this pathway. These were namely adrenocorticotrophic hormone (ACTH) and corticosterone. It is also important to note as vasopressin (VP) is a releasing factor for ACTH (Vander *et al.*, 1994) and as the SLOB transgene is driven off the VP promoter it is possible that ACTH levels may be affected, even though VP levels are unaffected (Wells, 1997). Pituitary ACTH levels in SLOB rats were not significantly different from normal animals (male SLOB 0.89±0.08μg/pit vs. normal 0.72±0.06μg/pit; female SLOB 0.52±0.07μg/pit vs. normal 0.75±0.08μg/pit). Plasma corticosterone released from the cortex of the adrenal gland and in response to ACTH is the major substance secreted in response to stress (Vander *et al.*, 1994). Plasma levels were measured in adult male and female rats and compared to levels in normal animals. Results showed great variances between animals and no significant difference between groups. As corticosterone has a biorhythmic secretion pattern (Windle *et al.*, 1998a, 1998b) and plasma concentrations are highly sensitive to stress,

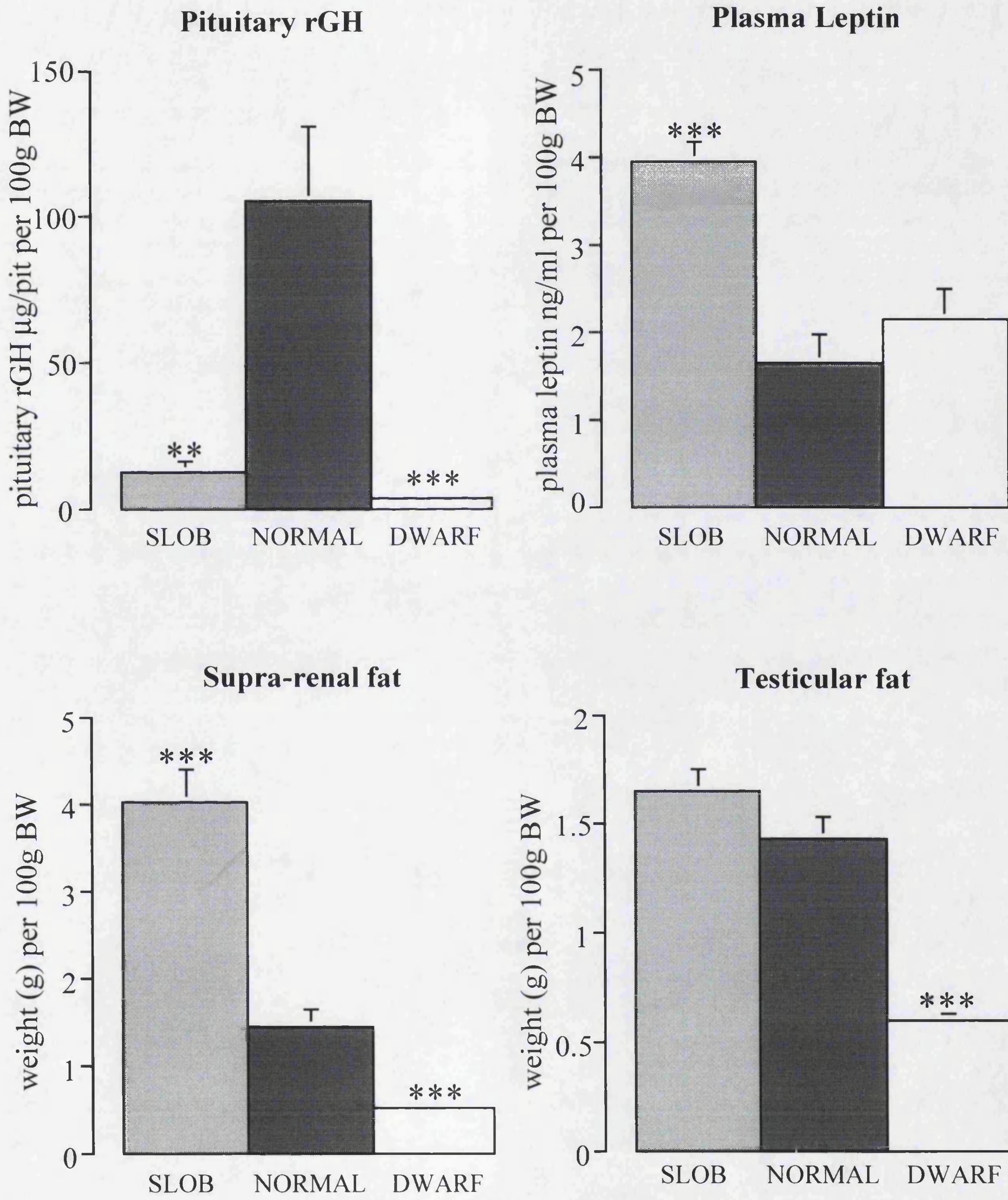


Figure 3.10 Pituitary rGH, plasma leptin, and fat pad weights in 1 year old SLOB, normal and *dwarf* animals. All values are corrected for per 100g bodyweight (BW), significances are shown for when compared with normal animals (**p<0.01, ***p<0.001, n=7).

sampling from conscious rats over a 24-hour period would give a more accurate assessment of corticosterone levels. **Figure 3.11** shows corticosterone levels over 24 hours in SLOB and normal male rats. Despite great variance between each animal and in view of the difficulties of measuring a stable level of corticosterone within a working animal unit, no major differences were found between SLOB and normal rats.

3.6 The diabetic status of SLOB rats

For the population of hyperglycaemic subjects older than 20 years, at least 90% are more than 10% overweight (Norman & Litwick, 1997). Thus, the most commonly associated physical finding with maturity-onset diabetes is obesity. Many of these individuals are insulin resistant, thus having elevated insulin levels and an inappropriately high blood glucose level. Many of the previously characterised rodent obesity models also show disturbances in insulin and glucose handling as will be discussed in 3.8. To evaluate the diabetic status of the SLOB rat I measured fasting plasma glucose and insulin levels. Results for 1 year old SLOB male and female rats are shown in **Table 3.1**.

Table 3.1 Plasma glucose and insulin levels in SLOB and normal rats

	Plasma Glucose (mg/dl)	Plasma Insulin (ng/ml)
SLOB male	114.7±4.2	1.94±0.9
Normal male	121.0±3.4	2.80±1.9
SLOB female	126.3±3.3	2.51±0.6
Normal female	135.4±6.7	2.54±2.3

No significant differences were found between SLOB and normal glucose or insulin levels in either male or female rats. GH exerts direct insulin-antagonistic effects even after the administration of physiologic doses of GH (Nam and Marcus, 2000). In fact, GH has been considered to be the principal factor in the decrease in insulin sensitivity

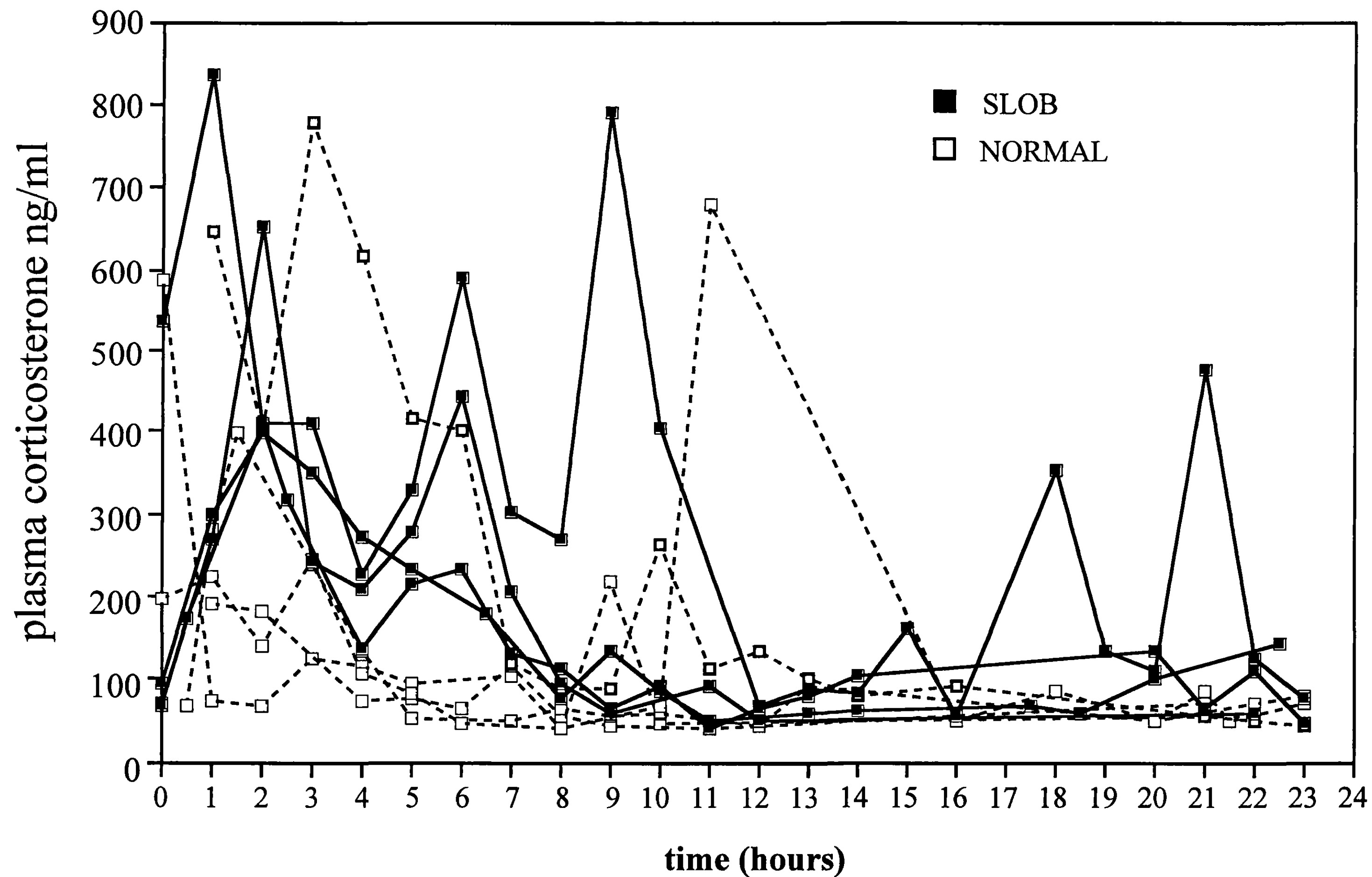


Figure 3.11 Plasma corticosterone levels in SLOB and normal rats over 24 hours. Individual corticosterone levels are shown for 5 normal and 4 SLOB male rats. Animals were sampled every 30 minutes over 24 hours. Corticosterone was measured by radioimmunoassay in half of the samples (every hour).

observed in the early morning, the so-called 'dawn phenomenon' (Bolli *et al.*, 1984) and the insulin resistance following hypoglycemia (Fowelin *et al.*, 1989). GH also has a direct effect on adipose tissue where it promotes lipolysis (Richelsen, 1997). When abdominally obese individuals are treated with GH it is thought that although their fat pad depots may be reduced their diabetes may be worsened. In fact, their insulin resistance is lessened due to the GH effect on removal of fat over-riding the GH effect on muscle and other tissues (Berneis and Keller, 1996). I considered the possibility that reduced levels of rGH might have a role in protecting SLOBs from diabetes. In Chapter 5 I will show that GH treatment has no effect on abolishing fat mass in the GH-deficient SLOB rat. Subsequently, it would be interesting to measure plasma insulin levels in GH-treated animals to assess if replacing GH alters the diabetic status of SLOB rats. 20-22 week old SLOB and normal rats were treated for 4 weeks with recombinant growth hormone at a dose of 200μg per day, delivered using an osmotic minipump. Following treatment, blood was collected and analysis of plasma insulin revealed no change between SLOB sham and SLOB treated rats, that is GH had no effect on plasma insulin levels (SLOB sham 6.02±1.1ng/ml vs treated 6.85±0.9ng/ml).

3.6.1 Insulin tolerance test (ITT) and Glucose tolerance test (GTT)

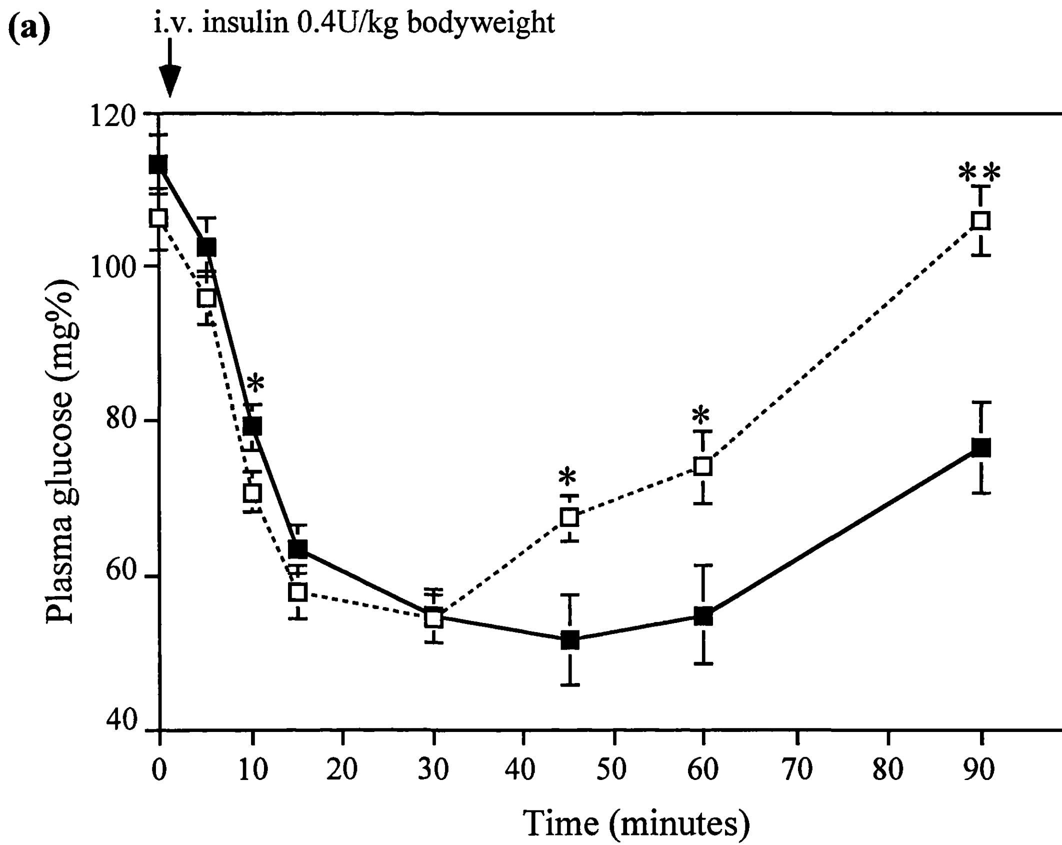
The resting glucose and insulin measurements shown above are static measures. A more insightful test of diabetic status is to carry out insulin tolerance tests (ITT) and glucose tolerance tests (GTT). An ITT was performed on 6 SLOB and 6 normal rats of just over a year in age when SLOB rats show severe obesity. Each received a dose of insulin at a concentration of 0.4U/kg bodyweight. This dose of insulin was chosen as it lowers blood glucose by 50% in *dwarf* rats (Clark *et al.*, 1996). Animals were sampled for 90 minutes following insulin administration, and for each time-point glucose was measured immediately following centrifugation to separate plasma (**Figure 3.12 (a)**). For the first 30 minutes both normal and SLOB animals appear to respond the same, at 30 minutes plasma glucose concentrations are equal in both groups (55mg/% glucose). After 30 minutes, normal animals start to clear the insulin load and glucose levels start returning to normal. However, in SLOB animals glucose levels remain low for a further 30 minutes after which they too increase. These results indicated SLOB rats were not insulin resistant and if anything were more

sensitive to the effects of insulin. It must be noted GH deficiency together with IGF-I deficiency may have a role in this blunted insulin response. To investigate this the ITT was repeated using GH treated SLOB rats; these rats were treated using osmotic minipumps as previously described. However, GH treatment had no effect on glucose response and was comparable to that in the untreated SLOB animals (**Figure 3.12(b)**).

Glucose tolerance tests were also performed in normal and SLOB animals of the same age, each receiving 0.5g/kg of D-glucose. This dose was chosen as it had previously been tested in corpulent (*cp*) rats (Russell *et al.*, 1999). In response to an intravenous glucose bolus, both SLOB and normal rats responded the same and shortly after administration began to clear the glucose load (**Figure 3.13 (a)**). Glucose levels continued to fall in both groups, although normal rat levels decreased at a faster rate than SLOB animals and had returned to normal by 60 minutes. However, there was no significant difference between SLOB and normal animals for any of the timepoints measured. Analysis of plasma insulin levels also showed little difference between SLOB and normal animals, a significant difference was only observed 5 minutes after treatment (**Figure 3.13 (b)**). From both ITT and GTT I concluded SLOB rats were neither diabetic nor insulin resistant. Thus, SLOB rats were able to respond to glucose and insulin loads in a normal manner and GH treatment did not have an effect on this response.

3.7 Lipid analyses

Although the relationship between heart disease, obesity and diabetes has long been recognised, the underlying mechanisms for the increased risk are poorly understood. However, perturbations in plasma lipids, which have been associated with the development of heart disease, are observed in some obese individuals (Sirtori and Vega, 1997). These include elevated levels of plasma triglyceride and low-density lipoprotein cholesterol (LDL-C) and decreased levels of high-density lipoprotein cholesterol (HDL-C). Several rodent models with varying severity's of obesity and diabetes have also been examined to determine whether they have high levels of plasma triglycerides and combined very-low-density lipoprotein and low-density lipoprotein cholesterol (VLDL/LDL-C) and decreased HDL-C levels, similar to those



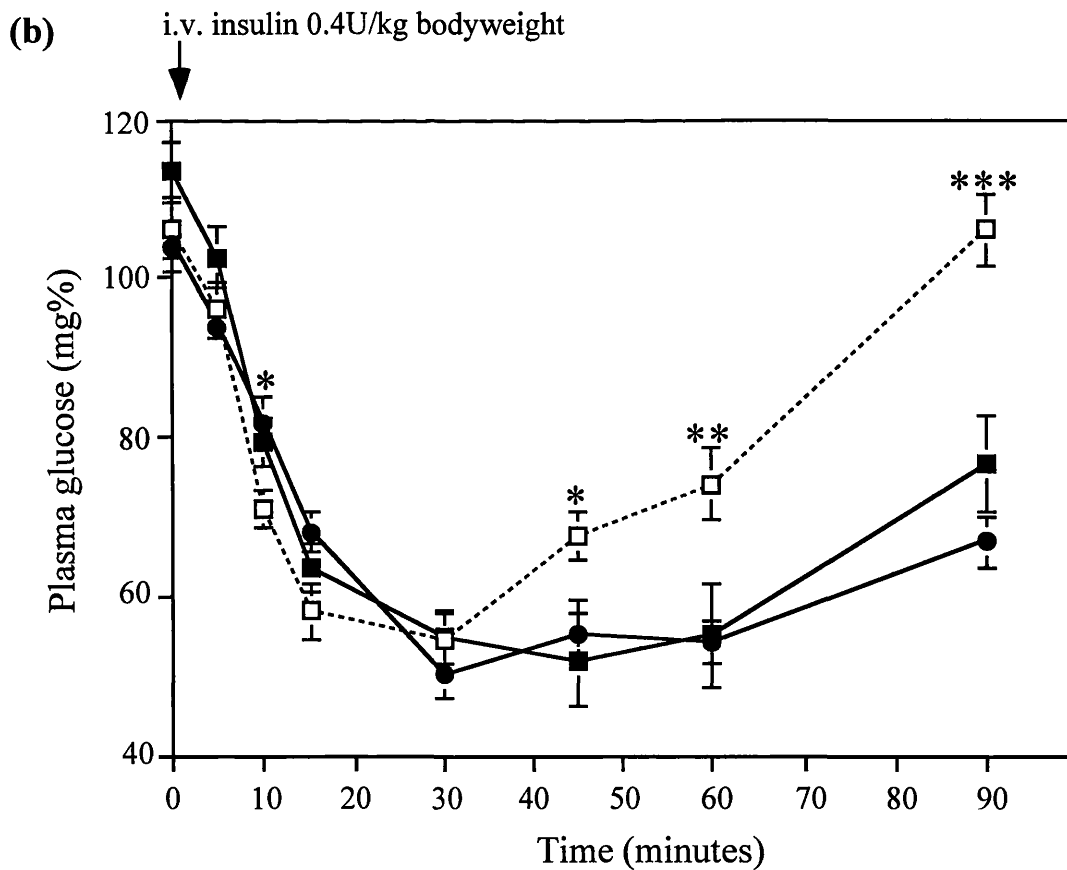
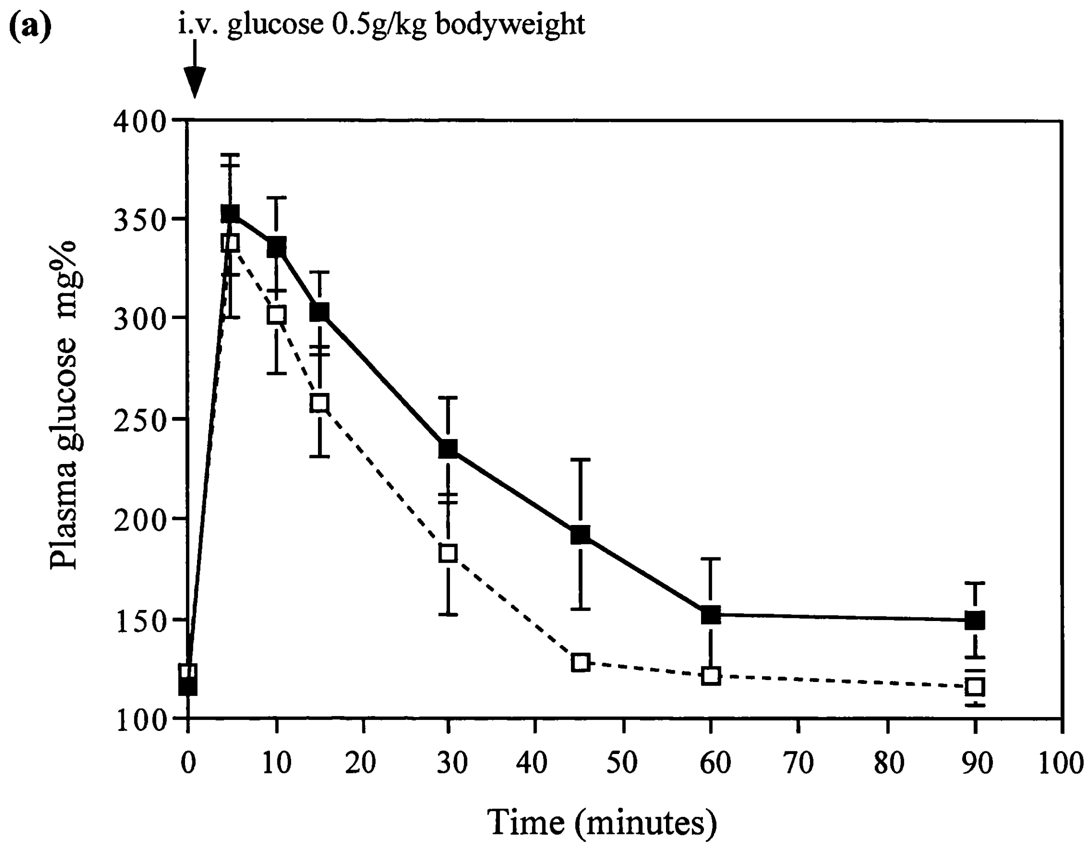


Figure 3.12 Insulin tolerance tests. Plasma glucose levels after 0.4U/kg bodyweight insulin challenge administered i.v. in (a) SLOB and normal male rats (n=6), (b) SLOB, SLOB-GH treated and normal male rats (n=6) (*p<0.05, **p<0.01) ■ SLOB, □ Normal, ● SLOB GH-treated.



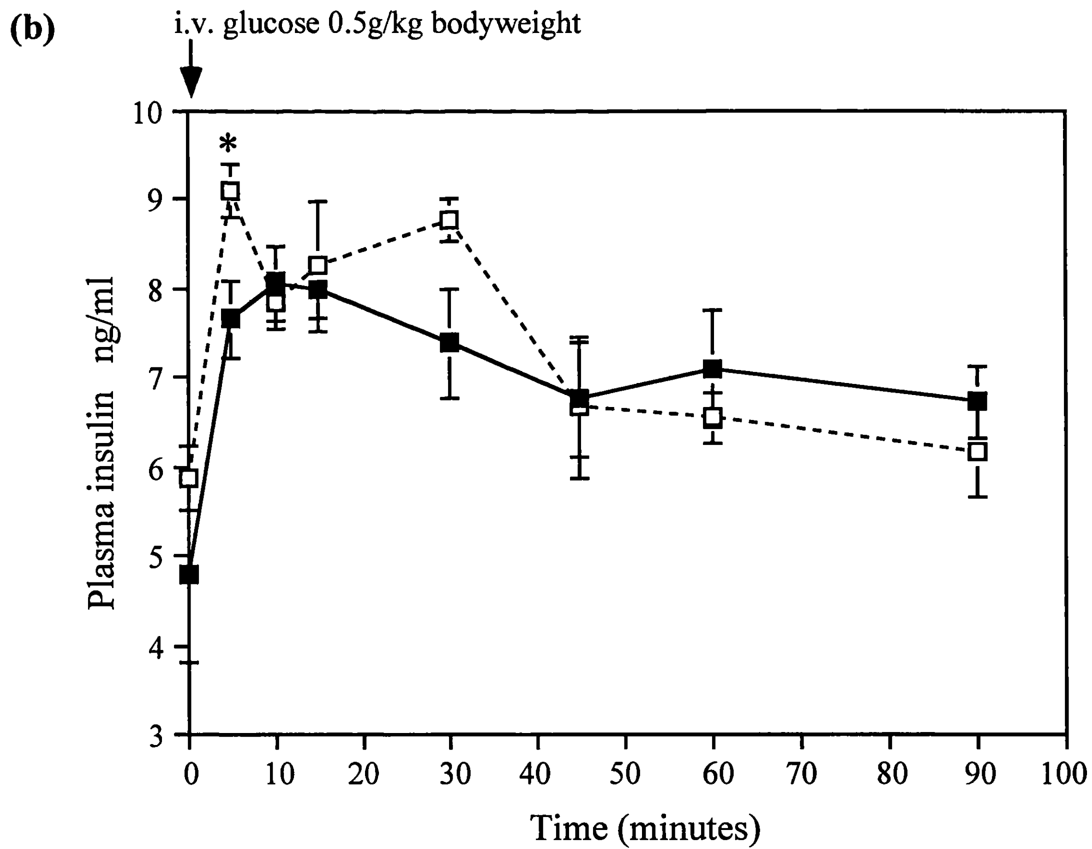


Figure 3.13 Glucose tolerance tests. 0.5g/kg bodyweight of glucose was administered i.v. to SLOB and normal male rats (n=6) (a) plasma glucose levels (b) plasma insulin levels (*p<0.05). ■ SLOB □ Normal

observed in human diabetics. These results are discussed in 3.8. Presented here are similar measurements and other lipid analyses carried out in SLOB male rats.

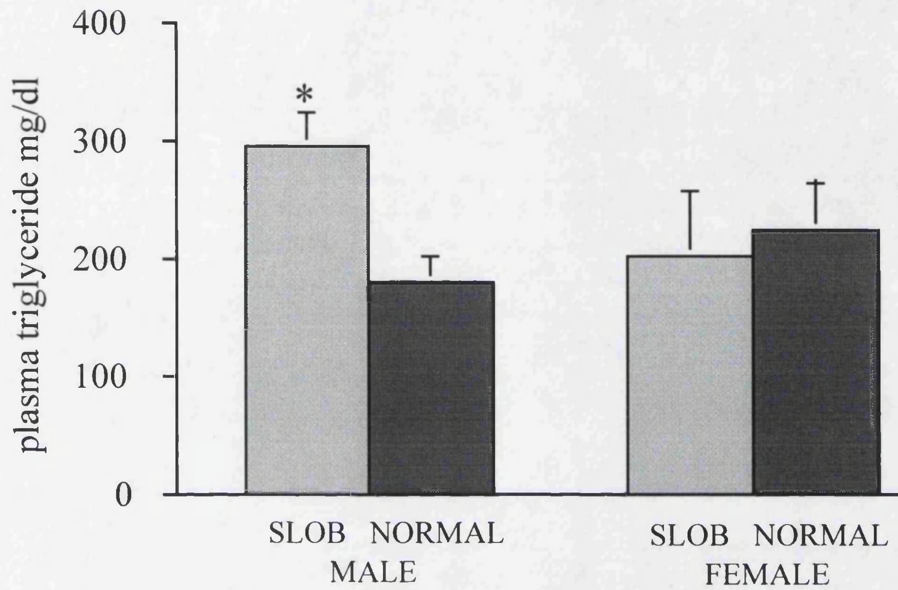
3.7.1 Plasma triglyceride and cholesterol levels

Compared with control mice, *ob/ob, db/db, tubby*, and *agouti* mice have triglyceride levels that are elevated 1.5 to twofold to those of controls (Nishina *et al.*, 1994). Elevated plasma cholesterol levels are also observed in these models, and are mainly due to an increase in HDL-C (Nishina *et al.,* 1994). As triglyceride amount is a direct measure of adipose tissue mass, I would also expect an elevated triglyceride level in SLOB males. **Figure 3.14(a)** shows SLOB males have a 1.6-fold elevation in triglyceride levels compared to normal males. However, in female SLOB rats the levels are normal, coinciding with the sex specific phenotype. Analysis of plasma cholesterol levels in male and female SLOB rats showed no difference compared to normal rats (**Figure 3.14(b)**). At first, this seems quite unusual and another parameter that differs from the previously characterised rodent obesity models (Nishina *et al.,* 1994). However, work carried out by Fredrik Frick at the University of Gôteborg in Sweden later revealed when lipoprotein profiles are examined, the nature of the lipid abnormality in terms of triglyceride and cholesterol becomes apparent. Fast protein liquid chromatography (FPLC) analysis in both male and female rats showed a marked increase in both triglyceride and cholesterol VLDL (**Fig 3.15**). HDL cholesterol also appears a little higher in SLOB animals compared to normal animals, however this analysis has since been repeated and the increase in HDL-cholesterol not noted.

3.7.2 Fat cell, size and number

An increase in adipose tissue mass is due to an increase in cell size, hypertrophy, or an increase in cell number, hyperplasia, or a combination of the two. Investigations in fat cell number and size have rarely been carried out in rodent obesity models (Bray and York, 1979). This could possibly be as the cause of obesity in many of these models is known and it is therefore assumed adipose tissue differentiates in a normal manner. As a cause for the SLOB phenotype remains unclear I assessed fat cell number and diameter in both supra-renal fat and testicular fat (**Figure 3.16**).

(a)



(b)

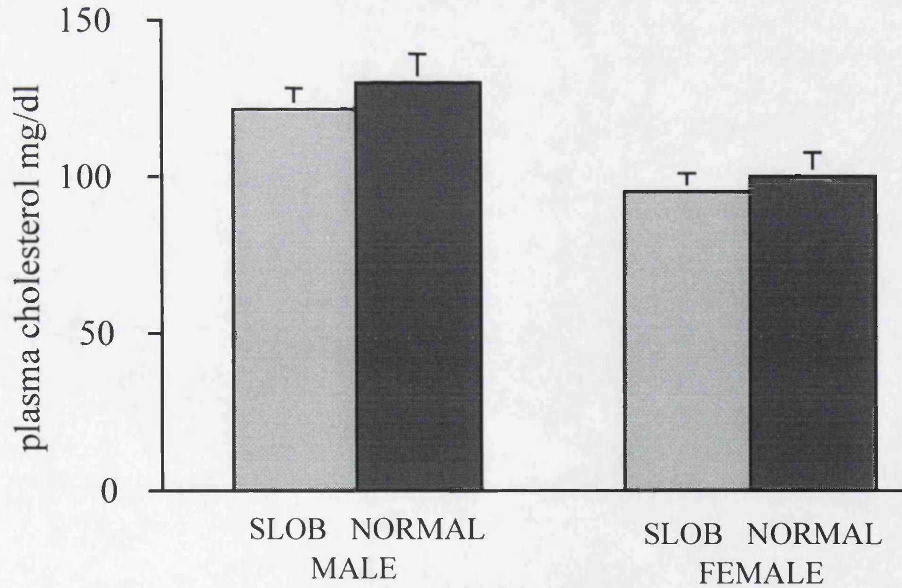
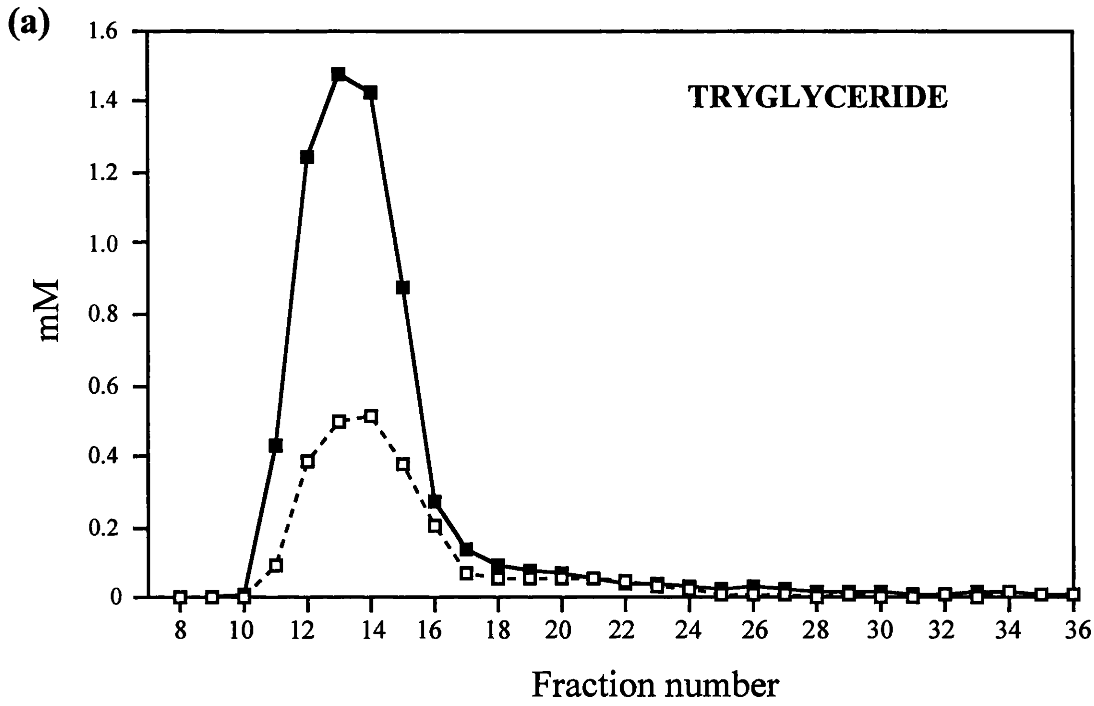


Figure 3.14 Plasma triglyceride and cholesterol levels. Measurements were taken in male and female rats at 1 year of age (n=6). (a) plasma trigyceride levels (*p<0.05) (b) plasma cholesterol levels.



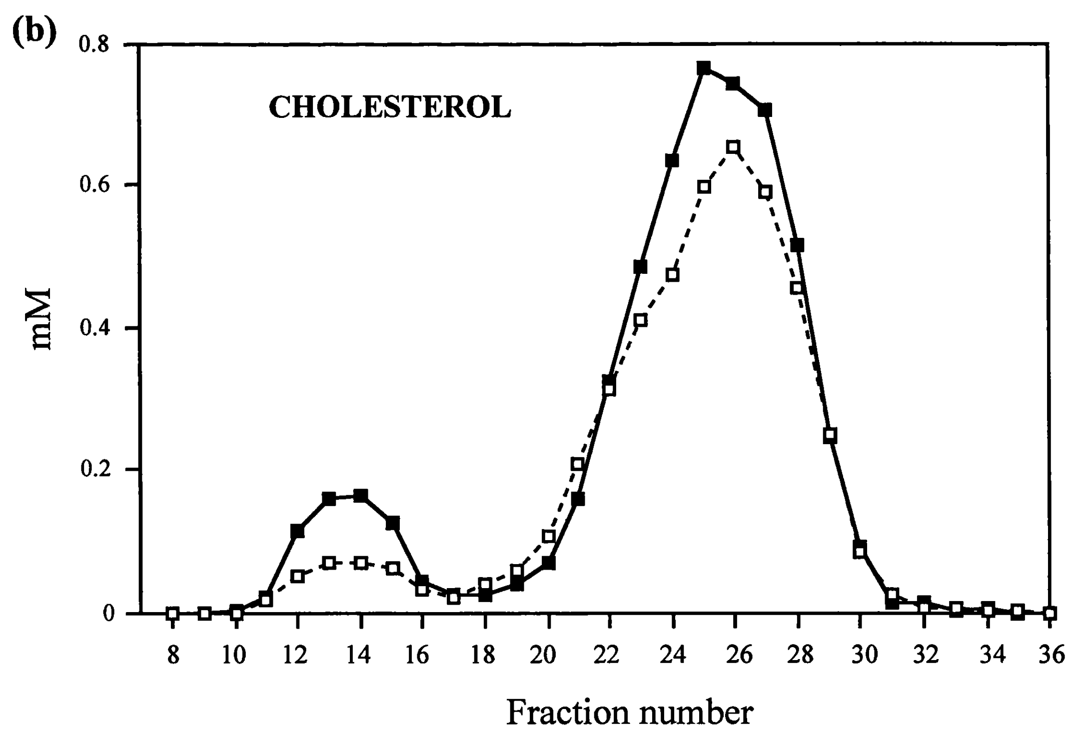
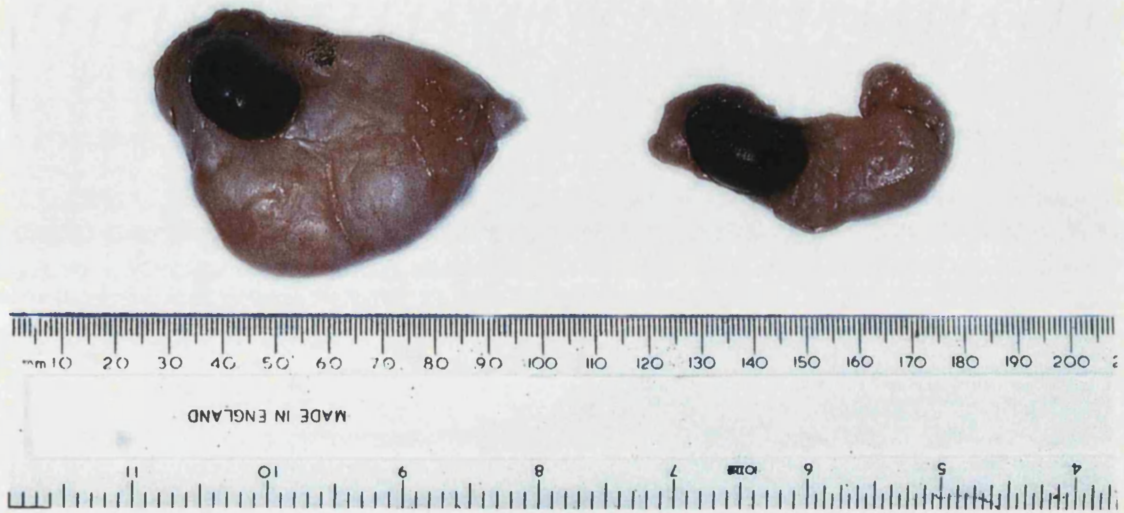


__Fig 3.15__ **Triglyceride and cholesterol lipoprotein profiles.** FPLC analysis of serum (a) triglycerides and (b) cholesterol lipoproteins. Serum was pooled from 3 rats, (n=2). ■ SLOB □ Normal

(a)



(b)

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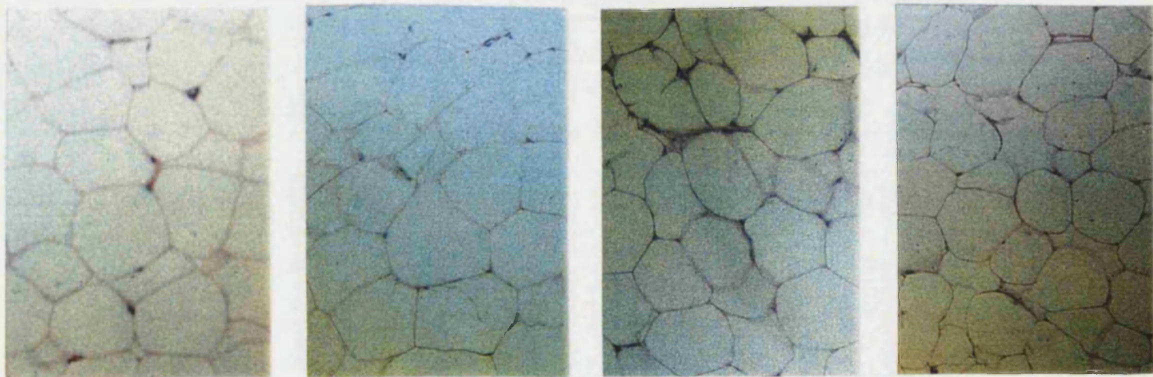


NORMAL TESTICULAR	SLOB TESTICULAR	NORMAL SUPRA-RENAL	SLOB SUPRA-RENAL

cell number x 0.9 (NORMAL TESTICULAR vs SLOB TESTICULAR)

cell number x 2.6 (NORMAL SUPRA-RENAL vs SLOB SUPRA-RENAL)

Figure 3.16 **Fat cell analysis in SLOB and normal rats.** (a) Supra-renal fat pad from a SLOB (left) and normal (right) male rat both 2 years of age. (b) Microscopical analysis of fat pads from male animals at 1 year of age.

Adult (7 month) male SLOB and normal animals were used to assess fat cell size and number. Following routine histological procedures fat was analysed using light microscopy and the computer package 'NIMH Image Analysis', which allows cells to be counted within a known frame area and also allows cell diameters to be easily measured. SLOB rats were found to have approximately 2.6 fold more cells in their supra-renal fat pad compared to normal rats (mean fat pad weight, SLOB 26.3±1.2g vs normal 7.5±0.7g). There was an insignificant difference found in cell number when testicular fat pads were analysed despite SLOB males having a greater fat pad mass compared to normal males in this group of animals (mean fat pad weight, SLOB 16.8±0.5g vs normal 12.3±1.1g). Mean cell diameter in supra-renal and testicular fat was not significantly different for SLOB and normal rats (supra-renal fat SLOB 0.29±0.007mm vs normal 0.27±0.013mm; testicular fat SLOB 0.10±0.004mm vs normal 0.09±0.003mm). Fat from animals used for this analysis was also used to harvest adipocytes. 100 cells from 2 representative animals from each group were viewed under the microscope in a haemocytometer chamber and diameters recorded using an eyepiece graticule. These results also showed no difference in cell size between SLOB and normal supra-renal or testicular fat adipocytes. Thus, the increase in abdominal adipose tissue in SLOB rats does not appear to be due to an increase in cell size but moreover an increase in cell number. However, factors such as apoptosis and amount of other cell types have yet to be analysed.

3.7.3 *In vivo* hepatic VLDL-TG production

Having identified disturbances in lipoprotein metabolism, to further elucidate the way in which SLOB rats process and release VLDL I carried out studies evaluating hepatic VLDL-TG production. VLDL-TG secretion over a four hour period in 8 SLOB and 8 normal animals was assessed. Triton was used as the test substance as it blocks lipoprotein lipase activity, stopping the degradation of VLDL, which remains in the circulation for analysis in blood samples (Hayashi *et al.,* 1981; Sato *et al.,* 1995). Blood samples were taken every hour, serum separated immediately and then tested for triglyceride amount as a measure of VLDL in the circulation. At all time points measured, VLDL amount in SLOB rats was significantly greater than in normal animals (**Figure 3.17**). SLOB rats were therefore secreting more VLDL from the liver, this result coinciding with higher VLDL-triglyceride and VLDL-cholesterol

HEPATIC VLDL-TG PRODUCTION

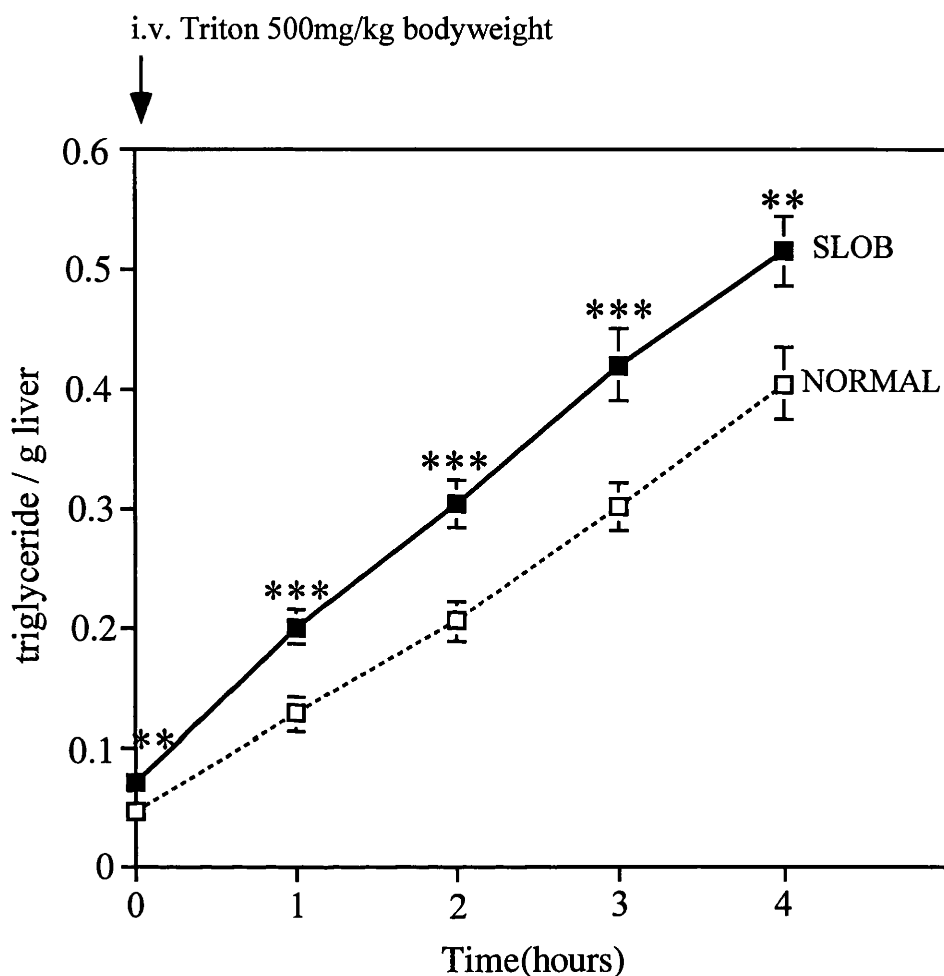


Figure 3.17 Hepatic VLDL-TG production in SLOB and Normal rats. 500mg/kg bodyweight Triton was administered to SLOB and normal male rats (n=8). After collection of a basal blood sample, hourly samples following treatment were taken and tryglyceride measured. Values plotted above are correlated to per g liver weight.

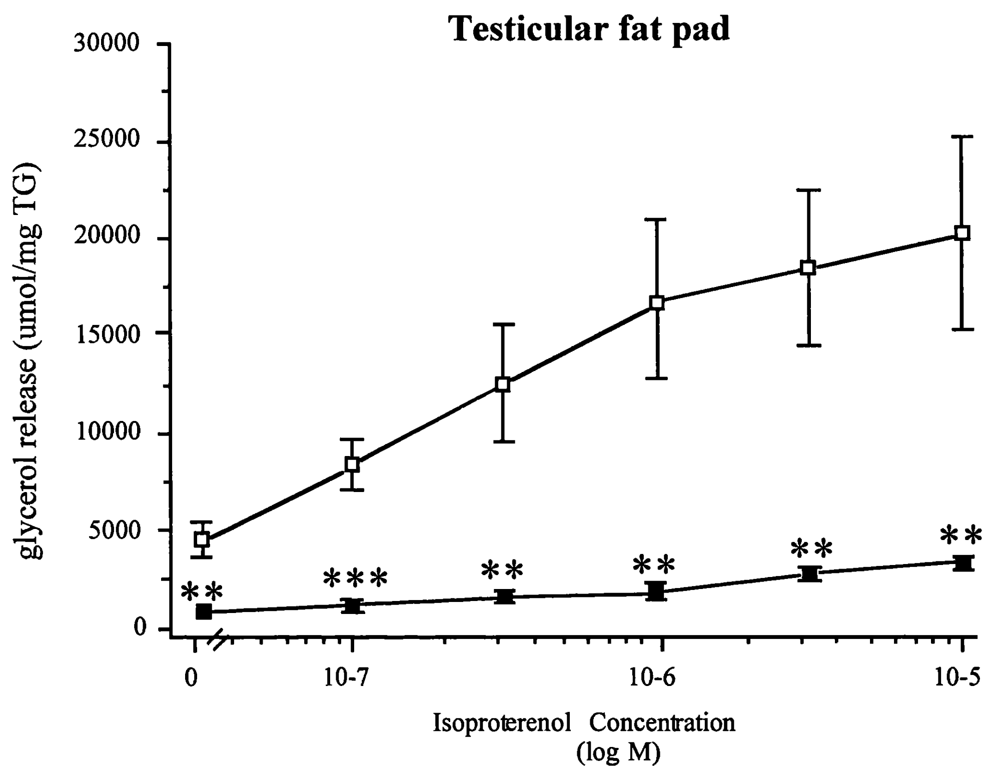
levels as evident in the previously shown triglyceride and cholesterol lipoprotein profiles.

3.7.4 Rate of lipolysis and lipogenesis

In adipose tissue, lipid mobilisation can be initiated by the stimulation of β-adrenergic receptors, which are coupled to adenylate-cyclase by a nucleotide binding protein (Lafontan and Bertan, 1993). This stimulation causes cyclic AMP (cAMP) production. cAMP activates a cAMP-dependent protein kinase that, in turn, activates hormone sensitive lipase (HSL) leading to lipolysis and the release of free fatty acids (FFA) and glycerol (Lafontan *et al.*, 1995). Isoproterenol is a β-adrenergic agonist which induces lipolysis, and glycerol output can be used as a measure of this lipolysis (Morimoto *et al.,* 2000). I tested 6 SLOB and 6 normal animals from which both supra-renal and testicular fat pads were excised. Analysis revealed that SLOB males breakdown fat at a slower rate than normal animals, reflected in the shift towards the right in the dose response curve (**Figure 3.18**). This result was true for both supra-renal and testicular fat pads, with the greatest effect observed in testicular fat; the implications of which will be discussed in 3.8.

3.7.5 Factors involved in adipogenesis – LPL, PPARs, apoE and apoB

I have shown that SLOB rats show abnormal lipoprotein profiles, have an increased production of hepatic VLDL-TG, and a decreased rate of lipolysis. These results clearly reveal SLOB rats have lipid disturbances, but whether these effects are primary or secondary to the SLOB phenotype remains to be determined. Measurements of some of the factors involved in adipogenesis may provide suggestions to the nature of these effects.



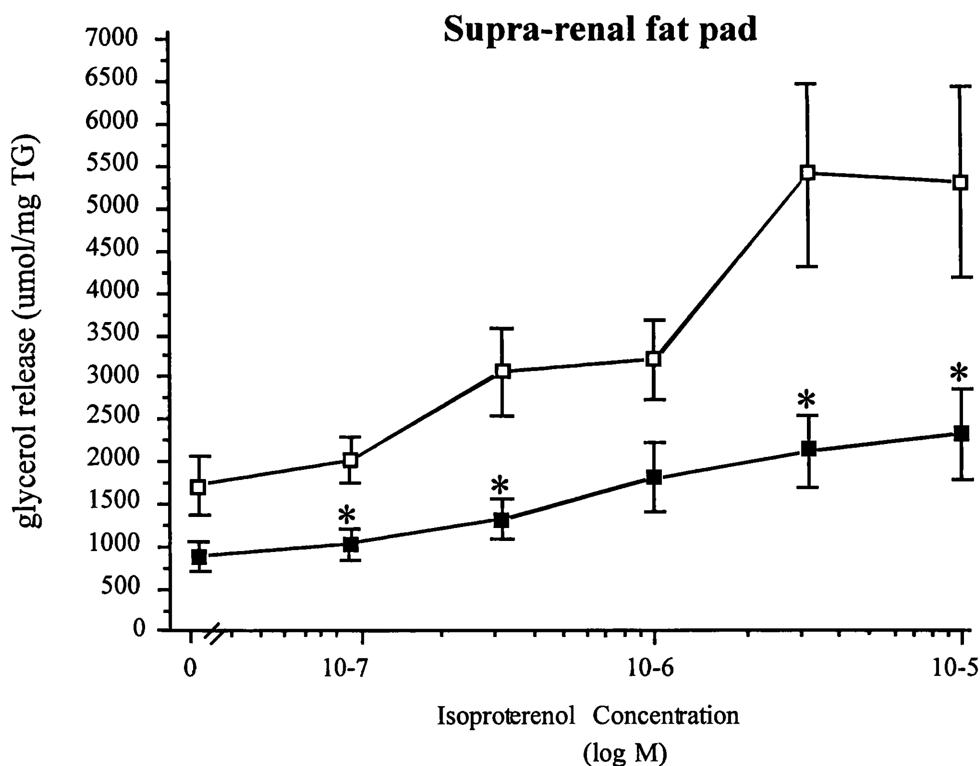


Figure 3.18 Lipolysis in SLOB and normal supra-renal and testicular fat pads. β-adrenergic (isoproterenol) stimulated fat cells were analysed from 6 SLOB and 6 normal male rats (7 months old) to assess the rate of lipolysis. Shown above are dose dependent glycerol release curves for (a) adipocytes from the testicular fat pad, (b) adipocytes from the supra-renal fat pad. ■SLOB □Normal.

Lipoprotein Lipase

Lipoprotein lipase (LPL) is a key enzyme in the regulation of the flux of fatty acids and affects levels of circulating lipoproteins, especially serum triglycerides and is rate limiting for the supply of triglyceride-derived fatty acids to skeletal muscle tissue (Levak-Frank *et al.*, 1995). Two different adipose depots, the supra-renal fat pad and testicular fat pad, and two muscular tissues, the heart (cardiac muscle) and soleus (skeletal muscle) were tested for LPL activity in SLOB and normal animals. Each group contained tissues from 6 male and 6 female rats. **Figure 3.19** shows LPL activity is increased in SLOB male and female supra-renal adipose tissue, and in female SLOB ovarian tissue but shows a non-significant change in testicular fat. Activity in the heart was significantly reduced in female SLOB rats, and in the soleus muscle was significantly reduced in male SLOB rats. The ratio of LPL activity in muscle and fat is very sensitive and a key contributory factor to adipose state (Eckel, 1989). The difference in LPL activity in the tissues tested may therefore contribute to SLOB obesity and the lipid abnormalities reported.

Apolipoproteins B and E

The apolipoproteins are also important determinants of lipid metabolism (Siest *et al.*, 1995). ApoE levels differed only in SLOB females (SLOB 65.1±3.7% vs normal 100±6.4%). SLOB males were found to have lower amounts of apoB compared to normal males (SLOB 60.4±5.7% vs normal 100±3.2%; female plasma not yet analysed). This is highly surprising as I previously found VLDL triglyceride to be increased. Each VLDL particle released from the liver carries one apoB particle; the amount of apoB is therefore usually equal to the amount of VLDL. In SLOB males the liver is perhaps secreting very large VLDL particles, accounting for the increase in VLDL fraction concentration but each particle still having one apoB, accounting for the decrease in apoB. Although this hypothesis is likely it has never previously been described and has not been observed in any characterised rodent model to date. However, such an occurrence could have important consequences for the uptake of such particles and consequently the amount of lipid stored.

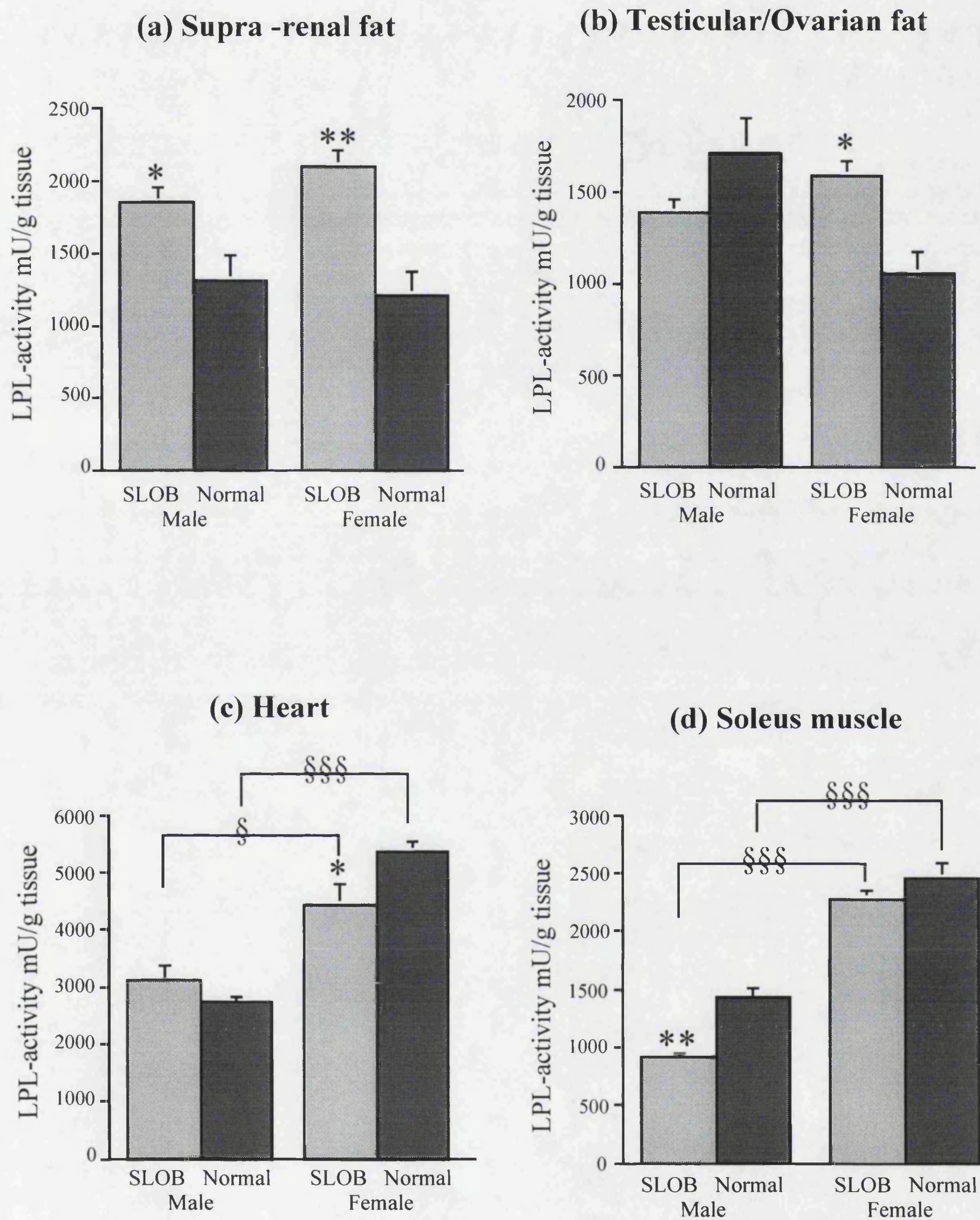


Figure 3.19 Lipoprotein lipase activity in heart, muscle and adipose tissue. Analysis carried out on tissues taken from male and female rats at 1 year of age (n=6, */§p<0.05, **p<0.01, ***/§§§p<0.001).

Peroxisome proliferator-activated receptor α

Peroxisome proliferator-activated receptors (PPARs) control the expression of a large array of genes involved in adipocyte differentiation, lipid storage and insulin sensitisation (Spiegelmen and Flier, 1996). Preliminary RPA analysis carried out by Fredrik Frick (Gôteborg, Sweden) of PPARα mRNA in samples I sent to him, revealed SLOB rats have a reduced expression in liver with the greatest effect seen in SLOB males (results expressed as a ratio of phosphorimager counts between PPAR-α and control ribosomal 18S; male SLOB 0.50±0.042 vs normal 0.78±0.02; female SLOB 0.35±0.02 vs normal 0.43±0.07). Free fatty acids (FFA) are activators of PPARs (Spiegelman and Flier, 1996); however, although SLOB males were found to exhibit a slight elevation in serum FFA levels, this rise was not enough to increase PPARα levels.

3.8 Discussion

3.8.1 SLOB obesity

This chapter shows there are four principal characteristics that together make the SLOB rat quite unique from any of the previously characterised rodent obesity models. These are, obesity-onset, fat distribution, sex-specificity, and an overall normal food intake. Obesity is evident in *ob/ob* and *db/db* mice by 2-3 weeks of age, in *fat/fat* mice by 5-8 weeks and in *tubby* mice by 9-12 weeks (Coleman and Eicher, 1990). The *agouti* mouse exhibits a maturity-onset obesity that peaks between 8 and 17 months of age (Dickie and Woolley, 1946; Roberts *et al.,* 1984). SLOB males show gross obesity at about 200 days (28 weeks) of age, thus like the *agouti* mouse and to some extent the *tubby* mouse, can be classified as exhibiting a maturity-onset obesity. However, my studies assessing if obesity is actually apparent at an earlier age in terms of fat pad weights and leptin levels, showed SLOB rats do in fact have an incipient obesity phenotype at 80 days (11 wks) of age, and at this stage obesity extends to both central and peripheral stores. How early the phenotype in this respect can be measured has yet to be investigated.

Plasma leptin levels were elevated in both young (80 days) and old (365 days) male and female SLOB rats. This is not really surprising, as leptin is released from adipose stores (Zhang *et al.,* 1994; Frederich *et al.,* 1995; Friedman, 1997a) and assuming there is no abnormality in leptin release and elimination, an increase in adiposity would result in a direct increase in plasma leptin concentration. However, although female SLOB rats do not show an obesity phenotype until a much later age, it is interesting to note that younger female SLOBs are already hyperleptinemic. The nature of this sexual dimorphic phenotype has yet to be determined but will be explored further in Chapter 5. This trait is another unique feature to this model; other rodent obesity models, although show some sexual dimorphism in, extent of obesity, lipid abnormalities and diabetic status (Coleman and Eicher, 1990; Nishina *et al.,* 1994; Pomp, 1999), do not show quite as marked an effect in sex-specificity of overall obesity as the SLOB rat, which clearly shows a male-specific phenotype.

In the *Zucker* rat excess weight gain extends to fat stores throughout the body rather than being confined to the axial and inguinal regions as is typically observed by the pear-shaped body conformation of *ob/ob* and *db/db* mice (Coleman and Eicher, 1990). The excess of weight gain in SLOB males is further concentrated in the central (visceral) supra-renal adipose depot, thus demonstrating another aspect of the phenotype unique to SLOBs and one similar to that seen in the obese human population (Bjontorp *et al.,* 1992; Kuczmarski *et al.,* 1994). Visceral fat stores have been reported to be the most important depot in terms of insulin resistance (Banerji, 1997) and cardiovascular disease (Larsson *et al.,* 1984). The blood flow from this depot is drained via the portal vein to the liver, in contrast to other fat depots that are drained to the systemic circulation. Visceral tissue also has a higher turnover rate of fat, in both men and women, than any other adipose tissue depot (Mårin *et al.,* 1992). Both lipid accumulation, by the action of lipoprotein lipase (LPL), and the lipolytic response to catecholamines, are elevated in this depot (Rebuffé *et al.,* 1989, 1990). The increased lipolytic activity of visceral fat combined with its anatomical localisation mean that the liver is exposed to higher concentrations of free fatty acids (FFA) than any other organ. FFA have an important influence on the liver metabolism and have been found to be slightly elevated in SLOB rats. Increased levels of FFA attenuate the hepatic clearance of insulin and the secretion of very-low-density-lipoproteins (VLDL) from the liver (Fukuda *et al.,* 1984; Svedberg *et al.,* 1991). Therefore, with enlarged visceral adipose tissue depots, these effects on the liver would be expected to cause peripheral hyperinsulinemia, hyperglycaemia, and elevated levels of VLDL. In SLOB rats, an increase was observed in VLDL but SLOB rats have normal insulin and glucose levels. Thus, although increased visceral fat and increased FFA may promote increased VLDL in some respect, the majority of the VLDL elevation is probably due to another cause. Lipid abnormalities in SLOB rats are discussed further in 3.8.7.

Excessive food intake promotes progressive weight gain if it is not accompanied by a compensating increase in energy expenditure. This is largely what happens in *Zucker*, *ob/ob*, *db/db* (Spiegelman and Flier, 1996) and *agouti* (Yen *et al.,* 1976) mutants. All these animals exhibit an uncontrolled feeding behaviour and a positive energy balance resulting in obesity. When assessing food intake per rat, SLOB males consumed the

same amount of food as normal male rats irrespective of age and bodyweight. However, when assessed per 100g bodyweight, food intake was slightly but significantly increased in young (100 day) SLOB males, but not in the old (365 day) SLOB males tested. Conversely, if food intake were calculated per 100g bodyweight in severely obese SLOB males this would probably show a reduced food intake compared to normal animals. I concluded from my food intake experiments that overall, SLOB males are not hyperphagic and consume about the same amount of food as their normal littermates.

It is possible, as food intake is not increased in these rats a major determinant of obesity may be an enhanced efficiency of calorie utilisation. In other words, these rats seem more proficient at storing their consumed calories as fat rather than utilising those calories for physical activity or for producing heat (thermogenesis) and for the elimination of energy by uncoupling mechanisms (Lowell and Spiegelman, 2000). Deciphering exactly what causes this increased energy storage has yet to be substantiated. Data on SLOB lipid abnormalities, together with data presented in further chapters, may aid in determining why despite normal food intake, SLOB males still develop severe abdominal obesity. It is important to note that although my data suggests SLOB rats are not hyperphagic at an old age, I discussed in chapter 1 that very minor changes in food intake over a long period of time can still cause obesity to develop. My analysis of food intake was carried out in separate groups of animals at two different age groups. It maybe more reliable to follow food intake in the same group of animals throughout their life. Then to determine exactly when and if at which point their intake exceeds that of normal littermates and how this cumulative food intake may contribute to their final obesity. These studies have yet to be carried out.

3.8.2 SLOB fertility

Wells (1997) concluded that SLOB males were infertile, however I have shown litters can be produced from SLOB males if animals are bred at an early age. SLOB females are fertile and do not show any abnormalities in reproductive function. As SLOB male and female rats have the same transgene insert, unless the products of this transgene or where it has integrated are sex specific in function, the genetic makeup

of these rats is similar. Other rodent obesity models also show sexual dimorphism in their fertility phenotype. Homozygous *fat* and *tubby* mice are infertile, but not sterile. As in SLOB males, litters can be obtained from homozygous *fat* and *tubby* males and females if they are mated before obesity develops at 5-8 weeks of age in the *fat* mouse and up to 12 weeks in the tubby mouse (Coleman and Eicher, 1990). Sterility is also a recognised feature of the *ob* mutation (Olefsky *et al.*, 1982). *ob/ob* males can occasionally reproduce if maintained on a restricted diet whereas *ob/ob* females are always sterile (Zuniga-Guajardo *et al.*, 1986). However, treatment with human recombinant leptin corrects the sterility defect in homozygous *ob/ob* female mice (Chehab *et al.*, 1996), which suggested a role for leptin in reproductive function. Further studies show administration of leptin to pre-pubertal mice accelerates puberty (Rogol *et al.*, 1998). In addition, human cases of leptin deficiency or leptin-insensitivity also show abnormalities in puberty onset and reproductive function (Montague *et al.*, 1997b; Clement *et al.*, 1998). These studies suggest a lack of leptin causes disturbances in reproductive function, but SLOB rats exhibit an excess of leptin. It is possible that this excess leptin has no function in SLOB fertility at all, or possibly that although SLOBs have elevated plasma leptins, they are resistant to its effects; this will be explored in the next chapter. The cause of SLOB infertility after about 12 weeks of age remains at this point an unknown problem. As well as testes morphology being normal, testosterone levels were also normal in these animals as were sperm count and motility.

It has been proposed that reproductive function is sensitive to the amount of adipose tissue stores, such that infertility occurs if a minimum body fat level is not maintained; conversely, ample fat stores should permit normal reproductive function (Frisch *et al.*, 1990). This theory is not tenable in light of all the evidence showing that diet-induced obesity and many forms of inherited obesity (e.g *ob/ob*, *db/db*, *tub/tub*, and *agouti* mice, *cp/cp* and *Zucker* rats) are all associated with delayed puberty and/or infertility (Bray 1992). In addition, from what I have shown, if this were the case SLOB males would not be infertile. Also arguing against the minimum-fat hypothesis are observations that refeeding food-restricted animals restores gonadotropin secretion and ovulation before adipose tissue stores return to normal levels (Bronson and Manning, 1991; Wade *et al.*, 1996). These observations together with the fact that obese SLOB males are infertile suggest that abundant body

fat stores do not sustain reproduction if the stored energy is not readily available for use.

3.8.3 Reduced growth hormone levels – role in obesity

In human obesity, growth hormone (GH) secretion is impaired and is reversed upon weight loss and thus is considered a consequence rather than the cause of the obese state (Williams *et al.,* 1984). Despite low GH levels, normal or increased IGF-I levels have also been observed in some obese subjects (Frystyk *et al.,* 1995; Nam *et al.,* 1997). This is thought to be due to the fact that as GH receptors are present in adipose tissue and even though GH hyposecretion may reduce the generation of IGF-I in each adipocyte, increased amounts of IGF-I may be secreted from the excessively enlarged amounts of adipose tissue. However, the relationship between GH and IGF-I levels in obesity have been conflicting, due to clinical differences in age, sex and degree and type of obesity (Nam *et al.,* 1997).

In the SLOB rat reduced pituitary GH levels and reduced plasma IGF-I levels are observed. Upon treatment with GH, IGF-I levels in SLOB rats returned to normal (Chapter 5) thus the impaired levels in non-treated rats are probably simply the result of endogenous GH-deficiency. The obese *Zucker* rat also expresses reduced levels of both growth hormone and growth-hormone-releasing hormone (Ahmad *et al.,* 1989, 1993).

Although the primary cause of SLOB obesity is most probably not GH-deficiency (Chapter 5), this characteristic may have a role in modifying some aspects of the developing phenotype. Growth hormone has a role in central obesity and treatment of abdominally obese men reduces abdominal fat mass, improves glucose and lipoprotein metabolism, and reduces diastolic blood pressure (Johannsson *et al.,* 1997). As the obesity in SLOB rats is predominantly present in the central adipose stores, it is possible GH-deficiency is driving fat storage to this depot. Also, in 80 day old SLOB males, both testicular and supra-renal fat pad weights are elevated, but at 1 year of age only supra-renal fat pads are elevated; this may also be due to developing GH-deficiency. Growth retardation is seen in terms of bodyweight in young SLOB rats who weigh less than normal littermates of the same age. However, bodyweights

of most SLOB rats are indistinguishable from normal littermates after about 200 days of age. If SLOB rats were not GH-deficient it is possible the development in gross bodyweight gain may be evident at an earlier age.

3.8.4 Metabolic studies

An assessment of maintaining body heat is one way of assessing calorie utilisation. I showed body temperature in SLOB males did not differ to that in normal males, however these measurements were crude and further analysis is required before any abnormalities in thermogenesis are determined. Challenging SLOB rats by exposure to a cold environment and then measuring body temperature over a period of time and then once animals are returned to normal room temperature, may show disturbances in SLOB rats being able to maintain normal body temperature. Abnormalities in thermogenesis have been identified in a number of the previously characterised rodent obesity models. For example, *ob/ob* mice have a marked cold intolerance, which is believed to be associated with their defective brown adipose tissue thermogenic responsiveness (Hogan and Himms-Hagen, 1981).

Pituitary TSH levels remained normal in SLOB rats, however analysis of thyroxine ($T_3/_4$) would give further clues as to the metabolic state of these animals. York *et al.*, (1978) reported an early abnormality in low circulating levels of T_3 in *ob/ob* mice. Later, Soomee and Kaplan (1994) discovered early treatment with triiodothyroxine (T_3) increased oxygen consumption and temperature and decreased body fat content in these mice.

My studies assessing SLOB metabolism have been very limited, further studies on energy intake, expenditure and output are warranted. These would provide a more accurate assessment of SLOB metabolism and possibly suggestions to the etiology of SLOB obesity.

3.8.5 Analysis of the HPA axis

The HPA axis has been implicated in obesity and many studies have reported a relationship between glucocorticoids and plasma leptin levels. Glucocorticoids have

been found to increase leptin production *in vitro* (Wabitsch *et al.,* 1996; Kolaczynski *et al.,* 1996) and exogenously administered glucocorticoids produce a sustained increase in circulating leptin levels in humans (Miell *et al.,* 1996; Larsson and Ahren, 1996; Papspyrou-Rao *et al.,* 1997). However, it should be noted that data on the role of leptin and the hypothalamic-pituitary-adrenal axis is inconsistent and has yet to be clarified (Cizza *et al.*, 1997; Bornstein *et al.,* 1997).

Endocrine abnormalities are commonly observed in many of the different mouse lines exhibiting obesity, with changes in adrenal corticosterone being particularly noteworthy. Adrenal corticoids are elevated in *ob/ob* and *db/db* mice compared with lean controls (Coleman and Burkart, 1977; Dubuc *et al.,* 1976a), but levels remain unchanged in the *agouti* mouse (Wolff and Flack, 1971). Likewise I also found levels were unaltered in SLOB males. Adrenalectomy in *ob/ob* mice (Solomon and Mayer, 1973; Saito and Bray, 1984) and *Zucker* rats (Stern *et al.,* 1983), eliminates hyperphagia and excessive weight gain in these rodents. Adrenalectomy in *agouti* mice normalises hyperglycaemia (Shimizy *et al.,* 1989) and reduces fat deposition, but does not completely prevent the relative obesity of *agouti* vs lean littermates (Jackson *et al.,* 1976). Therefore, although corticosterone levels are normal in *agouti* mice, the adrenals are necessary for the full expression of the obesity syndrome in these animals. It is therefore possible the adrenals may also have a role in the SLOB phenotype; adrenalectomy studies have yet to be conducted.

3.8.6 Diabetic status and insulin resistance

The pathogenesis of type 2 diabetes is characterised by two major features: peripheral insulin resistance and impaired insulin secretion from pancreatic β-cells (Kadowaki *et al.,* 1984; Taylor *et al.,* 1994). Hyperinsulinemia and elevated glucose levels are evident in *agouti* mice by 6 weeks of age (Frigeri *et al.,* 1983), by 10 days in *db/db* and by 15 days in *ob/ob* mice (Bray and York, 1979). Insulin promotes nutrient partitioning into adipose tissue and stimulates adipocyte growth and development (Kahn and Flier, 2000), thus hyperinsulinemia in these mice may contribute to their obesity. Leptin also has a role in insulin regulation. This is shown in leptin treated *ob/ob* mice, who within hours of treatment exhibit a fall in glucose and insulin levels (Halaas *et al.,* 1995). Leptin also has a clear insulin-sensitising effect acutely and also

after chronic administration to normal rodents (Halaas *et al.*, 1995; Campfield *et al.*, 1995; Pelleymounter *et al.*, 1995). The SLOB rat is quite unique in terms of its insulin and glucose responses. Whereas the other models mentioned clearly show evidence of diabetes and insulin resistance, the SLOB rat has normal resting insulin and glucose levels and normal ITT and GTT responses. Furthermore, GH treatment had no effect on the response to an ITT. Thus, whatever the underlying mechanism of the SLOB phenotype, this only effects obesity and not diabetic status as is often associated with this condition.

3.8.7 Lipid analyses

The primary defect in some forms of obesity may be an abnormal partitioning of lipid fuels toward storage, making these calories unavailable to oxidative tissues (Greenwood, 1984, 1985). SLOB rats clearly have some lipid abnormalities that may favour this hypothesis. Favouring adipose tissue storage would be an increase in lipogenesis and decrease in lipolysis. This may arise due to changes in individual factors and enzymes involved in adipogenesis, such as, an increase in adipose lipoprotein lipase (LPL), and a decrease in hormone sensitive lipase (HSL).

Triglyceride levels are elevated in *ob/ob*, *db/db*, *tubby* and *agouti* mice (Nishina *et al.*, 1994). These mice also have increased plasma cholesterol levels, mainly due to an increase in high-density lipoprotein cholesterol (HDL-C) (Nishina *et al.*, 1994). In SLOB rats, I also found plasma triglyceride levels to be elevated, again an expected result as adipose tissue mass is elevated. Whereas, in SLOB rats total plasma cholesterol levels were normal. However, analysis of individual lipoproteins showed an increase in VLDL-C as well as an increased VLDL-TG level. HDL and LDL were unaltered in SLOB rats compared to normal rats. Lipoprotein compositions are thought to be of importance in assessing diabetic and cardiovascular risk (Grundy *et al.*, 1979; Kesaniemi *et al.*, 1985; Egusa *et al.*, 1985; Siest *et al.*, 1995; Sirtori and Vega, 1997). Even though SLOB rats are not diabetic, the lipoprotein profiles observed in these rats are more typical of that in the human obese and diabetic population, than the profiles in most mouse obesity models. On the other hand, a recent study by Kobayashi *et al.*, (2000) characterised the diabetic dyslipidemia in the *db/db* mouse by analysis of various lipoproteins and concluded that mice fed a

western diet had a plasma lipoprotein phenotype that showed some similarities to that in patients with type 2 diabetes mellitus.

A reduction in the rate of lipolysis would promote fat accumulation. My results showed that the rate of lipolysis was reduced in SLOB rats compared to normal rats in both the testicular and supra-renal fat pads, with the greatest reduction occurring in the testicular fat depot. Data from normal rats showed there was already a difference between the degree of stimulation between testicular and supra fat, usually when carrying out this type of experiment there should be no obvious difference between different fat depots. Thus, the response in testicular fat in SLOB rats may also be blunted and lower than one would normally expect. Why such reduced stimulation occurs in normal animals could be due to reduced cellular activity resulting from non optimum collagenase treatment. However, as both depots were treated on the same day, at the same time and as they responded to isoproterenol in a dose dependent manner, this seems unlikely. Therefore I propose at this stage that SLOB rats do have a severely reduced capacity to respond to β-adrenergic stimulated lipolysis in both testicular and supra-renal fat depots. Possible explanations for this may be, a reduction in the number of β-adrenergic receptors in the adipose tissue, leading to a reduced ability to mobilise stored fat or, reduced levels of hormone sensitive lipase (HSL), leading to a reduced degree of hydrolysis of stored triglycerides. Both these effects would favour fat storage and thus elevate adipose tissue mass, however these parameters have yet to be tested in SLOB rats. If the rate of lipolysis in SLOB testicular adipose stores is as reduced as my results indicate, this suggests the primary adipose store should be in this depot and not in the supra-renal fat depot. However, it is possible for some reason the testicular store is able to compensate for this decreased lipolysis and increased accumulation by directing the fat storage to the supra-renal depots. Why and how this occurs and whether it is a primary or secondary consequence of the phenotype remains unclear. GH has also been proposed to possess a role in the regulation of metabolic effects on lipolysis and lipogenesis (Richelsen, 1997), as well as in the differentiation of the pre-adipocyte (Wabitsch *et al.,* 1995). Thus, in view of this and GH effects on abdominal adiposity, GH-deficiency in SLOB rats may have a role in diverting the fat storage to the supra-renal depot.

In order to determine the ratio of fat breakdown to fat production it would be useful to assess the rate of lipogenesis in rats of the same age. Unfortunately, problems in experimental procedure only yielded data for pilot animals from which I could not make any reliable conclusions, therefore accurate lipogenesis studies have yet to be conducted in SLOB rats. Such studies have been carried out in other rodent obesity models and increased rates observed in young *agouti* (Yen *et al.,* 1976), *ob/ob* (Cawthorne and Cornish, 1979) and *db/db* (Trayhurn and Wusteman, 1990) homozygotes.

LPL knockout mice are normal at birth, but develop lethal hypertriglyceridemia within the first day of life, at which point they have markedly reduced intracellular lipid stores (Weinstock *et al.,* 1995). Transgenic mice that express high levels of human LPL in muscle show increased muscle free fatty acid concentrations and increased numbers of fatty acid-metabolizing organelles, namely mitochondria and peroxisomes (Levak-Frank *et al.,* 1995). Together these observations suggest that tissue LPL expression is a major determinant of fatty acid entry into cells. Adipose LPL is elevated in obesity (Eckel, 1989) and following weight loss is elevated even further (Kern *et al.,* 1990), this suggests attempts to maintain lipid stores during fasting and to replenish lipid stores during refeeding. In terms of distribution, feeding results in an increase in the enzyme in adipose tissue and a decrease in the enzyme in muscle tissue, thus muscle LPL is regulated inversely to adipose LPL, (Eckel, 1989). This inverse regulation of LPL maximises energy storage during times of food availability and maximises energy availability for muscle during periods of food-seeking behaviour. Thus, an increased adipose/muscle LPL ratio would partition dietary lipid into adipose tissue and would explain some of the variability in weight gain when humans are exposed to excess calories. In addition, exercise results in a decrease in adipose tissue LPL, along with an increase in muscle LPL (Simsolo *et al.,* 1993).

Thus LPL activity has been considered to be rate-limiting for the removal of circulating lipoprotein triglyceride fatty acids from chylomicrons and VLDL (Eckel, 1989). In *Zucker* rats, adipose tissue LPL is increased, cardiac muscle LPL is decreased (Bessesen *et al.,* 1991), and skeletal muscle LPL activity is decreased

(Hartman, 1981). Similarly in SLOB rats, LPL activity was increased in central adipose tissue (supra-renal and ovarian fat), decreased in cardiac muscle (females) and decreased in skeletal muscle (males). Both these models favour lipid storage in adipose depots and disfavour energy mobilisation by muscle tissue. In particular, it is interesting to note that LPL activity was only increased in the supra-renal fat pad of male SLOB rats and not in the peripheral testicular fat pad. This coincides with the fact that obese SLOB rats have excessive adipose weight gain in the central region and not the peripheral region. In female SLOB rats, LPL activity was increased in both the supra-renal and ovarian fat pad stores, again both central adipose stores. Both SLOB and normal rats also showed significant differences between male and female animals in LPL activity in cardiac and soleus muscle, not seen in adipose tissue. Gender differences in LPL activity have previously been shown in Wistar rats, but these researchers found differences in both adipose and muscle tissues (Galan *et al.,* 1994). The difference in male and female LPL activity between the two muscle groups in SLOB rats are unclear. However, as LPL activity was significantly reduced in soleus muscle in male SLOB rats, a representative skeletal muscle, indicates the major muscle group in male rats shows indication of favouring fat deposition to adipose tissue rather than utilisation, coinciding with the sex-specific phenotype.

It is possible muscle tissue has an inability to process free fatty acids (FFA) presented to it and as LPL activity is low, the FFA are redirected to adipose stores where LPL activity is high. GH is known to increase heart and skeletal muscle tissue LPL activity (Oscarsson *et al.,* 1999). As both SLOB and Zucker (Ahmad *et al.,* 1993) rats have reduced GH levels, it is possible this may contribute to the reduced muscle LPL activity seen in these models. However, as both male and female SLOB rats are GH-deficient, this does not explain the differences in LPL activity in the two muscle groups. The primary cause of LPL activity change thus remains unclear and measuring LPL activity in GH-treated SLOB rats might clarify the role of GH-deficiency in this result.

Finally, lipid analyses revealed a significant increase in hepatic VLDL secretion but a reduction in apoB in SLOB rats. As previously mentioned this finding is unusual and one that will require further analysis. As VLDL amount is usually proportional to the amount of apoB, this result indicates that perhaps SLOB livers are generating very

large VLDL particles with small apoB particles. This would account for the increased VLDL amount and reduced apoB concentrations. Examination of apoB secretion rates in *ob/ob* and *db/db* mice shows no overall change in apoB secretion (Xiaohua *et al.*, 1997).

3.9 Summary

This chapter has demonstrated the SLOB rat as a unique and interesting model of male-specific visceral obesity. The obesity in these males is most markedly demonstrated in terms of their large supra-renal fat pad weights and elevated plasma leptin levels. Although males initially presented as infertile, attempts to breed from younger animals have proved successful and a future homozygous line is possible. GH-deficiency is most likely not causal of the SLOB phenotype and will be examined further in Chapter 5. In terms of diabetic state and insulin resistance SLOB males are normal, an unusual finding when compared to previously characterised rodent obesity models. Lipid analyses have revealed a number of abnormalities. These include a marked increase in VLDL triglyceride and cholesterol, a reduced apoB concentration, decreased lipolytic activity and increased VLDL secretion.

In the next chapter, I further my characterisation of the SLOB rat by investigating the expression levels of some hypothalamic neuropeptides known to have a role in feeding behaviour.

Chapter 4

Hypothalamic Neuropeptide Analysis

4.1 Introduction

For many years the mediators and mechanisms involved in the hypothalamic control of feeding remained largely unknown. However, the past five years have witnessed an explosive increase in research on the regulation of feeding and energy-balance (Schwartz *et al.,* 1996a; Woods *et al.,* 1998b; Smith, 1999). This increase has largely stemmed from the identification of leptin and the genetic characterisation of various subsequent rodent obesity models, which have greatly stimulated the investigation of peptides acting in the brain (Zhang *et al.,* 1994; Friedman *et al.,* 1998). Research into these molecules and their neuronal systems, receptors and interactions, have begun to unravel the circuitry between peripheral adipogenic signals and hypothalamic effector pathways, all of which have provided valuable information in understanding energy homeostasis and the control of bodyweight regulation.

The cause of obesity in SLOB rats remains unknown but one candidate is the novel gene *5'OT-EST* expressed selectively in hypothalamic nuclei, which will be shown in chapter 6. As this is a new and unique model, I decided to measure by *in situ* hybridisation the expression of a number of previously characterised hypothalamic feeding neuropeptides and receptors in an attempt to provide clues to either the underlying mechanism of the SLOB phenotype or to the consequences of its development upon their expression. A large number of neuropeptides have been measured in previously characterised rodent obesity models, thus I have attempted to make comparisons between results in these models and those in the SLOB rat, to further characterise and generate any clues in relation to SLOB-type obesity. In addition, as SLOB rats are GH-deficient I also decided to analyse the hypothalamic GH-axis to investigate the extent of this aspect of the phenotype on hypothalamic hormone and receptor levels.

4.2 Leptin receptors

Leptin, as previously described, is thought to be the long-sought-after lipostat factor postulated to govern energy balance through a negative-feedback loop that originates in adipose tissue and acts on hypothalamic centres in the brain (Friedman *et al.,* 1998;

Elmquist *et al.,* 1999). Leptin reduces food intake in a mechanism not fully understood but the hormone is thought to influence, or interact with, many of the key peptidergic regulators of feeding (Friedman and Halaas, 1998). This is summarised in **Figure 4.1**, which shows a model of the pathways of leptin in the hypothalamus. Leptin receptors in the brain are wide spread and consist of both intracellular and extracellular types (Steiner, 1996; Friedman, 1997a). I have shown in chapter 3 that SLOB rats have greatly elevated plasma leptin levels. It is possible, SLOB rats exhibit an abnormality in the reception of this elevated leptin, that is SLOB rats may be resistant to leptin action. One obvious indicator would be an abnormality in the receptor concentration in the brain if such a resistance was present. *In situ* hybridisation analysis was carried out to quantify extracellular leptin receptor (OBR-EC) expression measured in the piriform cortex, choroid plexus and thalamus, areas previously shown to strongly express leptin receptors (Bennett *et al.,* 1998) (**Figure 4.2**). Riboprobes for OBR-EC and OBR-IC together detect all isoforms of the leptin receptor. The riboprobe for OBR-EC detects both full length (OB-Rb) and all the short isoforms of leptin receptor detected so far (OB-Ra/c/d/e). For all areas measured, OBR-EC mRNA levels showed no difference in expression in either male or female SLOB animals when compared with normal animals. Thus, elevated plasma leptin levels in SLOB rats and the accompanying obesity did not appear to effect total leptin receptor mRNA levels in any of the areas quantified. Unfortunately, the intensity of signal for the intracellular form of the leptin receptor (OBR-IC) was not high enough to be reliably quantified in my studies.

4.3 Neuropeptide Y (NPY)

Leptin inhibits NPY-mediated neuronal activity in the hypothalamus and reduces levels of NPY mRNA in the arcuate nucleus, paraventricular and dorsomedial nuclei

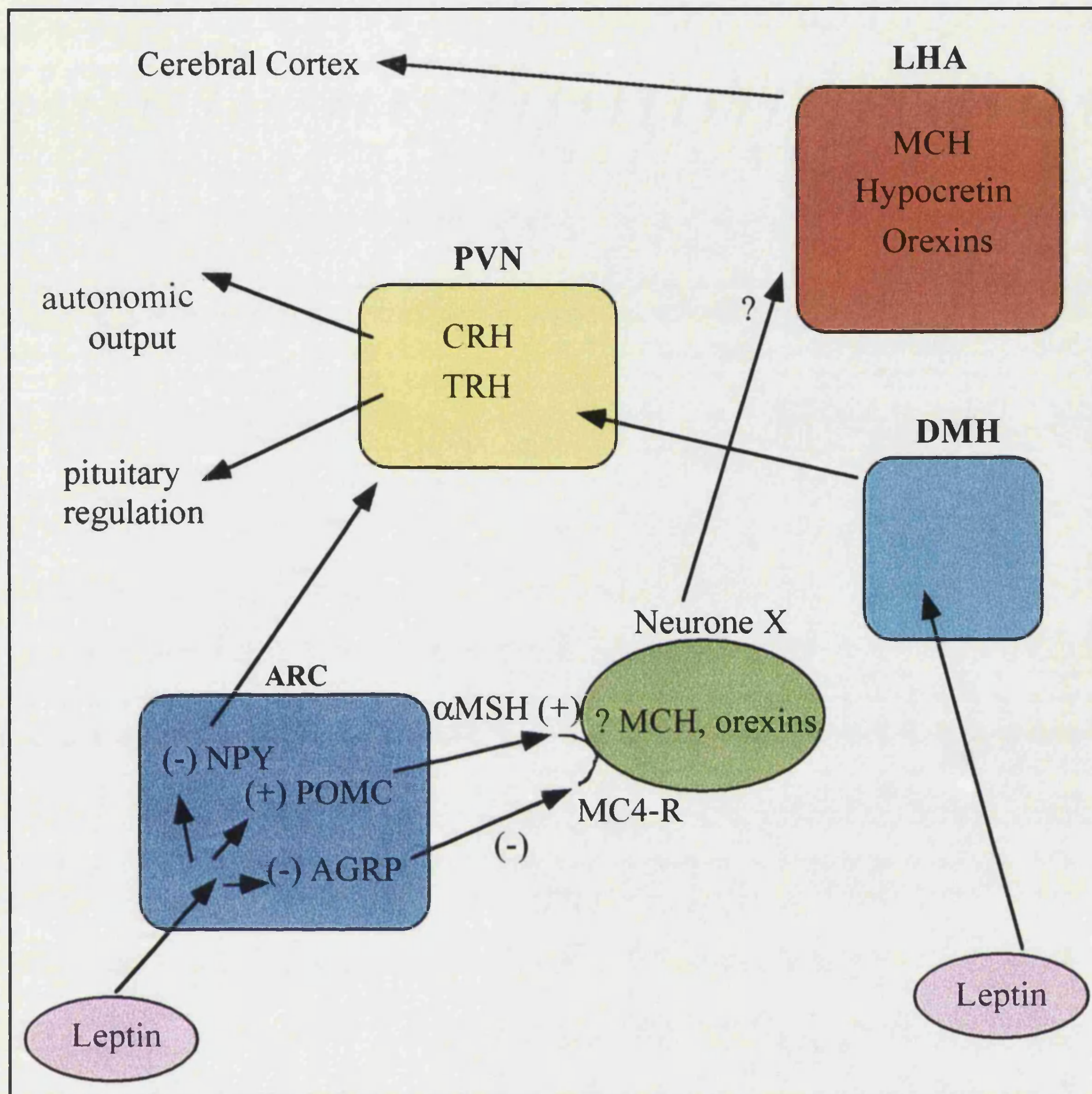


Figure 4.1 A Model of the pathways of leptin action in the hypothalamus. Through direct action on cell bodies in the arcuate nucleus (ARC), leptin positively regulates POMC (α-MSH) and negatively regulates NPY and AGRP. α-MSH neurones project to MC4 receptor-expressing neurones, AGRP-expressing neurones antagonise the α-MSH signal on these neurones. Directly or indirectly, this signal influences neurones in the lateral hypothalamus (LHA) that then influence hunger/satiety by mechanisms that may include long cortical projections involving MCH neurones. NPY projects to the paraventricular nucleus (PVN), which contains neurones expressing CRH and TSH (and other neuropeptides). The PVN influences anterior and posterior pituitary functions. The PVN also directly innervates autonomic preganglionic neurones, both sympathetic and parasympathetic. The dorsomedial hypothalamus (DMH) has neurones that are responsive to leptin and project to the PVN (adapted from Flier and Flier, 1998).

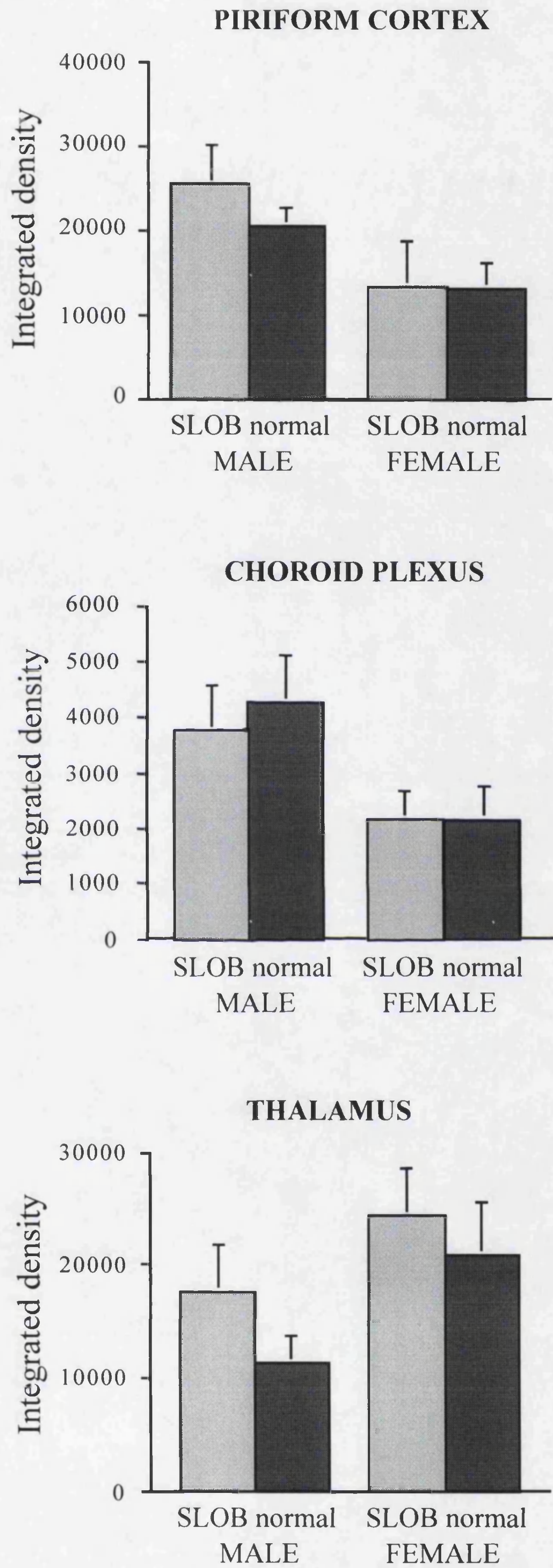


Figure 4.2 OB-EC mRNA levels in male and female rats. In situ hybridisation analysis of 3 different regions in SLOB and normal rat brains (animals 1 year of age n=6).

(Stephens *et al.*, 1995; Schwartz *et al.*, 1996b; Ahima *et al.*, 1996; Wang *et al.*, 1997). In obesity models that have a defect in leptin signalling, NPY mRNA expression and food intake are increased (Beck *et al.,* 1990; Stephens *et al.,* 1995). If leptin is the principal mediator of NPY gene expression and as SLOB rats have elevated plasma leptins I would expect NPY mRNA expression to be reduced in transgenic animals. *In situ* analysis showed NPY mRNA expression in the arcuate nucleus was decreased in SLOB male rats when compared to normal littermates (**Figure 4.3**). Whether this is primary or secondary is debatable; it could simply be a consequence of elevated plasma leptin levels (Smith *et al.,* 1998).

4.4 Corticotropin releasing hormone (CRF)

CRF is produced predominantly in the paraventricular nucleus (Morley, 1987; Kaiyala *et al.*, 1995). It is catabolic in nature and inhibits food intake, opposite to the effects of NPY. Leptin acts to stimulate expression of the gene encoding CRF (Schwartz *et al.*, 1996a) and basal CRF release in the brain (Raber *et al.*, 1997). The anoretic effect of leptin is attenuated by simultaneous administration of the CRF receptor antagonist, α-helical CRF (Uehara *et al.*, 1998), the weight loss induced by leptin is therefore thought to be likely to involve stimulation of hypothalamic CRF release. Therefore, due to elevated plasma leptin levels in SLOB rats I expected to find an increase in hypothalamic CRF expression. **Figure 4.4** shows that in fact CRF levels were significantly reduced in male SLOB rats.

4.5 POMC and AgRP

Arcuate nucleus expression of POMC (**Figure 4.5)** and AgRP (**Figure 4.6**), two peptides related to the melanocortin system were quantified in SLOB and normal rats. Rising leptin usually increases POMC mRNA expression, which then acts to reduce food intake (Thornton *et al.,* 1997). Again, if plasma leptin levels were the principal mediator of hypothalamic POMC expression, I would expect SLOB rats to have an increased arcuate POMC mRNA expression. However, no significant hypothalamic

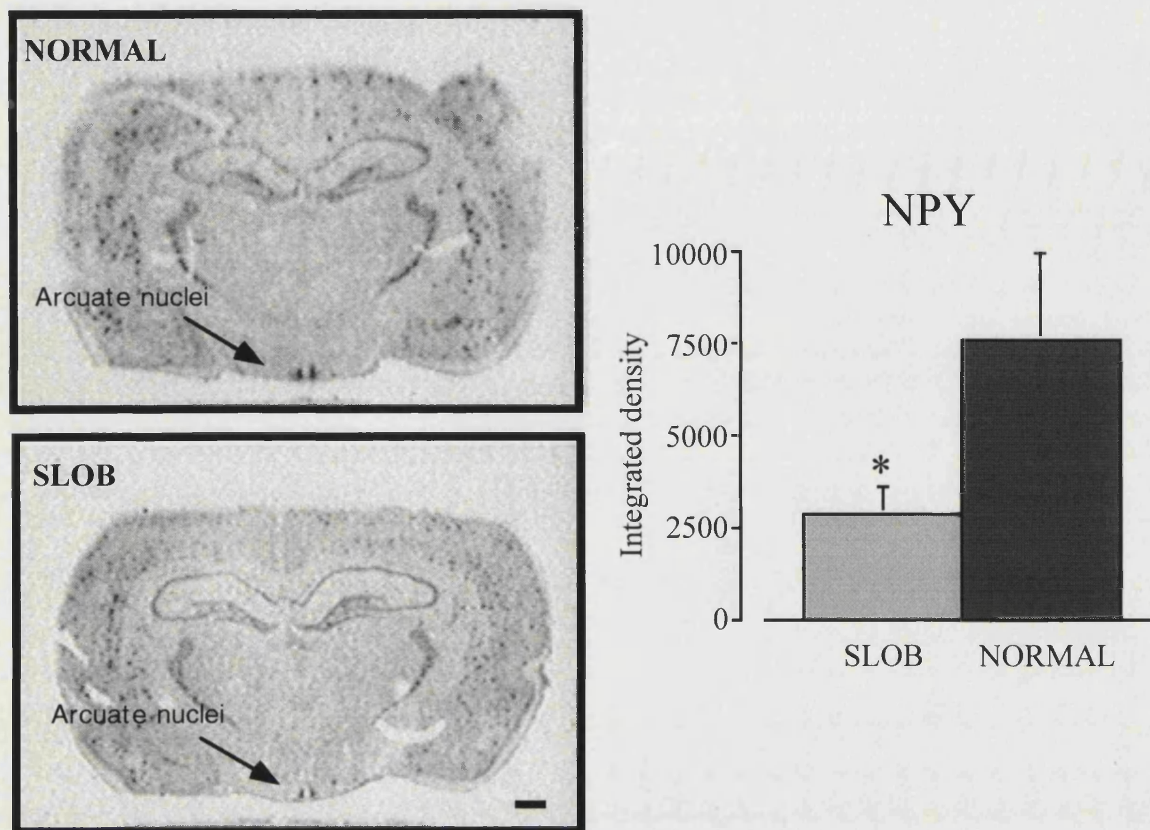


Figure 4.3 NPY mRNA levels in male rats. In situ hybridisation of arcuate sections from male SLOB and normal rats at 1 year of age (n=6 *p<0.05).

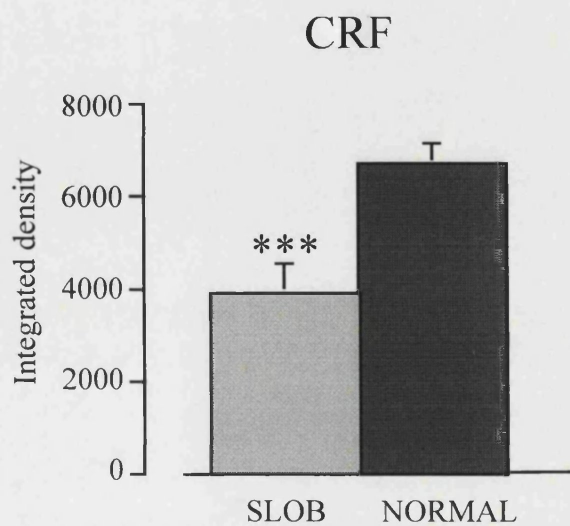


Figure 4.4 CRF mRNA levels in male rats. In situ hybridisation of paraventricular nucleus sections from male SLOB and normal rats at 1 year of age (n=6, ***p<0.001).

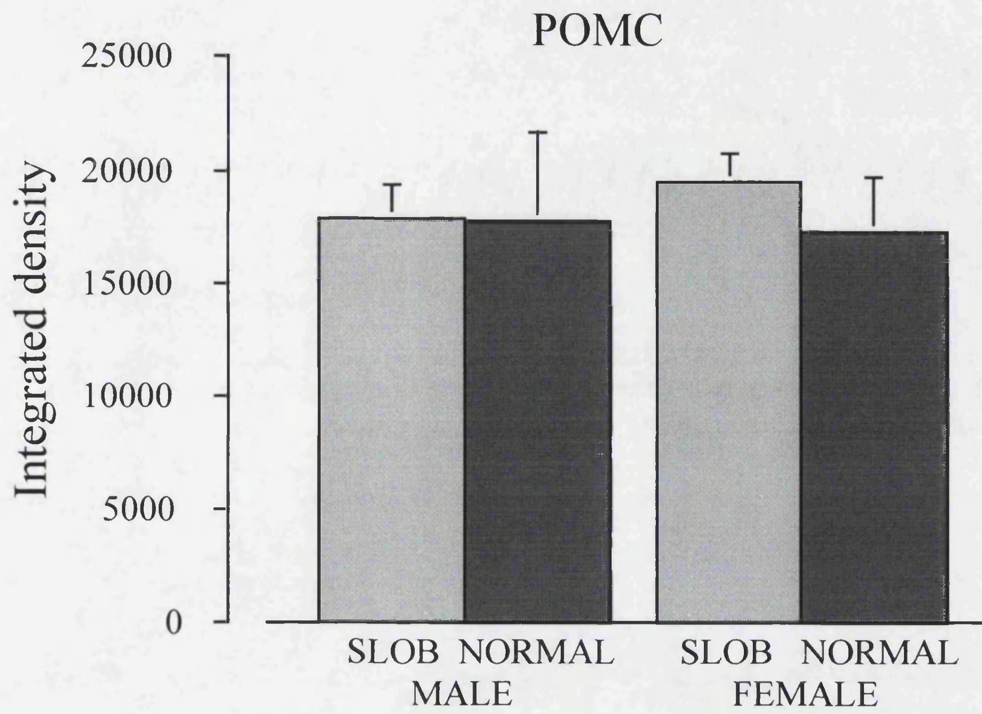


Figure 4.5 POMC mRNA levels in male and female rats. In situ hybridisation of arcuate sections from male and female SLOB and normal rats at 1 year of age (n=6).

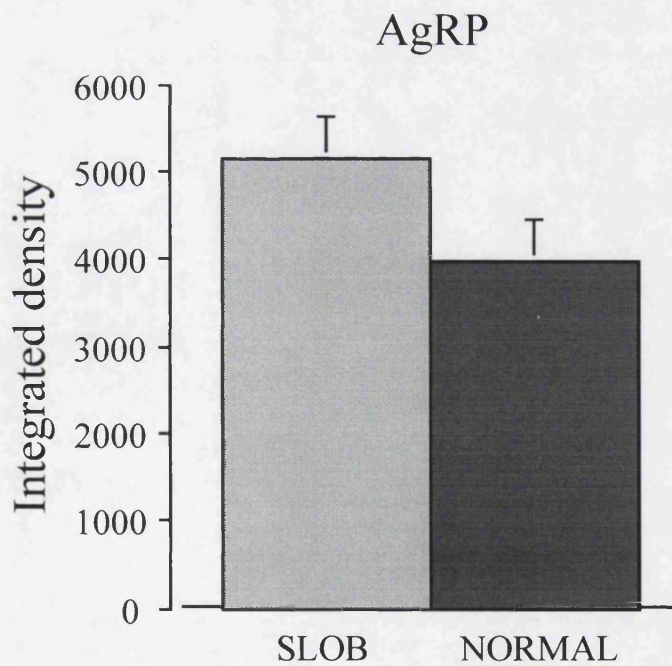


Figure 4.6 AgRP mRNA levels in male rats. In situ hybridisation of arcuate sections from male SLOB and normal rats approximately 1 year of age (n=6).

POMC mRNA differences were found between SLOB and normal animals. AgRP, which mimics the actions of the *agouti* protein by blocking melanocortin 4–receptors in the hypothalamus and causing an increase in food intake, was found to be slightly but not significantly higher in SLOB compared to normal male rats.

4.6 Tubby

Tubby is a relatively new neuropeptide and of particular interest to this thesis as the obesity in *tubby* mice is relatively late in onset and therefore resembles the weight gain in the human population and in SLOB rats. Also, tubby is expressed in the hypothalamus and testes (Noben-Trauth *et al.,* 1996), two areas in which the novel gene *5'OT-EST* is also expressed as will be explored further in chapter 6. To date, little work has been carried out on hypothalamic *tubby* expression patterns and presented in chapter 5 are data analysing *tub* mRNA levels in various experimental models such as high-fat feeding, ovariectomised, and oestrogen treated animals. Dr Paul Le Tissier at the NIMR cloned the rat *tub* gene and generated clones for riboprobe production. The 3'end of *tub* shows homology with the class of tubby like proteins (TULPs), however the probe generated by Dr Le Tissier does not cover this region and thus is specific for *tub* (covers bases 573-819 of rat tubby coding sequence). The data presented in this thesis on *tub* expression are the first to investigate hypothalamic mRNA expression in a rodent obesity model and in the experimental procedures mentioned.

tub is widely spread throughout the brain but particularly in the SON and PVN. A significant difference was found in the supraoptic nuclei (SON), in which SLOB males exhibited an increase in mRNA expression levels (**Figure 4.7(a)**). No significant differences were found in the paraventricular nuclei (**Figure 4.7(b)**), dorsomedial nuclei, ventromedial nuclei, or the hibenula. However, a sex-specific difference was observed in *tub* PVN mRNA expression between male and female normal rats, but was not quite significant between male and female SLOB rats.

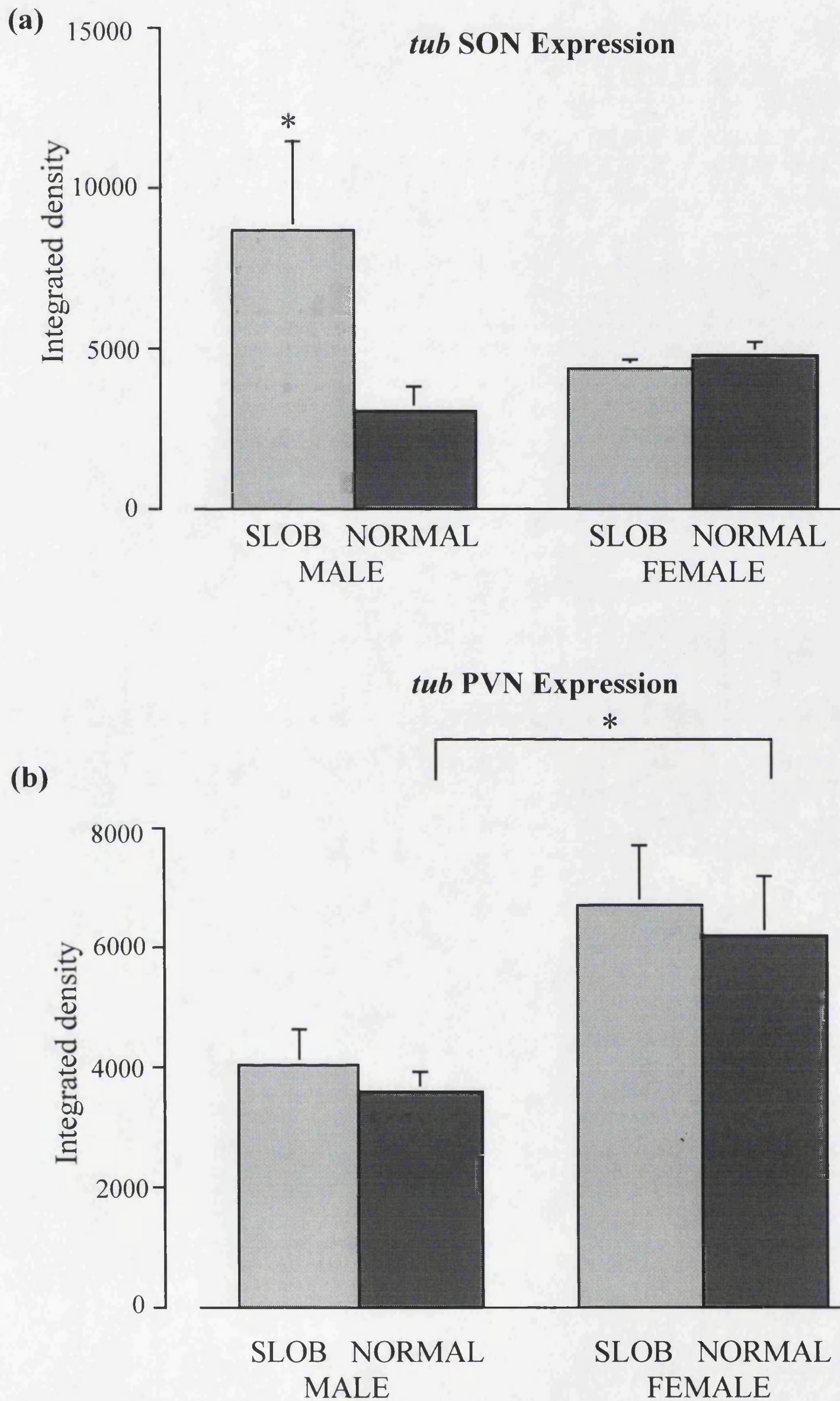
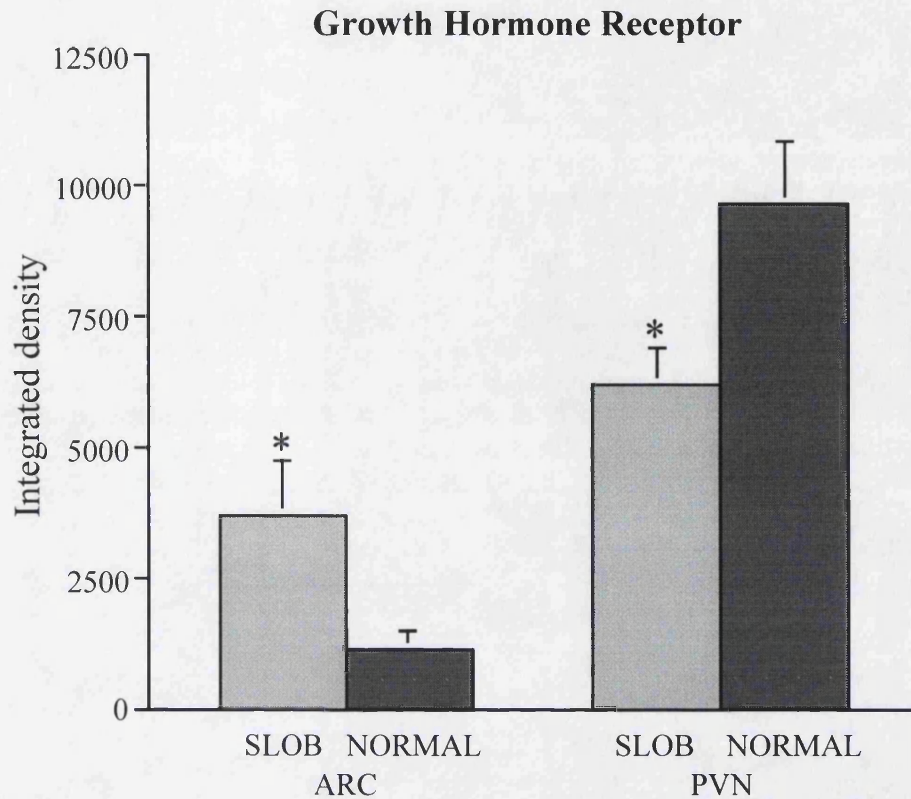


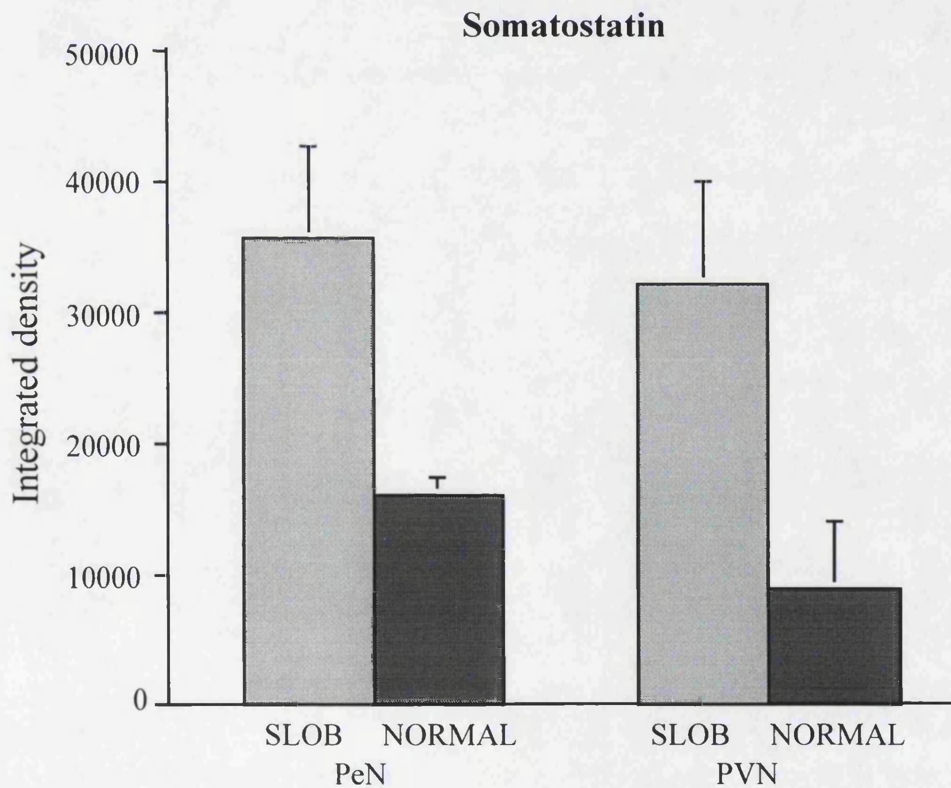
Figure 4.7 ***Tub* mRNA expression in SON and PVN nuclei.** (a) tub mRNA expression in the SON of male and female, SLOB and normal rats at 1 year of age. (b) tub mRNA expression in the PVN of male and female, SLOB and normal rats at 1 year of age (n=6. *p<0.05).

4.7 GH axis

Growth hormone deficiency in SLOB rats may effect expression levels of neuropeptides involved in the GH axis, such as, growth hormone receptors (GHR), somatostatin (SS) and growth hormone releasing hormone (GHRH). *In situ* hybridisation revealed an increase in arcuate GHR expression and a decrease in paraventricular GHR expression (**Figure 4.8 (a)**). The different results may be due to the difference in the amounts of hGH the GH receptors in different nuclei perceive. Note that overall PVN GHR concentration is higher than arcuate GHR expression, this is because the PVN covers a larger area than the arcuate. hGH from the transgene is expressed in PVN and SON nuclei driven off the vasopressin promoter as reported by Wells (1997). For this reason I chose to measure GHR in the PVN as this is an area not normally known for GH regulation but GH receptors have been found here (P. Bennett, personal communication). A local increase in hGH levels in this area may therefore downregulate GH receptor concentration in the PVN. It has already been found that hGH administered by i.c.v causes an increase in GHR arcuate mRNA expression in *dwarf* rats, and a further higher dose downregulates GHR concentration in the arcuate nucleus (Bennett *et al.,* 1995). Therefore, the reduced PVN expression in SLOB rats may be attributable to high local hGH levels in the PVN. The GH deficient *dwarf* rat and hypophysectomised rats exhibit reduced arcuate GHR concentration (Bennett *et al.,* 1995). In contrast, in the GH deficient SLOB rat, I found arcuate GHR concentration was increased. As hGH from the transgene is present in the hypothalamus, this may be affecting arcuate GHR concentration in the same way as low dose hGH central administration as shown by Bennett *et al.,* (1995).

In GH deficiency, PeN SS expression is reduced whereas arcuate GHRH expression is increased, which would lead to increased GH production and release (Bertherat *et al.,* 1995). However, GH-deficient SLOB males appear to have an increased expression of SS mRNA in both PeN and PVN nuclei (although not significant), the opposite of that normally expected in a state of GH deficiency (**Figure 4.8 (b)**). Again, this may





<u>Figure 4.8</u> **Hypothalamic GHR and SS mRNA expression.** (a) GHR mRNA expression in the arcuate and paraventricular nuclei of male SLOB and normal rats at 1 year of age (n=6, *p<0.05). (b) SS mRNA expression in the periventricular and paraventricular nuclei of male SLOB and normal rats at 1 year of age (n=6).

be explained by increased local production of hGH in the hypothalamus causing an upregulation of SS in these nuclei. This result therefore implies there is hGH feedback and is evidence for GHR levels being GH mediated. Furthermore, local hypothalamic hGH production in the Transgenic Growth Retarded (Tgr) rat, who expresses hGH under the control of the rat GHRH (Flavell *et al.,* 1996), also exhibits increased SS PeN mRNA expression (Pellegrini *et al.,* 1997).

It is most probable that the differences observed in the peptides of the GH axis are related to the GH-deficient trait of SLOB animals. However, there is a possibility these effects may be attenuated or ameliorated by the effect of obesity on these peptides, the role of GH and SS in energy homeostasis are discussed in section 4.8.6.

4.8 Discussion

Neuropeptide analysis shows SLOB rats have significantly reduced NPY, and CRF mRNA expression, an increased supraoptic *tub* mRNA expression and normal POMC, AgRP and OB-EC expression levels. In addition, GHR expression is significantly increased in the arcuate nucleus and significantly decreased in the paraventricular nucleus, and SS expression is increased in both periventricular and paraventricular nuclei. These measurements are only a superficial survey of some relevant hypothalamic neuropeptide and receptor levels in the steady state of the SLOB rat. They give an overall impression of large changes occurring in mRNA levels in the SLOB hypothalamus, and are further discussed here. Clearly, there are many other peptide systems that need to be analysed to get a fuller picture of hypothalamic gene expression and how they relate to obesity in the SLOB rat.

4.8.1 Leptin receptor mRNA levels

The inhibitory effect of leptin on food intake is believed to be mediated by the long isoform of the leptin receptor OB-Rb in the hypothalamus (Tartaglia, 1997). It is therefore conceivable that a decrease in OB-Rb expression in the hypothalamus could blunt the leptin signal transduction pathway, ultimately leading to the development of obesity. In *tubby* mice changes in OB-Rb mRNA are not detected in the hypothalamus (Guan *et al.,* 1998). In SLOB rats no change was observed in OBR-EC, although it may be argued as this probe detects all isoforms of the leptin receptor and not specifically OB-Rb, a change may be present in individual isoforms but which is not detected with a non-specific probe. Thus, further analysis using specific isoform riboprobes may be warranted.

Deficiency in leptin function results in excess food intake and severe obesity in *ob/ob* and *db/db* mice (Zhang *et al.,* 1994; Chen *et al.,* 1996; Lee *et al.,* 1996). I have shown previously that obese SLOB rats are not hyperphagic, here I have shown that there are no changes in leptin receptor mRNA levels. If SLOB rats are not leptin resistant and have elevated circulating leptin levels, due to an increased adipose tissue mass, it is possible that leptin is acting to reduce food intake which is perceived as a

non-hyperphagic obese model. However, this potential reduction in food intake does not prevent obesity in SLOB rats.

For leptin to exert its effect, the delivery of leptin into the central nervous system seems to represent a crucial step toward the regulation of food intake and energy balance. Even though leptin receptor concentration may be normal, it has been proposed a potential locus of leptin resistance in obese individuals may lie in the transport of leptin through the blood-brain barrier (BBB), and saturable transport of leptin into the brain may be a rate-limiting step with respect to leptin action (Schwartz *et al.,* 1996c; Caro *et al.,* 1996). Frequently, leptin doses that have no effect when administered peripherally reduce food intake when administered centrally (Friedman, 1997b). In addition, the cerebrospinal fluid/serum leptin ratio is much lower in obese rats when compared with lean rats (Burguera *et al.,* 2000), indicating that a decreased transport efficiency across the BBB might well be a major factor that contributes to obesity. Conversely, the estimated half-life as well as the biological activity of circulating leptin is similar in both lean and obese humans; so abnormal serum leptin binding or abnormal leptin catabolism does not seem to be the underlying mechanism for the development of human obesity (Klein *et al.,* 1996; McGregor *et al.,* 1996).

The hypothesis of peripheral versus central leptin action is one that has only recently begun to unfold. How leptin gains access to specific regions in the brain is still a controversial issue, a report by Wiesner *et al.,* (1999) has also suggested the possibility that the human brain produces leptin. It is possible that defects in the transport of leptin may also exist in SLOB rats, if a defect was present and corrected, would SLOB's reduce their food intake further from an otherwise normal level? As obese SLOB rats are not hyperphagic, such a defect seems unlikely. However, this analysis may confirm that SLOB rats exhibit a normal leptin pathway in terms of; leptin release correlated with adipose tissue mass, leptin sensitivity correlated with normal hypothalamic receptor concentration and efficient leptin transport through the blood brain barrier.

4.8.2 Leptin and NPY

Chronic administration of NPY into the hypothalamus of normal animals mimics many physiological consequences of leptin deficiency, including obesity, hyperphagia, reduced thermogenesis, decreased fertility and inhibition of growth hormone production (Stanley *et al.,* 1986; Catzeflis *et al.,* 1993; Zarjevskit *et al.,* 1993). All these factors suggest NPY mediates many of the changes caused by leptin deficiency. Leptin administration inhibits NPY induced feeding in *ob/ob* mice (Smith *et al.,* 1998) and decreases NPY gene expression in the arcuate nucleus (Schwartz *et al.,* 1996b). This appears to be a specific effect of leptin and not a secondary effect due to an alteration in food intake or body weight. Leptin has no effect on NPY expression in *db/db* mice (Schwartz *et al.,* 1996b), indicating the unresponsiveness of diabetic mice to leptin. Both these findings suggest that leptin functions, at least in part, by inhibiting NPY synthesis and release. In view of this, NPY mRNA expression would be expected to be reduced in SLOB rats with high leptin.

However, it should be noted that leptin is able to exert its effects without NPY, demonstrated by the generation of *ob/ob* mice also deficient in NPY by Erickson *et al.*, (1996). In the absence of NPY, *ob/ob* mice are less obese because of reduced food intake and increased energy expenditure and are less severely affected by diabetes, sterility, and somatotropic defects. As these mice have apparently normal food intake and body weights, this suggests that other systems compensate for NPY's normal activities in energy homeostasis.

NPY mRNA levels have also been quantified in the *Zucker*, *agouti* and *tubby* rodent obesity models. In the *Zucker* rat NPY is elevated (Beck *et al.,* 1990, 1992b, 1993), coinciding with a defective leptin receptor in this model and a consequently abnormal leptin reception resulting in hyperphagia. To assess the role of NPY in the etiology of the *agouti* mouse, NPY deficient *agouti* mice were produced (Hollopeter *et al.,* 1998). However, no differences in bodyweight or food intake were found between these double mutant mice and normal *agouti* mice. These results therefore indicated that NPY was not required for the obesity caused by ectopic *agouti* expression. In old *tubby* mice (6 months and older), NPY is increased in the dorsomedial and ventromedial hypothalamic nuclei (Guan *et al.,* 1998). This difference is not present

in normal mice, which indicates the change is most likely due to an increase in obesity, as *tubby* mice experience late-onset obesity. In the arcuate nucleus, NPY is reduced in both young (2-3 months) and old *tubby* mice (Guan *et al.,* 1998). Presented in this thesis are data for NPY mRNA expression in the arcuate nucleus of SLOB rats, results of which are similar to the *tubby* mouse. As SLOB rats show a late-onset obesity as in *tubby* mice, further analysis at varying ages may reveal similar age and/or body mass-dependent changes in nuclei such as the ventromedial (VMH) and dorsomedial (DMH) nuclei. Although, it is not yet clear how NPY pathways originating from DMH and VMH affect feeding behaviour and energy balance, the age/body mass dependent differences in *tubby* mice indicate these regions may well have an important role in obesity etiology.

In terms of regulation, as well as leptin, glucocorticoids, insulin and growth hormone are also implicated in energy homeostasis by effects on NPY. Adrenalectomy attenuates the effect of fasting to increase both food intake and hypothalamic NPY gene expression, and these impairments are reversed by glucocorticoid administration (Stanley *et al.*, 1989; Green *et al.,* 1993; Sainsbury *et al.*, 1997). In addition, central injection of NPY results in increased plasma corticosterone levels (Wahlestedt *et al.,* 1987) and insulin (Billington *et al.,* 1991; Beck *et al.,* 1992a; Zarjevski *et al.,* 1993), promoting energy storage as fat. NPY containing nerve terminals are also in close association with CRF cell bodies in the PVN (Wahlestedt *et al.,* 1987). NPY also increases the content of CRF (Haas *et al.,* 1987) and the level of CRF gene expression (Suda *et al.,* 1993), suggesting the increase in plasma corticosterone is presumably the result of increasing hypothalamic release of CRF (Dallman *et al.,* 1995). These studies suggest a delicate interplay exists between NPY and CRF in the regulation of energy balance.

4.8.3 Leptin and CRF

Although most reports claim leptin stimulates CRF expression, the interactions between leptin, CRF and the HPA axis appear to be complex, and contradictory results have been reported (Heiman *et al.*, 1997; Huang *et al.*, 1998). Thus, the identification of reduced CRF mRNA expression and elevated plasma leptin levels in SLOB rats are not surprising as leptin may actually inhibit the activity of the

neuroendocrine CRF-containing neurones and the activation of the HPA axis in response to stress. On the other hand, as reported in the previous chapter, no change in plasma corticosterone secretion or pituitary ACTH levels are observed (or in adrenal size as observed by Sara Wells, unpublished data) in SLOB rats. Thus, the difference in hypothalamic CRF mRNA levels does not appear to effect hormones downstream in the HPA axis. However, I have yet to determine plasma ACTH levels and it is possible although pituitary levels are normal, the amount of release may be abnormal, however as plasma corticosterone levels are normal this seems unlikely.

Glucocorticoids have been found to increase leptin production in vitro (Wabitsch *et al.,* 1996; Kolaczynski *et al.,* 1996) and exogenously administered glucocorticoids produce a sustained increase in circulating leptin levels in humans (Miell *et al.,* 1996; Larsson and Ahren, 1996; Papspyrou-Rao *et al.,* 1997). In contrast, calorific restriction and glucocorticoid administration lower CRF expression (Imaki *et al.,* 1991), whereas involuntary overfeeding and adrenalectomy cause anorexia and stimulate CRF expression (Jingami *et al.,* 1985). These data again demonstrate that the role of leptin in the hypothalamic-pituitary-adrenal axis has yet to be clarified (Cizza *et al.*, 1997; Bornstein *et al.,* 1997). The source of the significant reduction in CRF mRNA levels in SLOB males therefore remains unclear.

As with NPY, CRF mRNA levels have also been measured in other rodent obesity models and in food restricted animals. In young and old *tubby* mice, CRF mRNA in the paraventricular nucleus is unaltered compared to normal mice (Guan *et al.,* 1998). Likewise, hypothalamic CRF mRNA is unaltered in *Zucker* rats (Pesonen *et al.,* 1992), but CRF protein is lower in these rats compared to lean controls (Nakaishi *et al.,* 1990, 1993). Although corticosterone levels in *ob/ob* mice are elevated, no significant differences are observed in hypothalamic CRF mRNA expression in these mice (Jang and Romsos, 1998). Following food deprivation, CRF mRNA expression is reduced in the PVN of lean rats (Brady *et al.,* 1990), but is increased in *Zucker* rats or *ob/ob* mice (Timofeeva *et al.,* 1997; Huang *et al.,* 1998). Thus SLOB rats show the same reduced CRF mRNA expression as observed in food restricted normal rats.

In the rat brain, CRF targets two classes of transmembrane receptors referred to as CRF type 1 (Perrin *et al.,* 1993) and CRF type 2α (Lovenberg *et al.,* 1995) receptors.

CRF also binds a CRF binding protein (BP) that is widely distributed throughout the brain. The exact function of this protein has yet to be fully defined, but there is strong evidence that suggests CRF-BP could block CRF activity (Behan *et al.,* 1995). In *ob/ob* and *Zucker* rodents, there is no marked significant difference between lean and obese rats and between *ad libitum* fed and food-deprived rats in the expression of CRF-BP for most of the regions examined (Timofeeva *et al.,* 1999). As SLOB rats show a significant decrease in CRF mRNA levels, a difference in CRF-BP is also possible. Further analysis of the components of this pathway may then shed light on the source of such reduced CRF mRNA expression levels.

CRF inhibits gonadotrophin releasing hormone neuronal activity and is shown to be a sex-dependent hormone (Rivest and Rivier, 1995). It is possible that the altered CRF mRNA expression in SLOB rats may contribute to the sex-dependent phenotype of this model. In a study by Rivest *et al.*, (1989) the effect of administering CRF on bodyweight gain and food intake were shown to be sex-dependent. Whereas body weight and food intake were reduced in male rats, no significant differences were found in female rats. Both sexes of SLOB rats have reduced CRF mRNA levels, though only the male results are shown in this chapter. In chapter 5 are shown CRF mRNA expression levels in intact, ovariectomised and oestrogen treated female rats.

It is also important to note that a relationship exists between CRF, somatostatin and GH secretion (Rivier and Vale, 1985). Central administration of CRF inhibits GH secretion in the rat (Rivier and Vale, 1984) and CRF induces the release of SS by cultured brain cells (Peterfreund and Vale, 1984). These two effects thus cause a reduced secretion of GH from the pituitary. In the GH-deficient SLOB rat it is therefore possible that in someway CRF mRNA levels are reduced in an attempt to increase endogenous GH secretion.

Much work has yet to be carried out before the role of CRF in energy homeostasis is clarified. The observation that no defects in feeding behaviour are seen in CRF knockout mice (Muglia *et al.,* 1995) has led researchers to question the role CRF has in obesity. Nevertheless, although all of the previously characterised rodent obesity models mentioned have normal hypothalamic CRF mRNA expression, the occurrence of a significantly reduced CRF mRNA level in SLOB rats, an observation similar to

that seen in food deprived normal animals, requires further analysis. Investigation of the CRF system, in terms of CRF receptor 1 and receptor 2, CRF-BP analysis, as well as adrenalectomy studies may aid in this analysis.

4.8.4 Leptin and POMC

Decreased POMC mRNA levels in the arcuate nucleus are found in negative energy conditions such as diabetes (Sipols *et al.*, 1995; Kim *et al.*, 1999), food restriction and deprivation (Kim *et al.*, 1996), and lactation (Kim *et al.*, 1997). A >60% reduction in hypothalamic POMC mRNA expression is observed after a 2 day fast in normal mice which is positively correlated with leptin mRNA and negatively correlated with NPY mRNA (Mizuno *et al.*, 1998). Due to POMCs connection with leptin Mizuno *et al.*, (1998) investigated POMC expression in leptin deficient (*ob/ob*) mice and leptin insensitive *db/db* mice. In fasted control and experimental mice POMC expression was reduced. In leptin treated *ob/ob* mice expression was increased three fold. It appeared therefore that like NPY, POMC was mediated by leptin and had an important role in feeding behaviour.

Obese *Zucker* rats also have reduced POMC mRNA levels in the arcuate nucleus relative to lean littermates (Kim *et al.*, 2000). Likewise, in old (6 months and over) *tubby* mice, POMC arcuate mRNA expression is also significantly reduced (Guan *et al.*, 1998). However, the expression of POMC mRNA in the equivalent region of juvenile (2-3 months), non-obese *tubby* mice is not observed, supporting the notion that increasing adipose tissue with age, with increasing plasma leptin levels act to suppress the hypothalamic POMC system. This hypothesis however, is not true for SLOB and *agouti* rodents. Both SLOB rats (in this thesis) and *agouti* obese mice (Kesterson *et al.*, 1997) show no change in POMC levels in the arcuate nucleus, indicating pathways independent of leptin may exist that enable POMC expression to remain normal irrespective of elevated plasma leptin levels.

4.8.5 Leptin and AgRP

Leptin receptors are localised on AgRP containing neurones and like NPY, AgRP is regulated negatively by leptin and stimulated by fasting in wild-type mice (Mizuno *et*

al., 1999; Wilson *et al.,* 1999). Arcuate expression of AgRP is elevated in *ob/ob* and *db/db* mice (Shutter *et al.,* 1997). In this same study dexamethasone failed to induce this induction, demonstrating that the up-regulation in AgRP was not attributable to hypercortisolemia secondary to obesity. Rather the elevated expression in *ob/ob* and *db/db* animals, but not in obese, nonmutant animals, suggested that impaired leptin signalling pathways were directly involved. Hypothalamic AgRP levels have also been assessed in *tubby* and *fat/fat* mice by Northern blotting techniques. Both these strains of mice have identical levels of AgRP mRNA compared with normal controls (Wilson *et al.,* 1999), however *in situ* hybridisation analysis of specific brain nuclei have yet to be conducted in these rodent models of obesity.

Because AgRP promotes anabolic activity, its regulation may be similar to the regulation of neuropeptide Y (NPY), which also promotes anabolic activity. In addition, NPY and AgRP are both expressed in the arcuate nucleus (Shutter *et al.,* 1997) and co-expressed in fasting activated hypothalamic neurones (Hahn *et al.,* 1998). NPY, as previously mentioned is inhibited by leptin in *ob/ob* mice (Mizuno *et al.,* 1998; Schwartz *et al.,* 1996b) and is stimulated by fasting in wild-type and *db/db* mice (Mizuno *et al.,* 1998). Identical trends are also found when quantifying AgRP in these experimental models (Mizuno *et al.,* 1999), suggesting that NPY and AgRP exert similar anabolic effects and are responsive to similar physiological signals reflecting nutritional status. In view of this, I would also expect a similar result in AgRP mRNA levels as with NPY mRNA levels in SLOB rats, however, a significant reduction was not observed in AgRP mRNA expression, which actually showed a non-significant increase in expression in SLOB males. This suggests AgRP and NPY may have some independent physiological regulatory pathways as well as some which may be similar.

As AgRP acts by blocking the MC4 receptor, although mRNA levels of this neuropeptide are unaltered, it would be interesting to see whether MC4 expression is changed in SLOB rats. Unfortunately, I have not yet optimised MC4 antisense:sense signal ratio, so this receptor has yet to be quantified properly in hypothalamic SLOB nuclei.

4.8.6 *Tub* expression

tub mRNA expression in normal mice is found to be highest in the paraventricular, ventromedial, and arcuate nucleus (Kleyn *et al.,* 1996). Since this report on *tub* hypothalamic expression by *in situ* hybridisation, little data has been generated on *tub* mRNA levels in other rodent obesity models. As mutation of the *tub* gene in tubby mice causes severe obesity, such studies would be interesting. Expression levels of *tub* protein in brain lysates assessed by Western blotting have been carried out in *ob/ob*, *db/db* and MC4-R deficient mice (Stubdal *et al.,* 2000). However, no significant differences in *tub* protein level were observed in any of these obesity models. Data presented in this thesis show for the first time that changes in hypothalamic *tub* expression are nuclei specific and that in SLOB males a difference is observed in *tub* mRNA expression in the supraoptic nucleus. I have also shown for the first time that *tub* mRNA expression in the PVN of normal rats shows a sexually dimorphic expression level. So far, the function of the *tub* protein remains unclear but it is possible in light of my findings on the SLOB rat, showing for the first time that *tub* expression is altered in a rodent obesity model; that this protein may have a role in the sex-specific obesity phenotype of SLOB rats. As further studies on the *tubby* mouse and *tub* protein evolve, the significance of this result may become clear. However, at present I cannot conclude the relevance of these findings.

4.8.7 The GH axis

Data presented in this thesis on mRNA levels of peptides of the GH-axis, probably arise primarily as a result of GH-deficiency caused by negative feedback from hGH present in the transgene. However, GH has an important role in energy homeostasis and alterations in obesity status, such as high fat feeding or food deprivation, markedly influence GH secretion (Dieguez and Casanueva, 1995). Therefore the changes observed could play a role in modifying the SLOB obese phenotype. Hypothalamic NPY appears to play a physiologically significant inhibitory action on GH secretion (Rettori *et al.,* 1990; Okada *et al.,* 1993) and leptin induced GH secretion is suppressed by NPY (Carro *et al.,* 1998). In addition, the severe growth hormone deficient *dwarf* rat has reduced arcuate NPY mRNA levels as in SLOB rats (Bennett *et al.,* 1997). It is possible therefore that as the *dwarf* rat is not obese and a

reduction in NPY levels would favour an increase in GH secretion, the reduced NPY levels observed in SLOB rats may be partly due to GH-deficiency as well as elevated plasma leptin levels.

Somatostatin (SS) has also been shown to have a role in feeding behaviour, although results have been confusing and both anorexic and orexigenic effects have been reported. Physiological doses of SS injected centrally increase feeding in the light period, but there is no effect when SS is administered during the dark phase (Feifel and Vaccarino, 1990). In SLOB rats, SS appears to be increased, although this result was not significant. Further analysis with an increased sample number is required before reliable conclusions can be drawn about SS mRNA expression levels in SLOB rats. SS mRNA levels have also been assessed in the genetically obese *Zucker* rat, a model in which the secretion of GH is also impaired (Ahmad *et al.,* 1989, 1993). Whereas these animals have reduced GHRH mRNA expression, SS mRNA expression is unaltered (Ahmad *et al.,* 1993; Tannenbaum *et al.,* 1996). Thus, if SS mRNA expression in SLOB rats is increased, this may be representative of excess local GH in the brain from the hGH transgene, rather than representative of peripheral and central GH deficiency as in *Zucker* rats.

4.9 Summary

Hypothalamic neuropeptide analysis in SLOB rats has generated some unique findings, many of which will require further analysis before their role in contributing to the SLOB phenotype can be defined. I have shown that NPY mRNA and CRF mRNA expression levels are significantly reduced and supraoptic *tub* mRNA expression is significantly increased in SLOB rats. Alterations in NPY levels are also observed in many of the previously characterised rodent obesity models, and are thought to be a result of altering plasma leptin levels. However, CRF mRNA alterations have not been reported in any of the previously characterised rodent obesity models mentioned and for the first time hypothalamic *tub* mRNA expression levels have been assessed in an obesity model (SLOB rat). Whereas, changes are observed in POMC and AgRP mRNA levels in some obesity models, these peptides were not significantly altered in SLOB rats.

It is difficult from the data to ascertain whether the observed neuropeptide changes are a cause or an effect of the obesity syndrome. As the SLOB rat is characterised further, such new data may provide a greater understanding of the SLOB phenotype. In addition, I will present in the next chapter how manipulation of the SLOB phenotype such as high fat feeding and ovariectomy, effects those neuropeptides that are altered in the static state SLOB rat model; namely NPY, CRF and *tub* mRNA expression.

Chapter 5

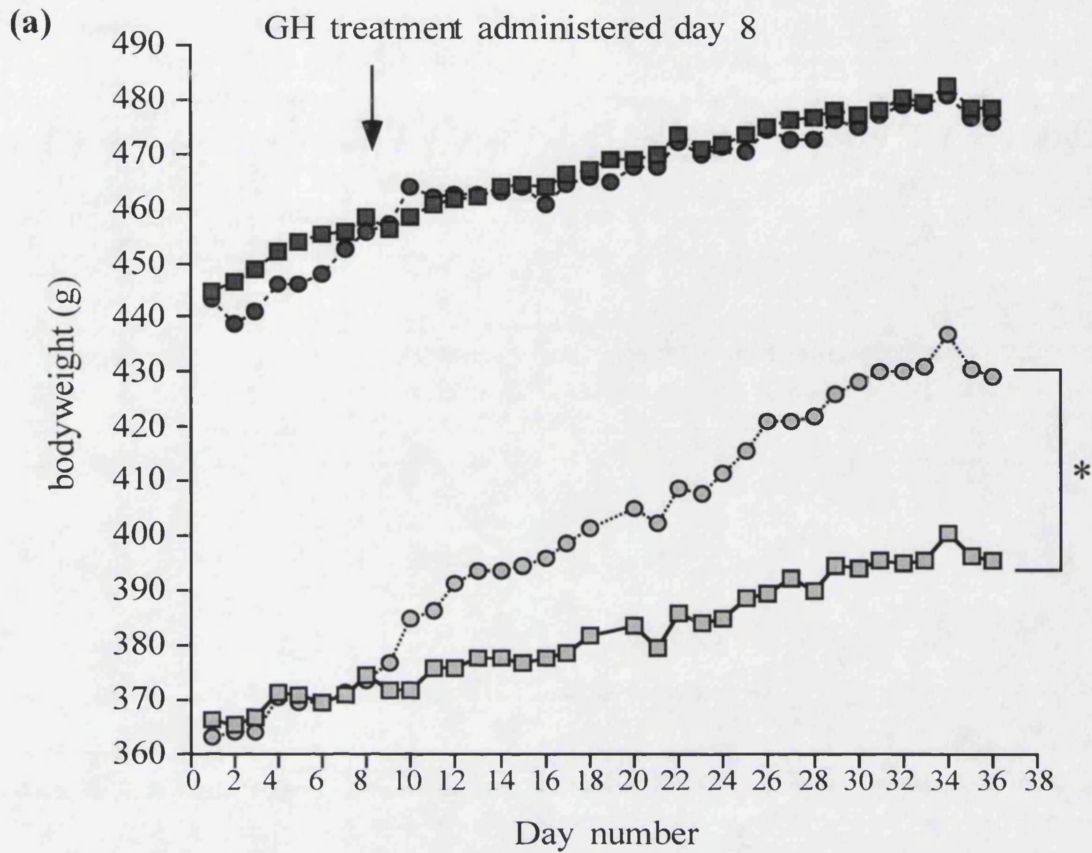
Manipulation of the SLOB Rat Phenotype

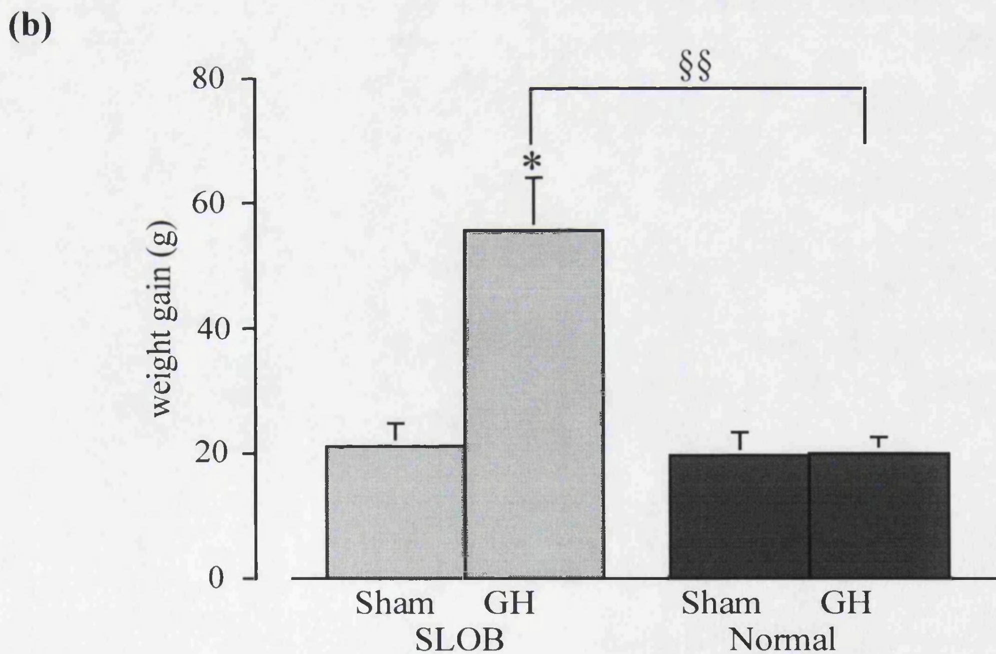
5.1 Introduction

Following my investigations in characterising the SLOB rat, I wanted to manipulate various parameters that might alter expression of the phenotype and assess how SLOB rats responded to these challenges. SLOB rats are GH-deficient; what effect would GH treatment have on obesity in these rats? High fat feeding and food restriction are two procedures often tested in rodent obesity models, what effect would these have in SLOB rats? In addition, why is the SLOB phenotype more prominent in SLOB males than in females? Are SLOB females protected from developing severe obesity? What effects may some of these challenges have on hypothalamic neuropeptide expression of NPY, CRF and *tub* mRNA, currently found to be altered in SLOB rats? This chapter describes a series of experiments attempting to address these questions.

5.2 Effect of growth hormone treatment in SLOB rats

I concluded in chapter 3 that isolated GH-deficiency per se does not account for SLOB obesity, as the more GH-deficient *dwarf* model does not develop the SLOB phenotype, however, GH-deficiency may still contribute to the phenotype. In order to pursue this further I decided to replace this GH-deficiency by treating SLOB males with recombinant growth hormone and observing the effects on the phenotype. Animals were treated for 4 weeks using osmotic minipumps filled with recombinant human growth hormone (rhGH). Each group contained between 5-7 male rats, each animal receiving 200μg rhGH per day. This dose was chosen as it is well established to cause a growth promoting effect in *dwarf* rats (Gabrielsson *et al.*, 1995; Bennett *et al.*, 1997). Animals used for this experiment were 20 weeks old and at this age SLOB rats weighed significantly less than age-matched normal rats. Results showed GH treatment in normal animals had no effect on overall body weight gain but GH-treated SLOBs gained more weight than untreated SLOBs and GH-treated normal rats (**Figure 5.1**). Measures of obesity such as supra-renal fat pad weight and plasma leptin concentration were unchanged in GH-treated SLOBs **(Figure 5.2)** indicating SLOB rats were still obese. However, GH treatment caused a significant increase in





<u>Figure 5.1</u> Effect of GH treatment on bodyweight in SLOB and normal male rats. Male rats were treated with 200μg/day of recombinant growth hormone. Shown above are (a) daily bodyweights over 4 week treatment period, and (b) overall weight gain at the end of 4 weeks (n=6, $^*p<0.05$, §§ $p<0.01$).
□ SLOB-sham, ○ SLOB-GH, ■ normal-sham, ● normal-GH.

spleen organ weight, usually reduced in SLOB rats. Although a tendency to an increased weight was also observed in soleus muscle and kidney weight, these results were not significant (**Figure 5.3**). I concluded therefore that GH treatment did not abolish the obese phenotype in SLOB males, but reversed the loss of weight in those organs effected by GH deficiency, thus increasing lean body mass; this effect being observed as an overall gain in body weight.

5.3 Effect of High Fat Feeding in SLOB rats

An imbalance in energy intake and energy expenditure is necessary to induce net body weight gain or a net body weight loss (Bray and York, 1979; Friedman and Halaas, 1998). However, variations in total energy intake of daily food is not the only factor in determining bodyweight; the macronutrient composition of the diet also plays a major role in the development of such an energy imbalance (Flatt *et al.*, 1988; Schutz *et al.*, 1995). This may then be compensated by changes in body composition even if there is no net change in energy balance. In this context, it has been suggested that dietary fat promotes adipose accumulation more effectively than dietary carbohydrate (Danforth *et al.*, 1985; Schutz *et al.*, 1989; Raben *et al.*, 1996). Thus, high-fat diets can induce body weight and adiposity increases in humans (Astrup *et al.*, 1994) and animals (Lim *et al.*, 1991). High fat diets also contribute to insulin resistance (Reaven *et al.*, 1967; Storlien *et al.*, 1986), impaired glucose metabolism (Glueck *et al.*, 1969), type 2 or non-insulin dependent diabetes mellitus (NIDDM) (Himsworth *et al.*, 1935, Kolterman *et al.*, 1979), stroke, and coronary artery disease (Lipid Research Clinics Program, 1984). Although the mechanisms underlying these issues are not clearly understood, many studies have tried to assess the effects of such diets on individual biochemical and metabolic parameters or regulators in order to aid in this understanding. Experiments were therefore carried out in order to assess what effect a high fat diet would have in SLOB male and female rats.

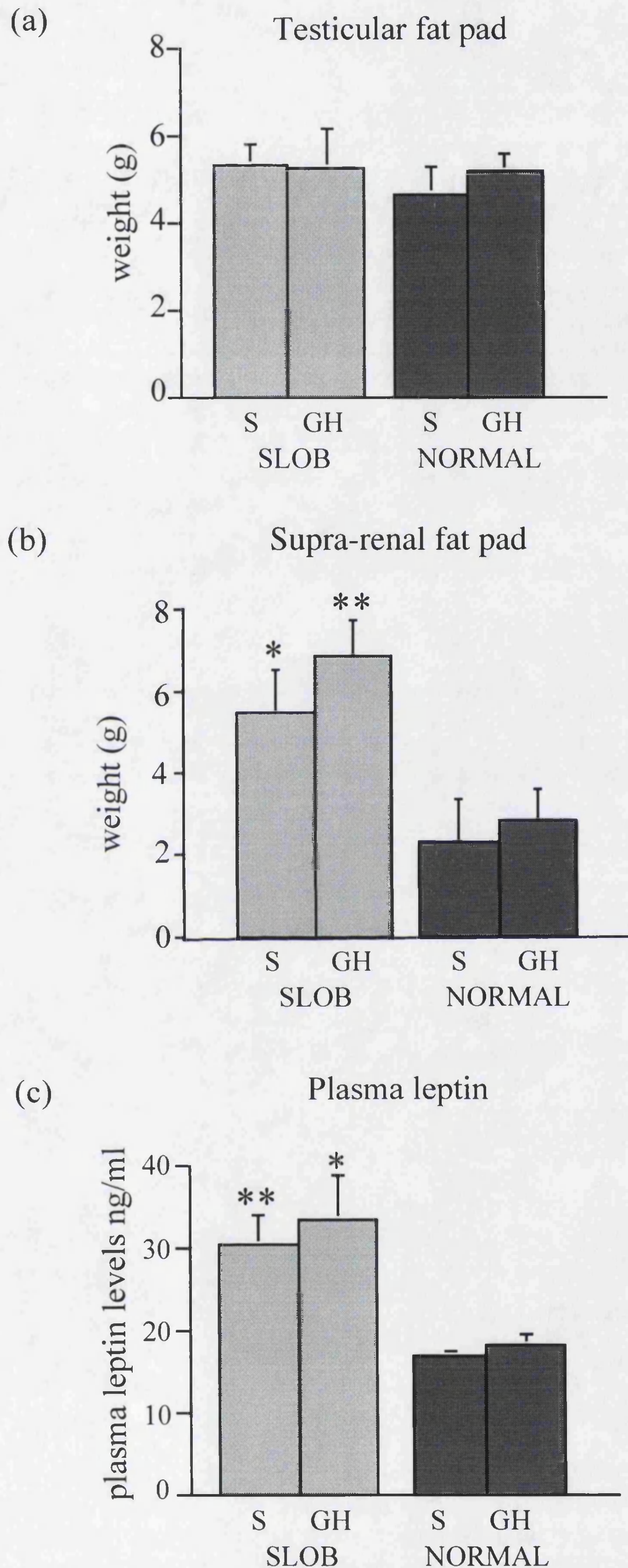


Figure 5.2 Effect of GH treatment on fat pad weight and plasma leptin levels in SLOB and normal male rats. Osmotic minipumps delivered 200µg/day of recombinant growth hormone for 4 weeks. Significances are shown for SLOB animals compared to normal animal following the same treatment procedure (S=sham, GH=GH-treated; *$p<0.05$, **$p<0.01$).

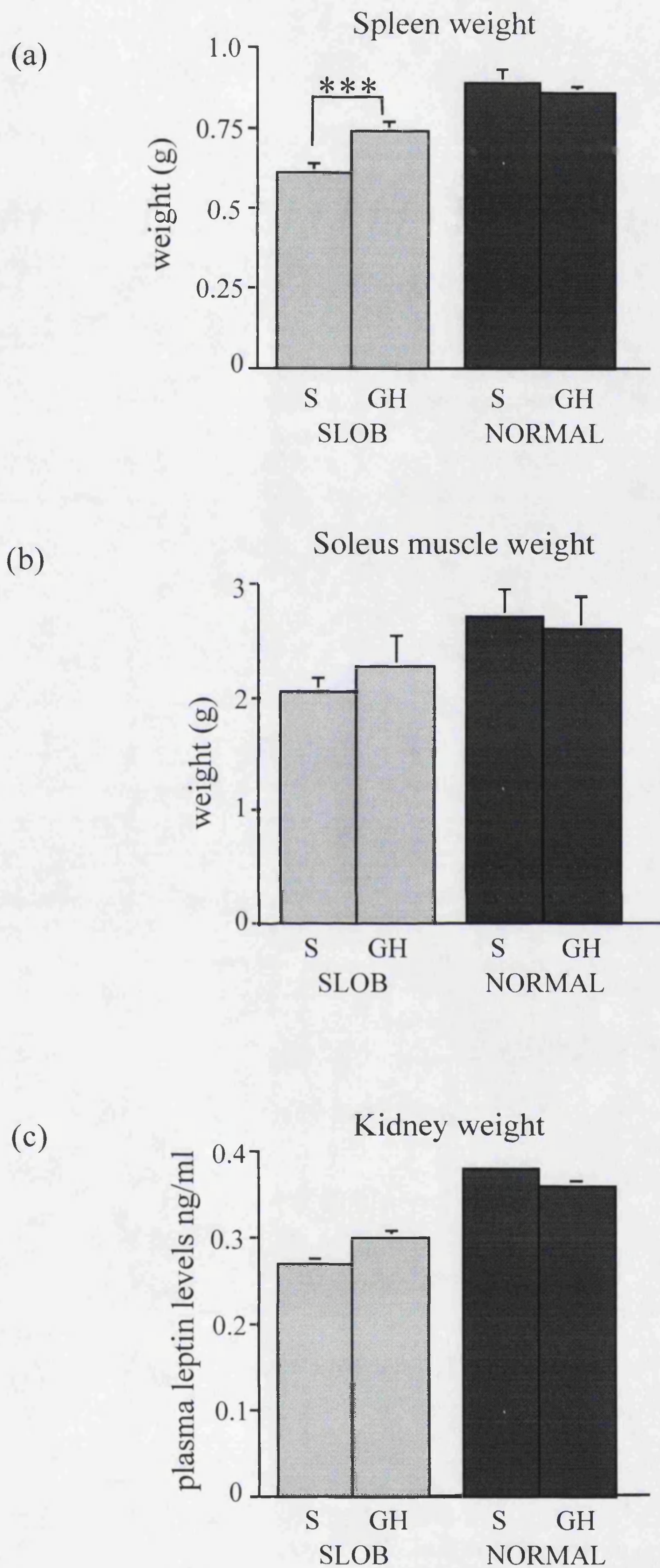


Figure 5.3 Effect of GH treatment on organ weights in SLOB and normal male rats. Organ weights taken after 4 weeks treatment with 200µg/day of recombinant growth hormone. (S=sham, GH=GH-treated; ***p<0.001, n=6).

For this study I felt it was important to choose a moderate elevation in fat-content in the high-fat fed SLOB and normal animal groups. Many researchers simply wanting to assess the maximum effect of feeding a diet of very high fat content choose diets containing between 40-65% fat (Gong *et al.,* 1990; Van-Heek *et al.,* 1997). My aim of high fat feeding SLOB rats was not really to increase fat intake to an absolute maximum, but more to alter the dietary composition to a more representative western human diet and observe how SLOB rats differ in their ability to respond to this challenge compared to normal rats. Laboratory animals at the NIMR are fed a standard chow diet consisting of 4% fat. To be more representative of the average western human diet, a diet consisting of 30% fat was selected for this study. It is also important to note that although fat and carbohydrate content differed in the two diets, protein content was kept the same to ensure effects of high fat feeding in SLOB rats were directly due to the macronutrient fat and not to protein.

100-day old rats were used and prior to the study all animals had been fed the normal chow diet. The reason I chose this age of animals was two-fold. First, the high fat diet would be made available prior to obvious obesity was apparent in terms of gross body composition in both male and female SLOB rats. In this way, I could determine the influence of diet on the development of obesity rather than simply its maintenance or exacerbation. Second, growing rats are known to be in a dynamic state of fat accumulation (Hirsch & Han 1969). I thought that the influence of dietary fat would therefore be more evident around this period. Separate male and female studies were conducted, each contained 4 groups of age-matched littermates (n=7). These groups consisted of (a) normal rats fed standard chow (4% fat), (b) normal rats fed high-fat (30% fat), (c) SLOB rats fed standard chow (4% fat) and (d) SLOB rats fed high fat (30% fat).

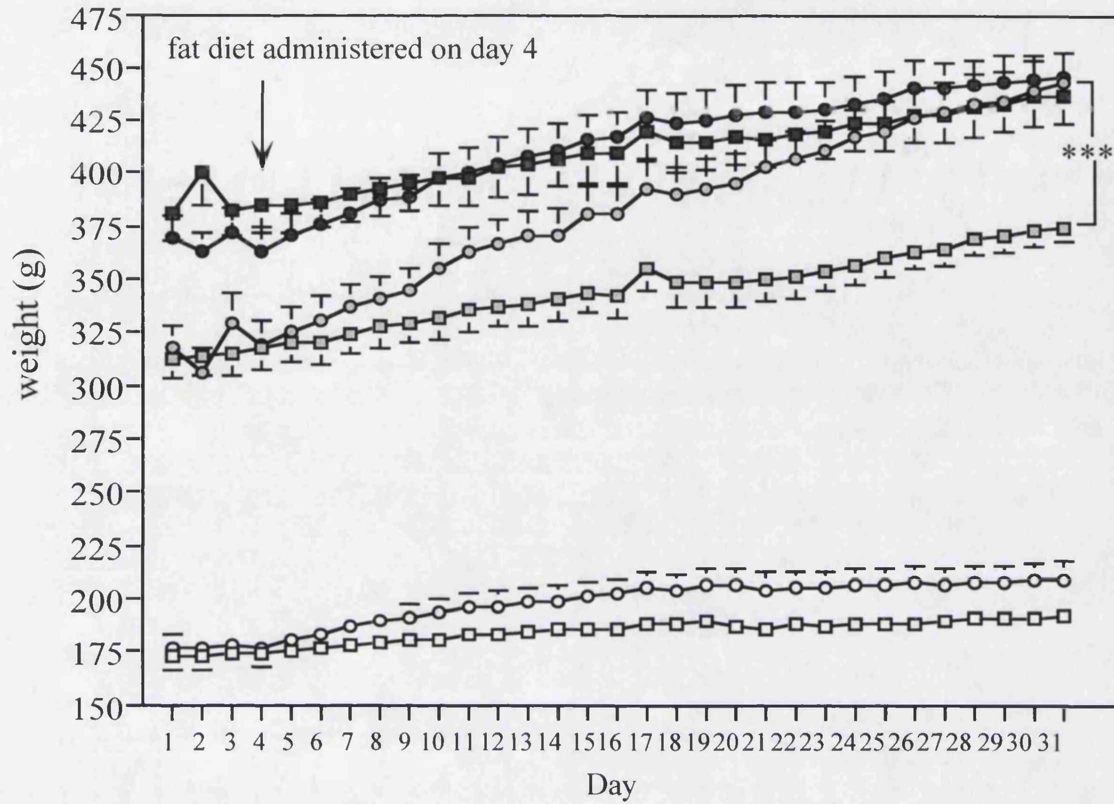
In addition, I also included *dwarf* rats in these experiments to serve as controls for the effects of GH-deficiency, since this is also present in SLOB rats. Thus, each experiment contained two additional groups of animals (n=5), (e) *dwarf* rats fed standard chow (4% fat) and (f) *dwarf* rats fed high fat (30% fat).

Animals were placed into individual metabolic cages, and allowed to acclimatise for 3-4 days before the start of the experiment. After switching food to 30% fat diet in

half the groups, food intakes and bodyweights were recorded at the same time each day for two weeks. Thereafter, animals were transferred to Bowman cages in groups of 5-7, with the same test diet and bodyweights recorded daily for a further 2 weeks.

5.3.1 Effect of high fat feeding in male rats

Usually, severe obesity in SLOB males is not evident until after 200 days of age, however, on an enriched (30%) fat diet, gross obesity rapidly became evident, even at this earlier age (**Figure 5.4 (a)**). Animals on the high fat diet gained more weight compared to chow fed animals of the same type (**Figure 5.4 (b)**), however, high-fat fed SLOB males gained over twice the weight compared to high-fat fed normal littermates or *dwarf* rats. A change to a higher dietary fat content would normally result in a decrease in food intake which may or may not fully compensate for calorie intake (Horvath *et al.,* 2000). As SLOB rats gained excessive weight on a high fat diet, it was possible these animals were therefore consuming more calories than normal animals on a high fat diet. **Figure 5.5** shows SLOB animals on a high fat diet did not significantly reduce their cumulative food intake whereas normal and *dwarf* rats did, this may in part explain the increased weight gain in SLOB rats. The large increase in bodyweight change was also reflected in plasma leptin levels (**Figure 5.6**) and fat pad weights (**Figure 5.7 (a), (b)**). Leptin levels were significantly increased in SLOB and *dwarf* high fat fed animals but not in normal animals. High fat fed SLOB animals showed a significant increase in plasma leptins compared to high fat fed normal animals. All high-fat fed animals showed a significant increase in supra-renal fat pad weight and again, high fat fed SLOB rats had larger supra renal fat pads compared to high fat fed normal animals. Results for testicular fat pad weights showed, high fat fed SLOB and normal animals had significantly greater fat pad weight compared to chow fed animals of the same type. However, high fat fed *dwarf* rats did not show a significant increase in testicular fat pad weight. Also testicular fat pads in high fat fed SLOB rats did not weigh significantly more than in high-fat fed normal animals. Thus, from the observations made, high-fat feeding in SLOB males



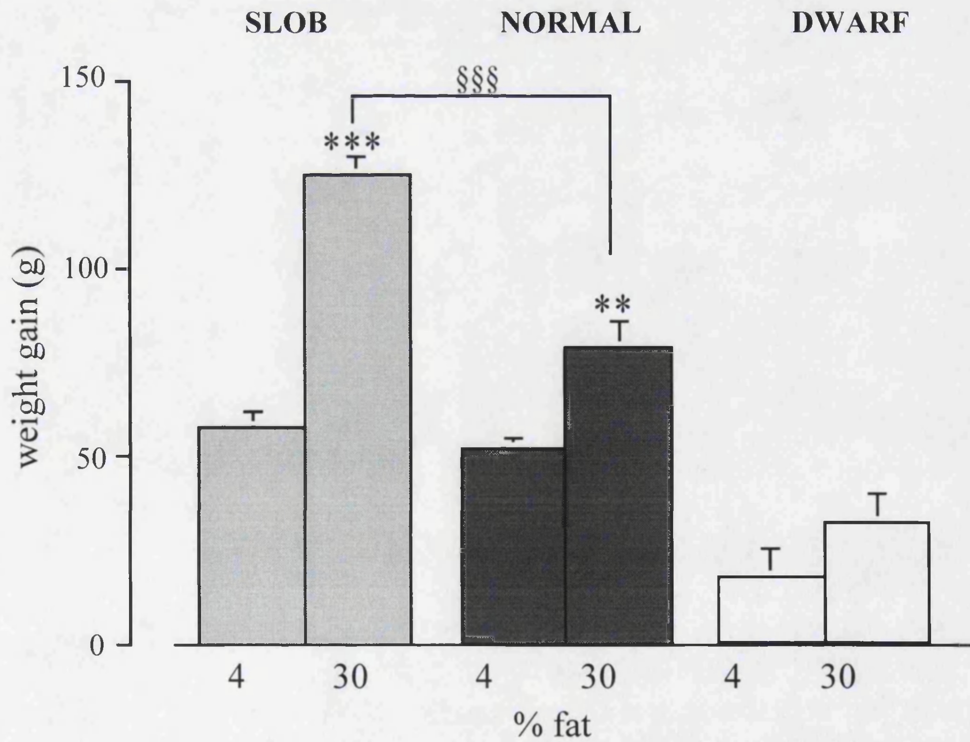


Figure 5.4 **Effect of high-fat feeding in male rats.** (a) Daily bodyweights during 4 weeks on CHOW or 30% fat diet. (b) Overall body weight gain after 4 weeks (n=5-7, **p<0.01, ***p<0.001).
■SLOB CHOW, ● SLOB fat, ■normal CHOW, ● normal fat, □*dwarf* CHOW, O*dwarf* fat

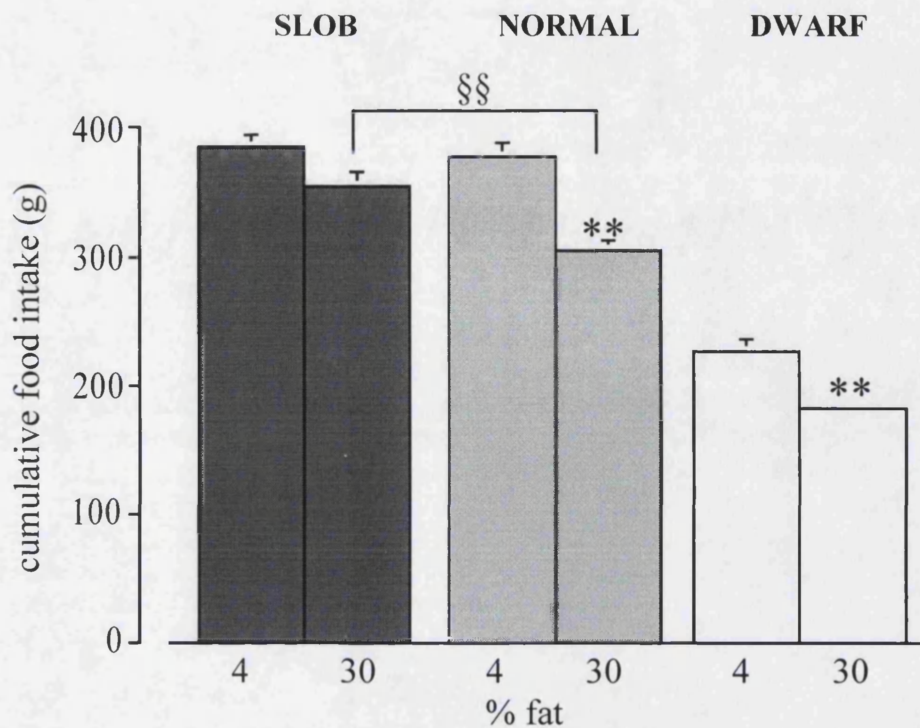


Figure 5.5 Cumulative food intake. Mean cumulative food intake over 14 days on 4% (chow) or 30% fat food, significances are shown in high fat fed animals compared to chow fed animals of the same type (n=5-7, **/§§ p<0.01).

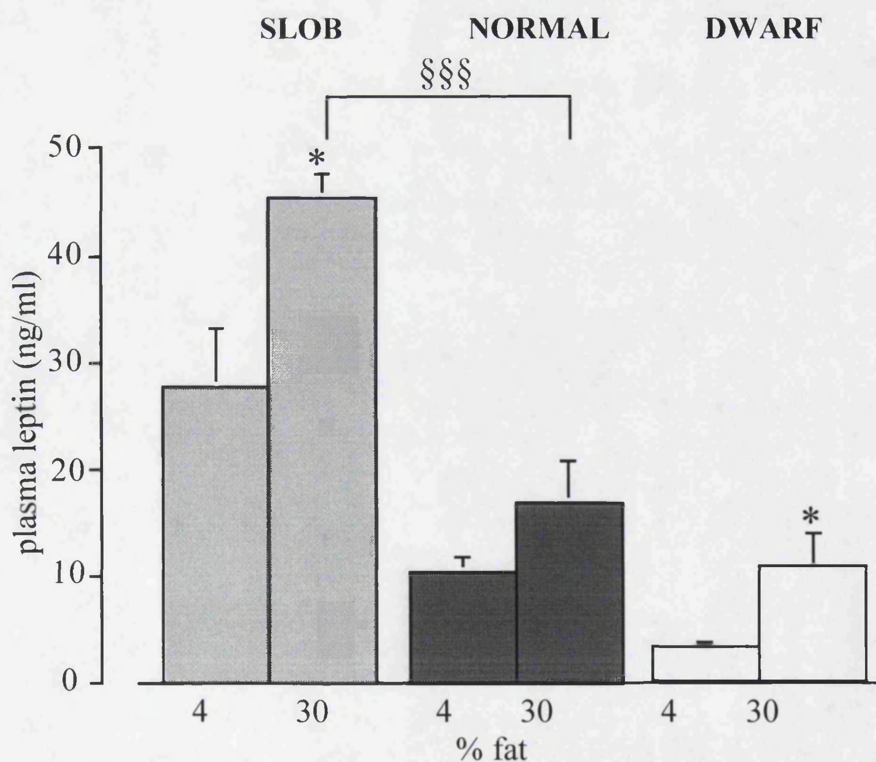
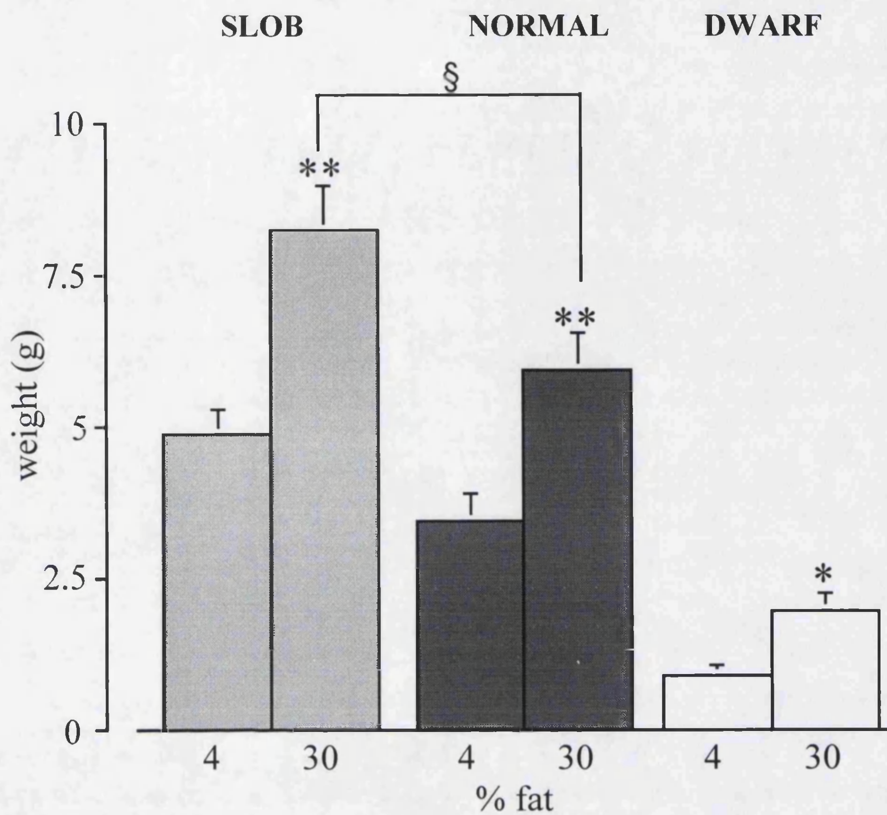
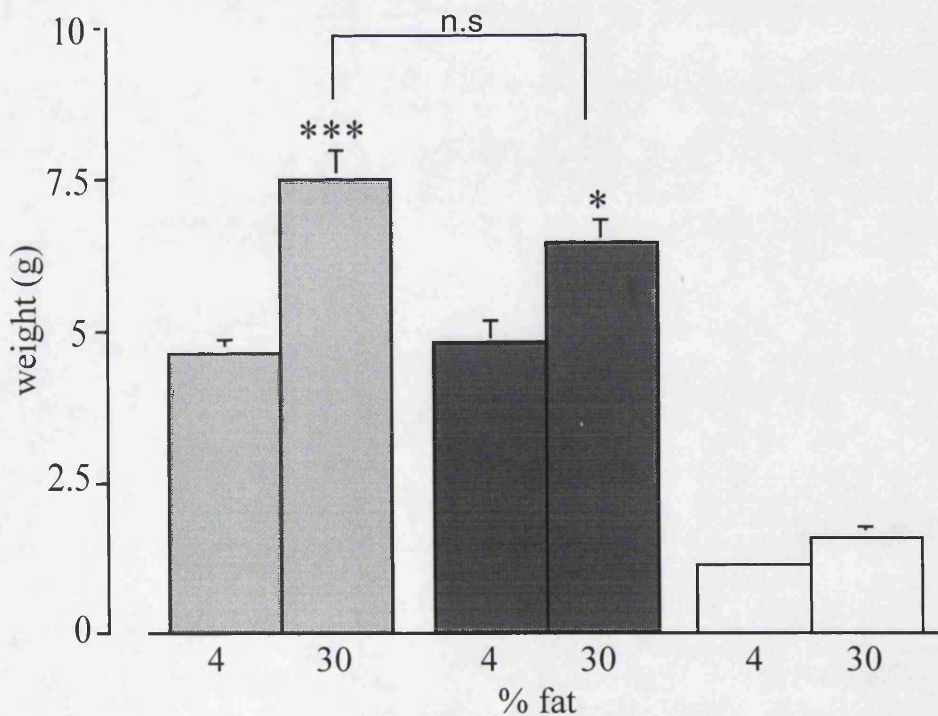


Figure 5.6 Plasma leptin levels. Leptin levels after 4 weeks on 4% (chow) or 30% fat diet. Significances are shown for high fat fed animals compared to chow fed animals of the same type (n=5-7, *p<0.05, §§§ p<0.001).

(a) Supra-renal fat pad weight



(b) Testicular fat pad weight



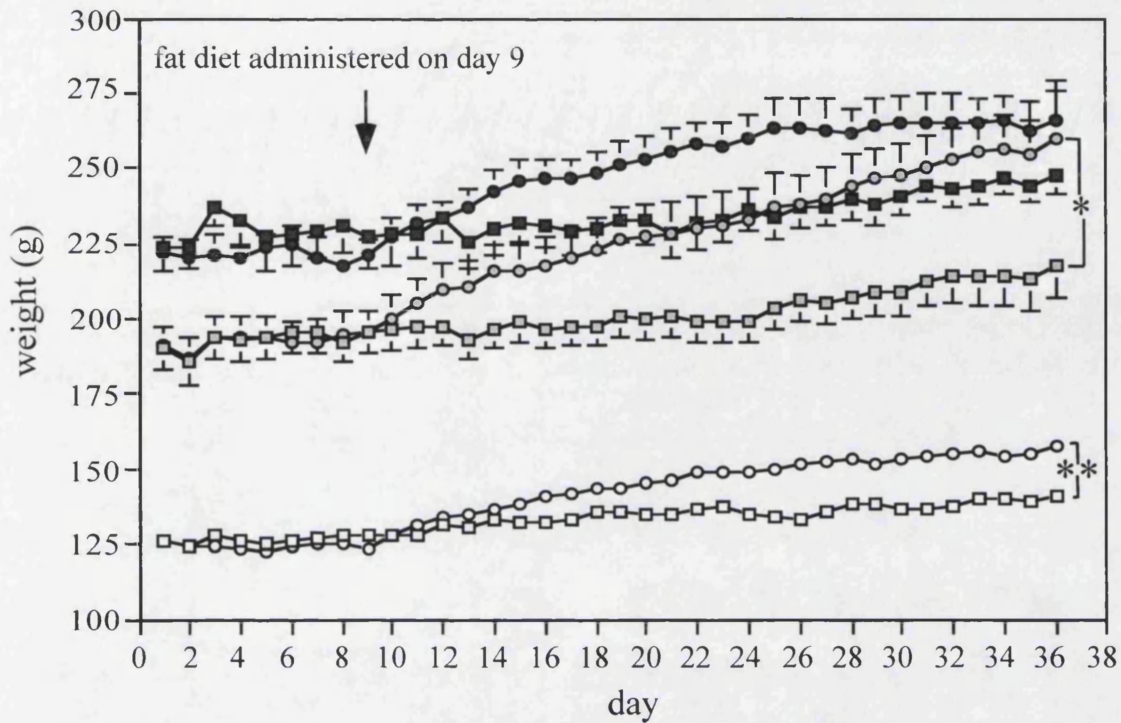
<u>Figure 5.7</u> Fat pad weights. (a) Supra renal fat pad weights in animals fed 4% (chow) or 30% fat diet for 4 weeks. (b) Testicular fat pad weights in animals fed 4% (chow) or 30% fat diet after 4 weeks. Significances are shown for high fat fed animals compared to chow fed animals of the same type (n=5-7, */§p<0.05, **p<0.01, ***p<0.001 n.s = not significant).

caused predominantly an increase in the central supra-renal fat depots rather than in the peripheral testicular fat depot. High fat feeding caused a reduction in food intake in normal and *dwarf* males but not in SLOB males. High fat fed normal males showed significantly increased supra-renal and testicular fat pad weights but a normal plasma leptin level. High fat fed *dwarf* rats showed significantly increased supra-renal fat pad weights and plasma leptin levels. From this study I deduced, a severe obesity phenotype comparable to that observed in older SLOB rats was induced in young (128 days) SLOB males and that high fat feeding normal and *dwarf* males did not induce a SLOB obesity phenotype over this period.

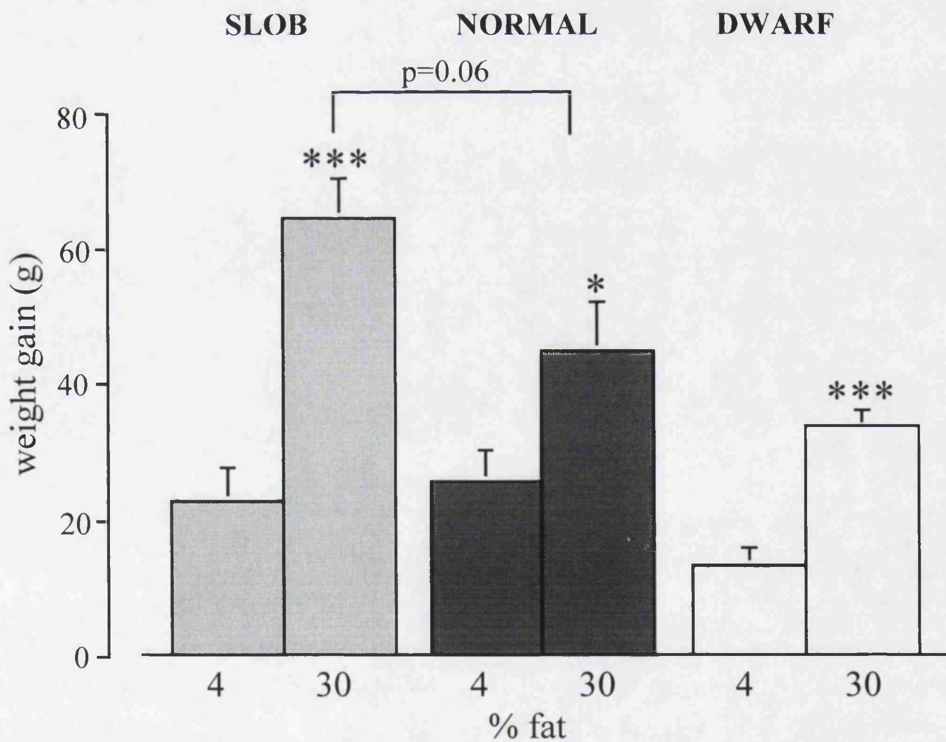
5.3.2 Effect of high fat feeding in female rats

When female *dwarf* rats are fed a high fat diet they become obese and insulin-resistant compared with chow-fed controls (Clark *et al.*, 1996). However, fat feeding in *dwarf* females has a greater effect on bodyweight than fat feeding in *dwarf* males. Although the severity of the SLOB phenotype is greater in males, there is evidence for a trend to obesity in females as shown by increased plasma leptin levels (Chapter 3, figure 3.8). Therefore, I decided to assess the effect of a similar high fat regime in normal, SLOB and *dwarf* female rats. **Figure 5.8 (a)** shows bodyweights over 4 weeks on chow or high-fat diet in SLOB, normal and *dwarf* females and overall weight gain in each group at the end of the experiment **(Figure 5.8 (b)**. As expected, all high-fat fed animals gained more weight than chow-fed animals of the same type. However, as with male SLOB rats, female SLOB's gained over twice as much weight on a high-fat diet compared to *dwarf* and normal females. Cumulative food intake was not reduced in high-fat fed SLOB females which would account for the increased gain in bodyweight, however, neither was it reduced in normal females, but a significant reduction was observed in *dwarf* females (**Figure 5.9**). Plasma leptin levels were elevated in all high fat fed animals (**Figure 5.10**), as were fat pad weights (**Figure 5.11**). Thus, like in young SLOB males, high fat feeding in young SLOB females causes an obesity phenotype which develops more rapidly than in high fat fed normal and *dwarf* females.

(a) Bodyweight chart



(b) Bodyweight gain



<u>Figure 5.8</u> Effect of high-fat feeding in female rats. (a) Daily bodyweights during 4 weeks on 4% (chow) or 30% fat diet. (b) Overall body weight gain after 4 weeks (n=5-7, *p<0.05, **p<0.01, ***p<0.001).

□ SLOB CHOW, ○ SLOB fat, ■ normal CHOW, ● normal fat, □ *dwarf* CHOW, ○ *dwarf* fat

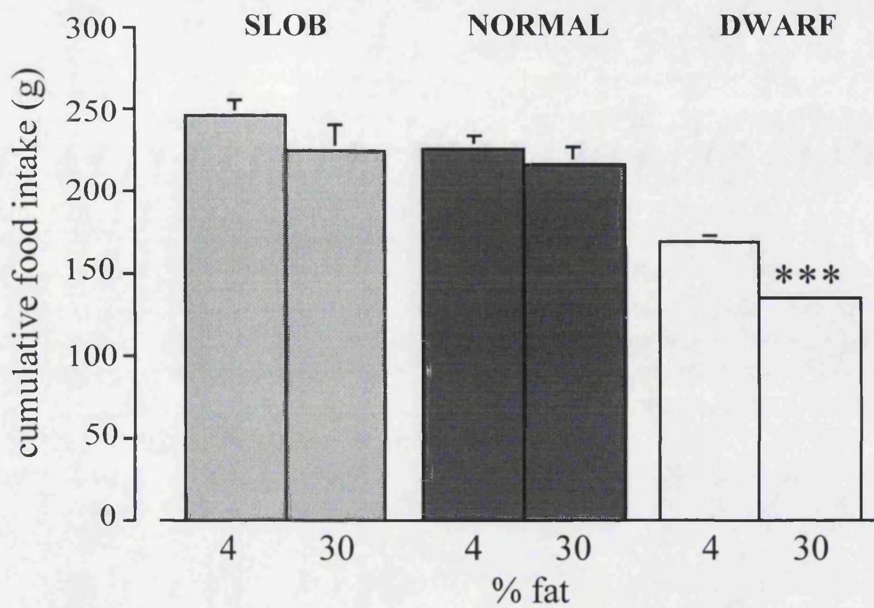


Figure 5.9 Cumulative food intake. Mean cumulative food intake over 14 days on 4% (chow) or 30% fat food, significances are shown in high fat fed animals compared to chow fed animals of the same type (n=5-7, ***p<0.001).

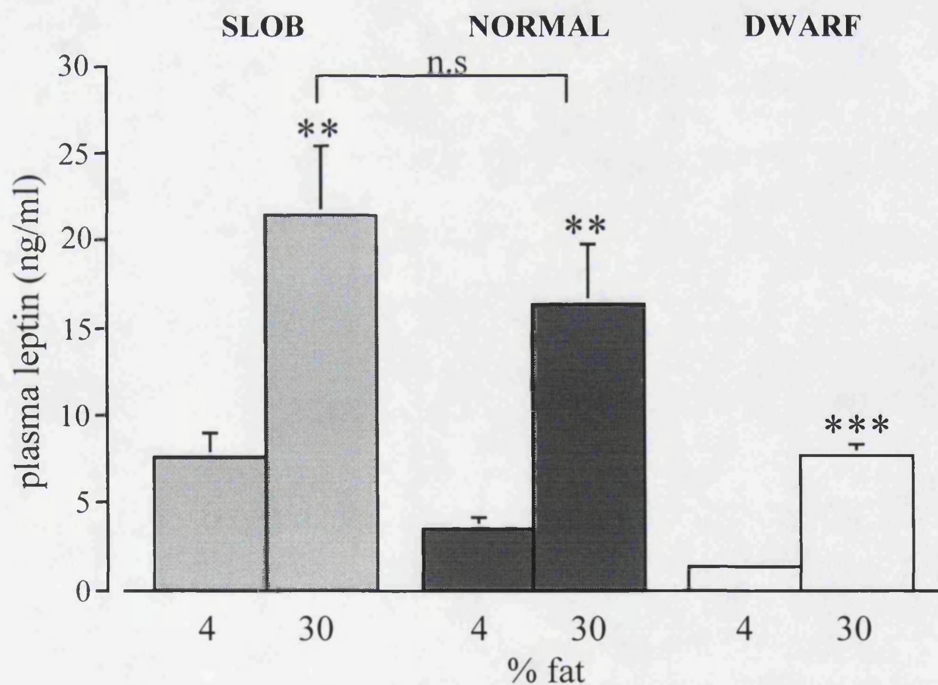
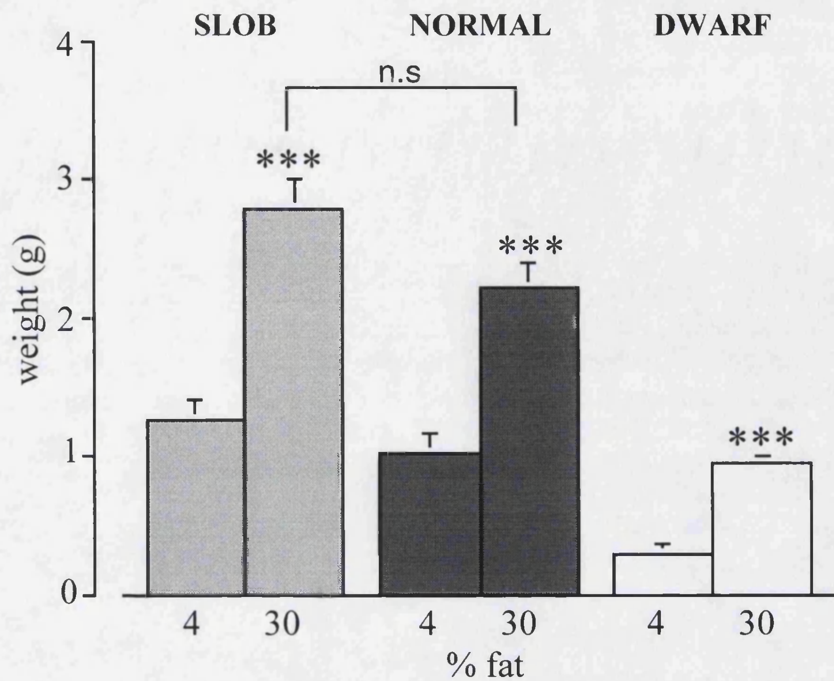


Figure 5.10 Plasma leptin levels. Leptin levels after 4 weeks on 4% (chow) or 30% fat diet. Significances are shown for high fat fed animals compared to chow fed animals of the same type (n=5-7, *p<0.05, **p<0.01, ***p<0.001, n.s = not significant).



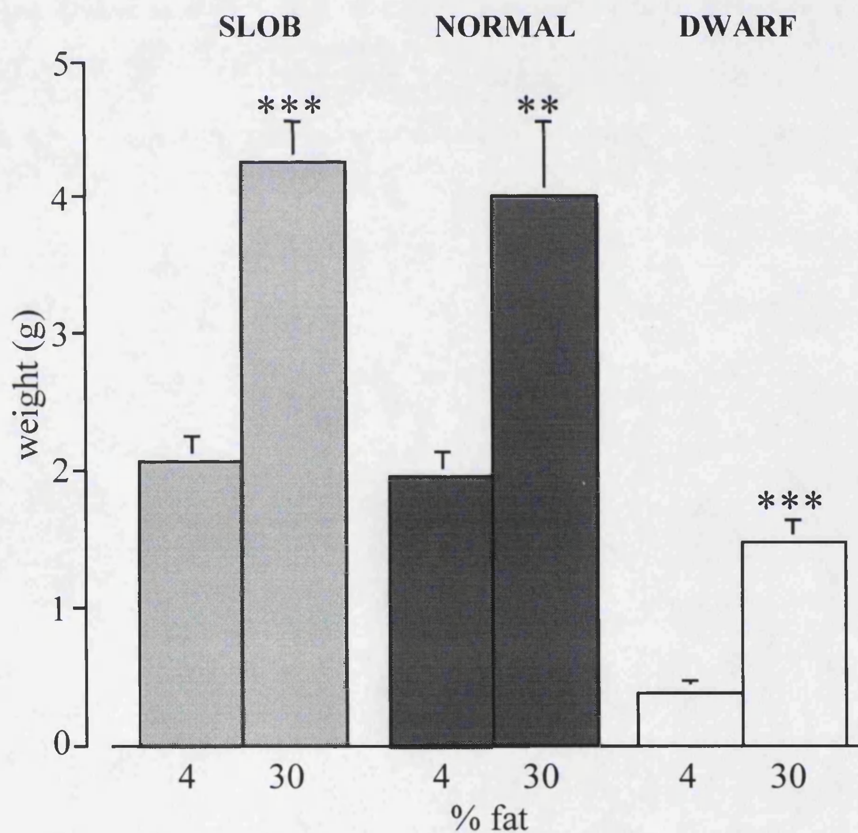


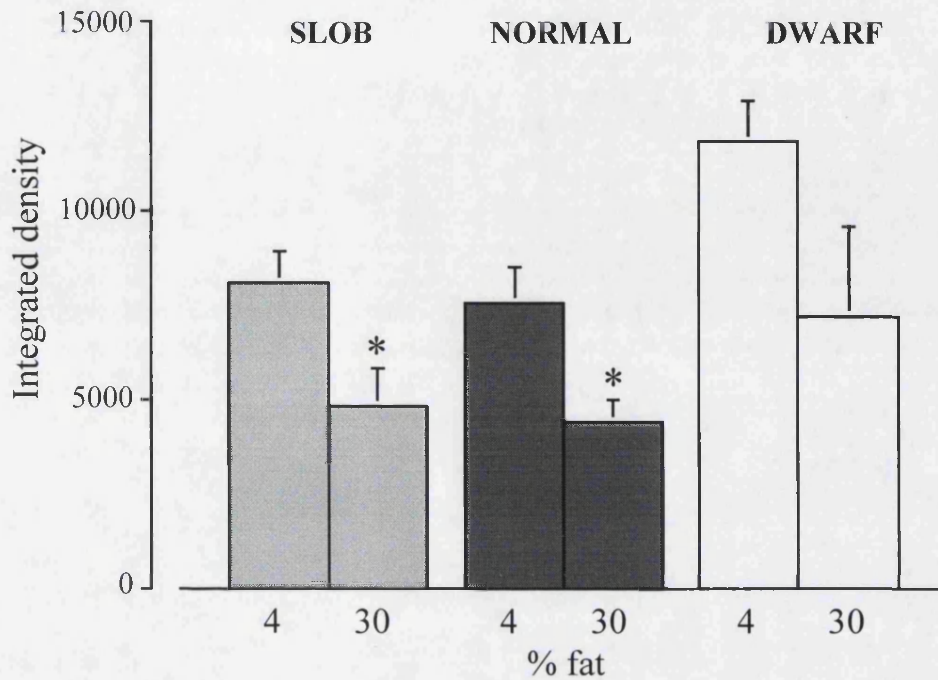
Figure 5.11 **Fat pad weights.** (a) Supra renal fat pad weights in animals fed 4% (chow) or 30% fat diet for 4 weeks. (b) Ovarian fat pad weights after 4 weeks on chow and high fat diet. Significances are shown for hih fat fed animals compared to chow fed animals of the same type (n=5-7, **p<0.01, ***p<0 001 n.s = not significant).

5.3.3 Effect of high fat feeding on hypothalamic neuropeptide expression

In chapter 4 I showed SLOB rats have an altered hypothalamic mRNA expression of CRF, *tub* and NPY. In this section I describe measurements of these neuropeptides carried out on brain sections from the previously described high fat feeding experiment in male rats. High fat feeding was found to cause a decrease in CRF mRNA expression measured in the paraventricular nucleus in all high fat fed groups **(Figure 5.12 (a))**, although this difference was only significant for SLOB and normal animals.

High-fat feeding significantly reduced NPY mRNA expression in normal and *dwarf* rats but had no effect on NPY mRNA levels in SLOB rats (**Figure 5.12**(b)), who already exhibit a reduced NPY mRNA expression. In normal animals high-fat feeding reduced the NPY mRNA expression to a level comparable to that seen in the chow or high-fat fed SLOB rats.

Tub mRNA expression was measured in paraventricular, supraoptic and dorsomedial nuclei. No difference in expression levels was observed between chow-fed and high-fat fed animals for either normal or SLOB rats. However significant differences were found in various nuclei in *dwarf* rats. High-fat feeding caused an increase in *tub* mRNA levels in the dorsomedial nucleus ($p<0.0001$), and a decrease in mRNA levels in the paraventricular nucleus ($p<0.002$) of *dwarf* rats. So far, no data has been published on *tub* mRNA expression levels in animals exposed to a high fat diet, therefore my studies are the first data in *dwarf* rats which show an interesting and significant expression in response to high fat feeding. As the *dwarf* rat is severely GH-deficient, it is possible that GH may have a role in *tub* mRNA expression in these animals.



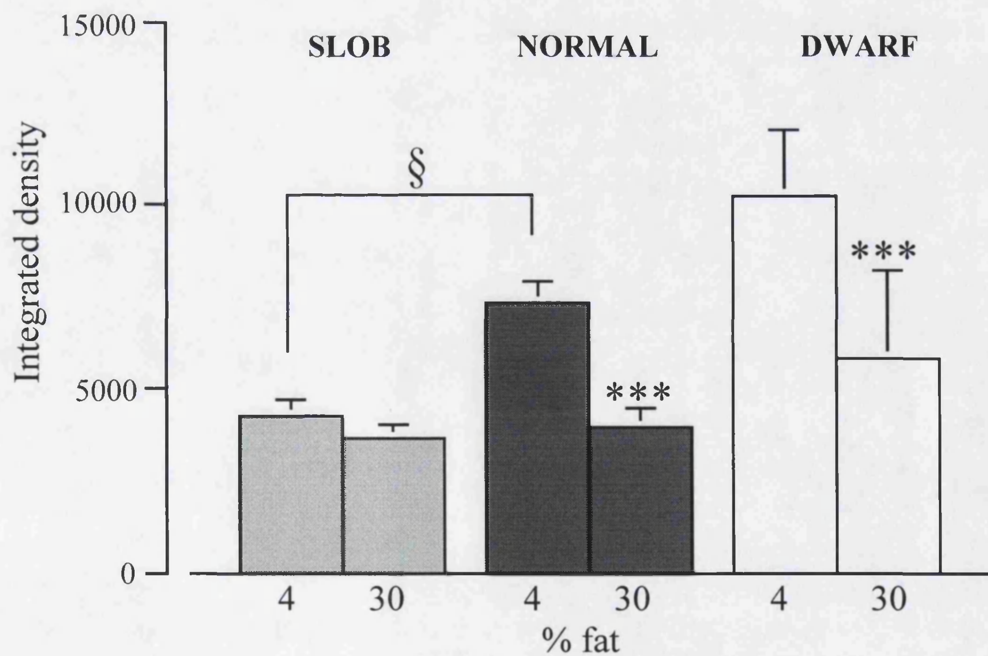


Figure 5.12 Hypothalamic CRF and NPY expression. (a) CRF mRNA expression in the PVN of 4% (chow) and 30% fat fed male rats. (b) NPY mRNA expression in the ARC of 4% (chow) and 30% fat fed male rats (n=5-7, */§p<0.05, ***p<0.001).

5.4 Effect of Food Restriction in SLOB Males

The development of most forms of obesity is associated with hyperphagia (Seidell, 2000), therefore the most commonly promulgated treatment for both prevention or amelioration of the obese condition, regardless of its origin, has been the prevention of hyperphagia by caloric restriction (Danforth, 1985; Wadden, 1993). Such studies investigating the effects of caloric restriction have been carried out for the past 30-40 years. Furthermore, the close association of the severe hyperplastic-hypertrophic form of early onset obesity (Hirsch and Knittle., 1970; Bjorntorp *et al.,* 1971; Brook *et al.*, 1972) has led to the suggestion that infants and children may become hypercellular and obese due primarily to early overfeeding, and that prevention of this early hyperphagia may prevent the later development of obesity. However, it is becoming increasingly clear that caloric restriction, while decreasing bodyweight, does not always decrease body fat to the desired extent (Zucker, 1967; Cox and Powley, 1977). For example, it is well known that long-term food restriction has little effect on body composition in mature *ob/ob* mice (Alonzo *et al.*, 1955; Dubuc *et al.*, 1976b). Neither caloric restriction from 17d of age nor continuing the pre-weaning milk diet until four weeks of age substantially affects the development of obesity or delays the onset of diabetes in these mice (Dubuc *et al.,* 1981). In the *Zucker* rat prevention of hyperphagia throughout life by continuous food restriction also has no effect on production of the complete obese phenotype (Cleary *et al.,* 1980).

One of the major differences between these animals and SLOB animals are that severely obese SLOB rats are not hyperphagic. The consequence of food restriction in SLOB rats is therefore not easy to predict and may be different to that seen in *ob/ob* mice and *Zucker* rats. Food restriction studies can be achieved by adopting various methods. For food restriction from birth, one can limit food intake during the suckling period by increasing litter size in a weaning cage. When an obese animal displays hyperphagia, food restriction can be achieved by pairfeeding to the intake of normal lean animals or reducing the intake to a certain percentage of daily consumption by normal lean animals. I showed in chapter 3 that old (365 days) SLOB rats are not hyperphagic, even when expressed per 100g bodyweight. I

decided to study old male rats (11 months old) that already show a severe obesity phenotype and assess the effects of any change in obesity, rather than the prevention of it. Because SLOB and normal animal food intake at this age are the same, food restriction could be chosen as 50% of the mean daily food intake per animal for all the animals. This was chosen as previous researchers had also used this in adult rats and produced a significant decrease in bodyweight with minimum stress to the animal (Moustafa *et al.,* 1980). 50% of normal food intake could also produce a sustained reduction over 2 weeks, rather than a drastic effect over a few days which results from a more pronounced food withdrawal

Four groups of age-matched males were selected, each group containing 6 animals; (a)SLOB–*ad libitum*, (b)SLOB-food restricted, (c)normal–*ad libitum*, (d)normal-food restricted. Animals were placed into individual metabolic cages with standard chow diet and tap water to drink. After acclimatisation food intake per animal was recorded daily for four days and mean daily food intake calculated. In this case I found the rats to consume an average of 20g of food per day per animal, therefore food restricted animals were allowed 10g/day. Food was rationed out daily at 19.00h, just before the dark period in the hope to maintain the normal rhythm of eating in rats, at the same time food hoppers for *ad libitum* groups were topped up and bodyweights for all animals recorded. After 2 weeks of food restriction, animals were culled, measurements taken and blood and organs collected for analysis.

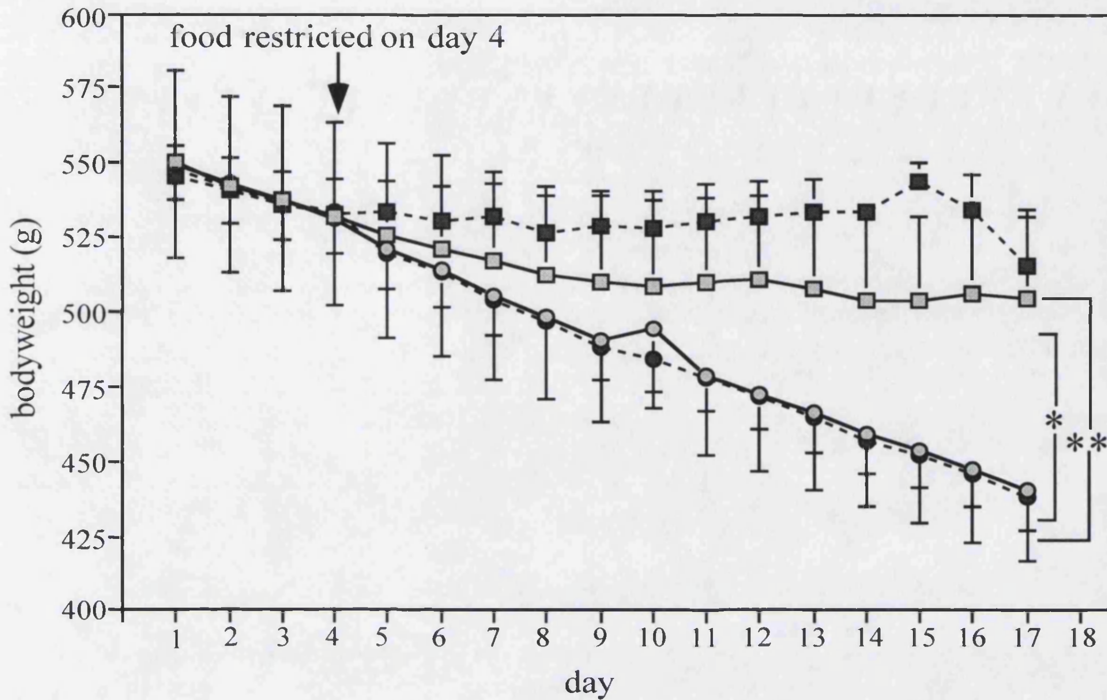
Figure 5.13(a) shows bodyweights for normal and SLOB rats during 2-weeks of food restriction or *ad libitum.* All groups lost weight, with as expected the greatest loss in bodyweight occurring in food restricted SLOB and normal animals. However, their bodyweight curves were almost identical therefore no difference in the rate of loss of weight between transgenic and normal animals was seen. The short initial loss of weight in *ad libitum* SLOB and normal groups was probably due to the fact that at this age these animals are quite large and perhaps take time to adjust to housing and feeding in metabolic cages. However, I ensured they could still access the food hoppers. As SLOB *ad libitum* animals lost more weight than normal *ad libitum* animals, I had to consider the possibility that their greater abdominal adiposity reduced their mobility or adaptation to this housing. However this weight loss had stabilised after 4 days and by the end of the study there was no significant difference

in weight loss between *ad libitum* groups. This was shown in both the final weight at the end of the study (day 17 **Figure 5.13(a)**) and the weight loss chart (**Figure 5.13(b)**). Fat pad weights, supra-renal, mesenteric and testicular depots were measured at the end of the experiment (**Figure 5.14(a),(b),(c)**). In normal animals, significant weight loss was observed in all three depots but in SLOB animals a significant loss was only observed in the supra-renal fat depot. As the effect of food restriction in SLOB rats appears to be adipose-depot specific, a measurement of plasma leptin levels would indicate a change in contribution from this depot in SLOB rats. Leptin levels are thought to reflect peripheral rather than central adipose stores (Lonnqvist *et al.,* 1997a; Friedman and Halaas, 1998), I would therefore expect relatively less change in plasma leptin levels in food restricted compared to *ad libitum* SLOB rats. However, plasma leptins were significantly reduced in food restricted SLOB rats compared to *ad libitum* SLOB rats (**Figure 5.14(d)**), suggesting that the increased central adipose depot in these rats is responsible for their elevated plasma leptin levels.

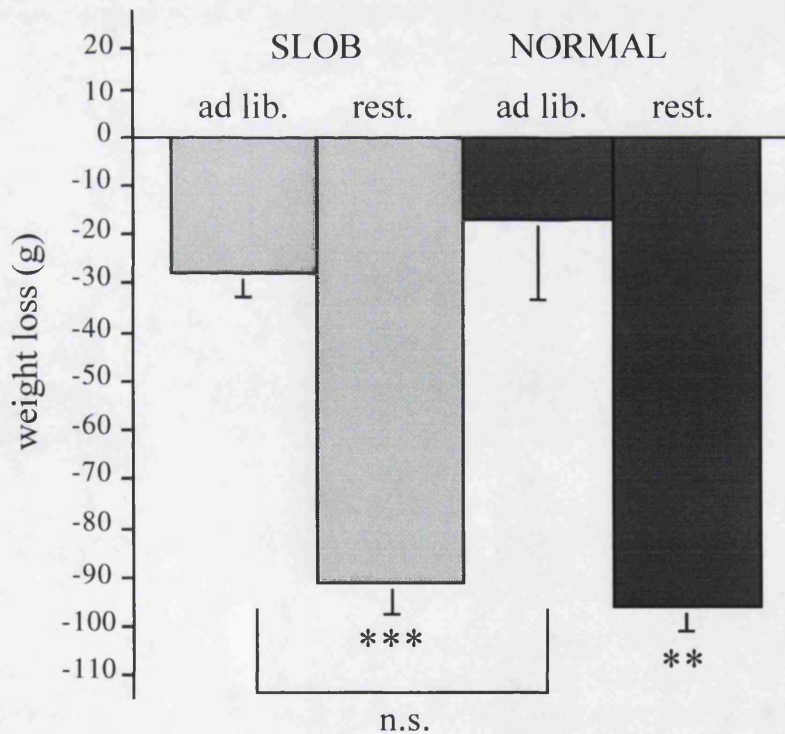
I also measured the effect of food restriction on various organ weights (**Figure 5.15**). For both the heart and soleus muscle, no change was found between *ad libitum* and food restricted SLOB or normal animals. This indicated both SLOB and normal rats utilised relatively more energy from their adipose depots, although the two-week restriction may not have been enough to mobilise energy away from muscle. Liver weights were significantly decreased in both food restricted SLOB and normal animals, whereas spleen weight was only reduced in food restricted normal animals.

As described in 5.2, SLOB organ weights are reduced in weight compared to normal rats, probably due to GH deficiency. Food intake affects plasma IGF-I levels and this subsequently affects organ growth (Frystyk *et al.,* 1995; Cattaneo *et al.,* 1996; Clark *et al.,* 1996; Nam and Marcus, 2000). I therefore decided to measure plasma IGF-I levels in these animals. **Figure 5.16** shows IGF-I levels were significantly reduced in food restricted normal rats, but food restriction had no effect on IGF-I levels in SLOB rats.

(a) Bodyweight change



(b) Bodyweight loss



<u>Figure 5.13</u> Weight change in SLOB and normal male rats fed *ad libitum* or on 50% food restriction. (a) Body weight change over 2 weeks on *ad lib.* or restricted diet. (b) Overall bodyweight loss after 2 weeks (n=6, **p<0.01, ***p<0.001, n.s=not significant). ☐ SLOB *ad libitum*, ◯ SLOB restricted, ■ normal *ad libitum*, ● normal restricted

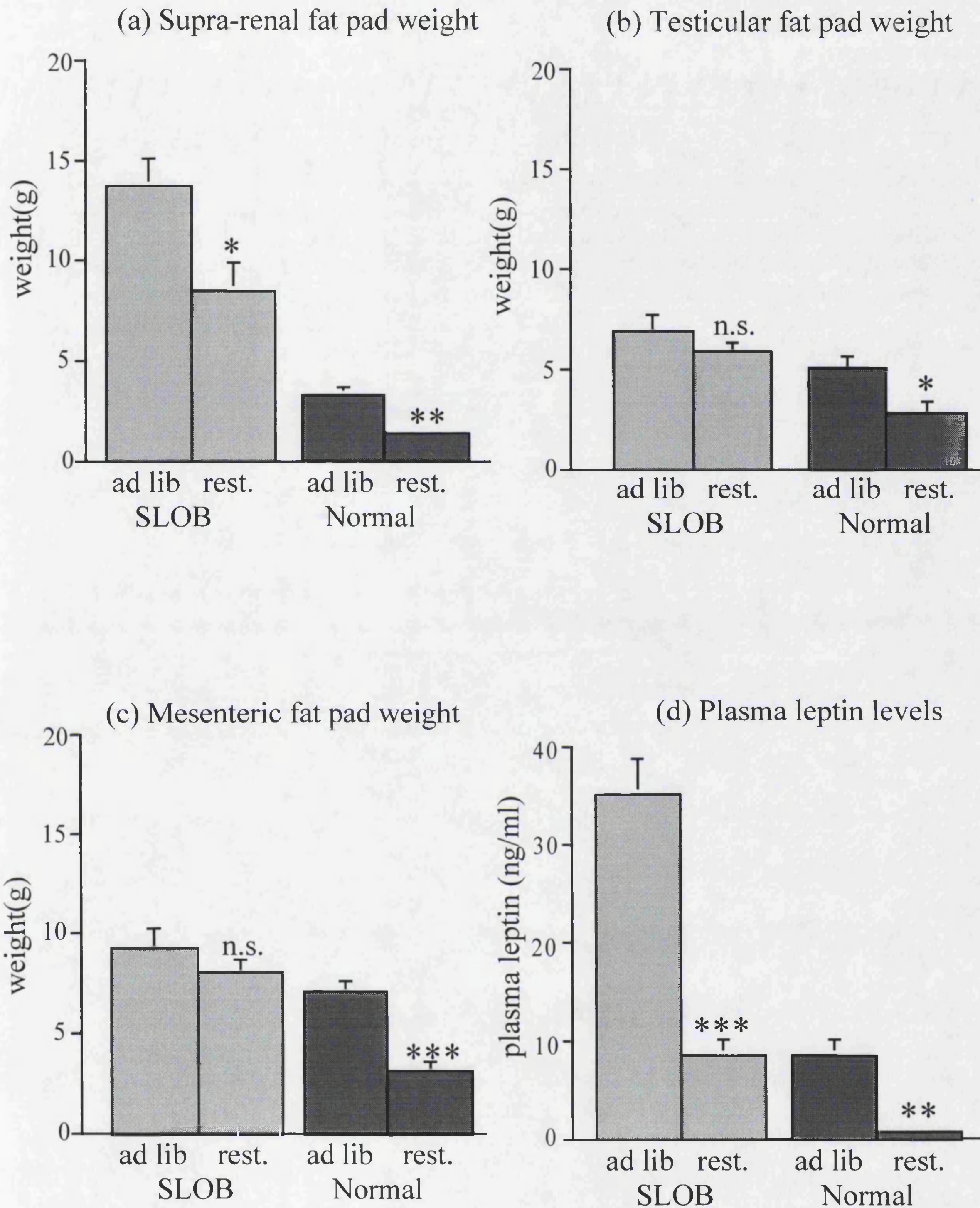


Figure 5.14 Effect of food restriction on fat pad weights and plasma leptin levels. Measurements taken after 2 weeks *ad libitum* or 50% food restriction (a) supra-renal fat pad weight, (b) testicular fat pad weight, (c) mesenteric fat pad weight, and (d) plasma leptin levels (n=6, 11months old, *p<0.05, **p<0.01,***p<0.001 n.s = not significant).

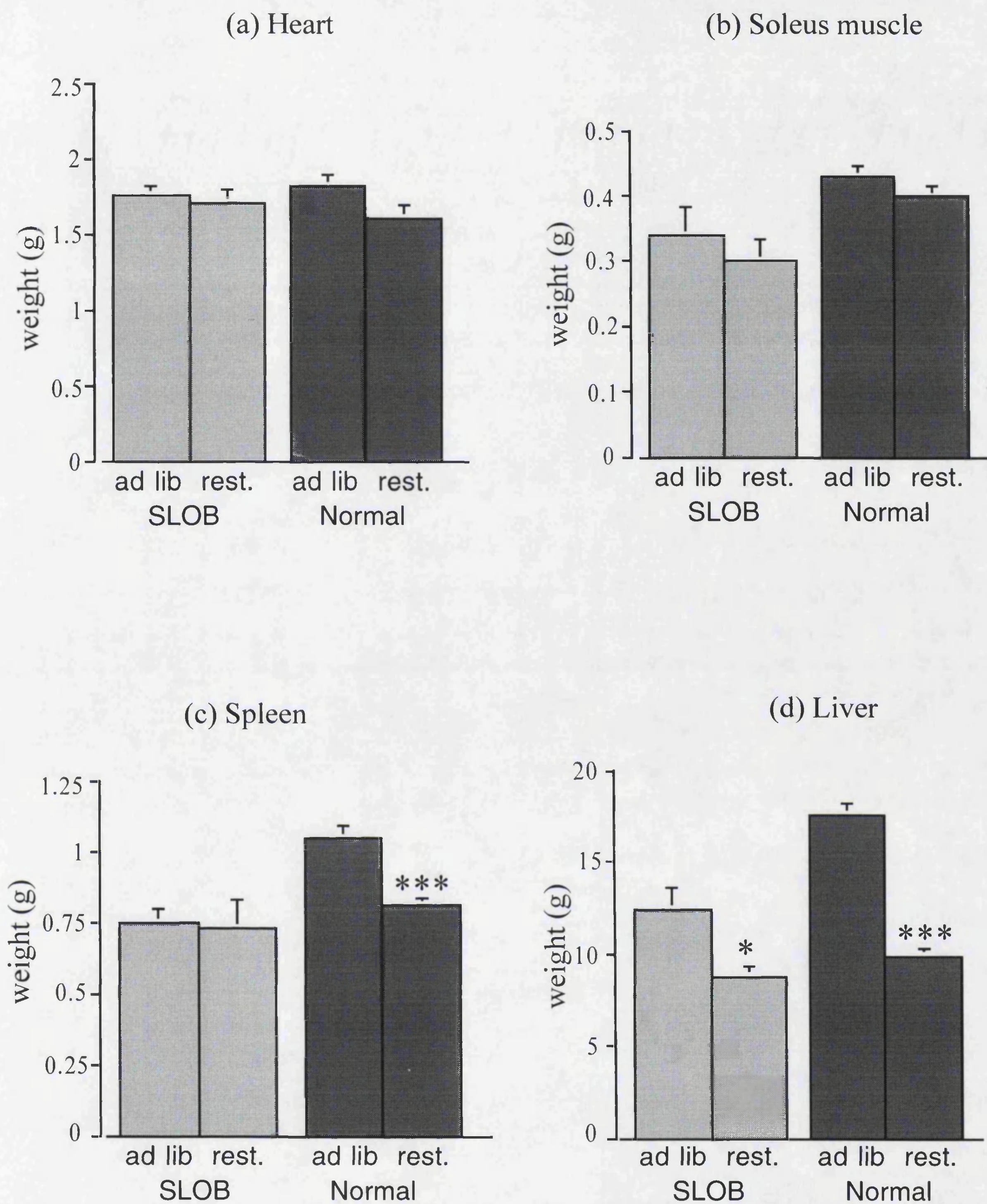


Figure 5.15 Effect of food restriction on organ weights. Measurements taken after 2 weeks on *ad libitum* or 50% food restricted diet in male rats 11 months of age (n=6, *p<0.05, ***p<0.001).

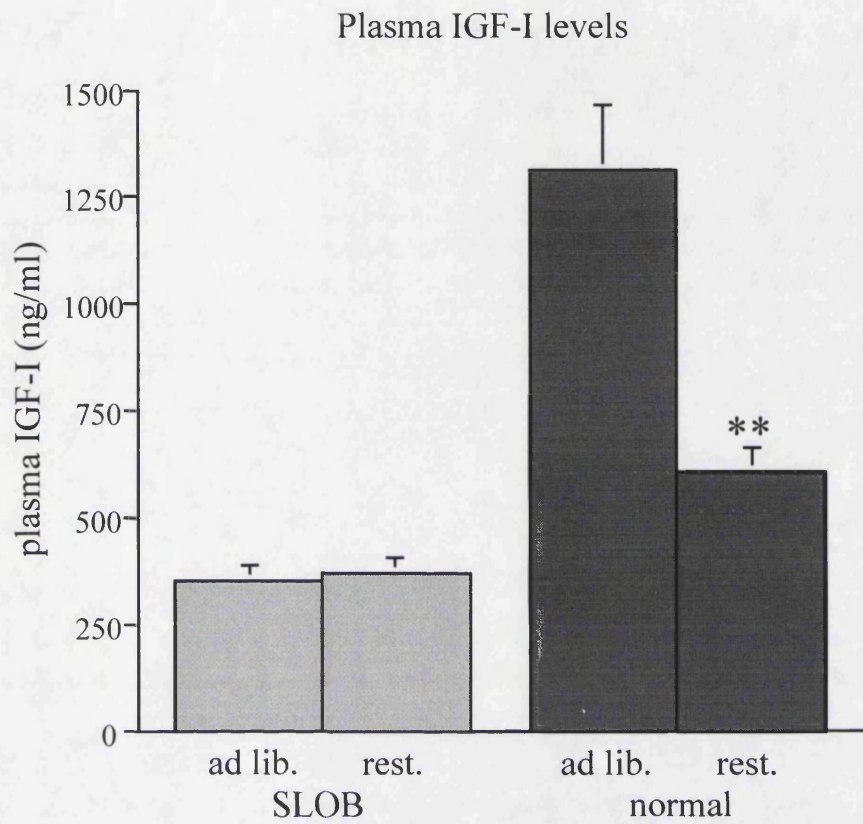


Figure 5.16 Plasma IGF-I levels. IGF-I levels measured by radioimmunoassay after 2 weeks *ad libitum* or 50% food restriction in male 11 month old rats (n=6, **p<0.01).

Splenic growth is particularly sensitive to circulating IGF-I levels (Clark *et al.*, 1993), thus the significant decrease in spleen weight seen only in food restricted normal rats correlates to the significant decrease in plasma IGF-I levels in this group. In SLOB rats IGF-I levels are already low in *ad libitum* animals, food restriction did not have a further effect on this already blunted IGF-I system and therefore does not cause a further reduction in spleen weight. The liver does not have IGF-I receptors but contains numerous GH receptors and its growth is sensitive to GH directly (Vander *et al.*, 1994). Pituitary GH levels were found to be unaltered in SLOB or normal *ad libitum* animals compared to SLOB or normal food restricted animals (SLOB: *ad lib* 82.2±9.7µg/pit vs food rest. 95.3±12.2µg/pit, Normal: *ad lib* 347.9±81.5µg/pit vs food rest. 344.2±101.5µg/pit). It should be noted that the effects of fasting on GH secretion in rodents are different from those in humans. Fasting normally suppresses GH secretion in rats (Tannenbaum *et al.,* 1979) whereas it markedly increases spontaneous GH secretion in humans (Dieguez *et al.,* 1995. The reduction in liver weight in both SLOB and normal animals may therefore be directly related to reduced secretion of GH, although pituitary GH levels remain unaltered.

5.5 Effect of Gonadectomy and Oestrogen Treatment

The origin of the sexual dimorphism in the severity of the SLOB phenotype remains unclear. As already discussed, SLOB females are fertile and normally show obesity at a much later age compared to SLOB males. However, high fat feeding induces severe obesity in young SLOB females, suggesting that SLOB females on a normal diet are somehow protected from developing severe obesity until a later age. I suspected that oestrogen may have a role in obesity etiology in SLOB rats.

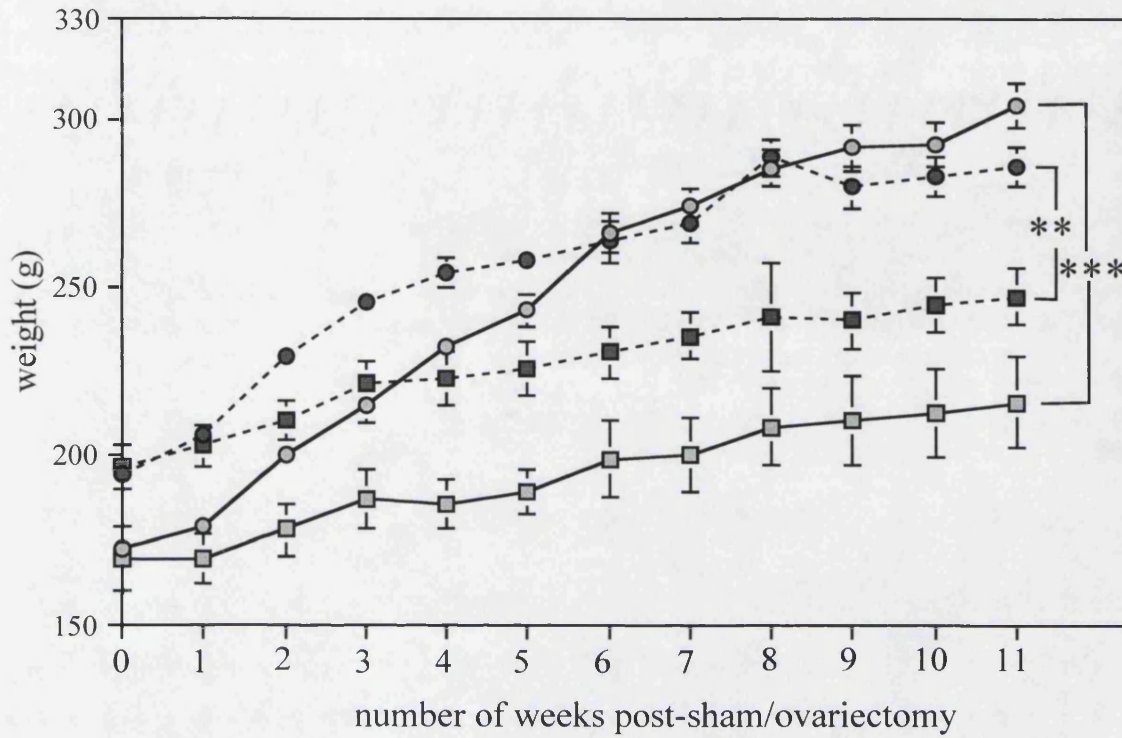
Two experiments were carried out, both using animals 10 weeks of age at the start of each study. The first included 14 SLOB females, 14 normal females, 14 SLOB males and 14 normal males. From each group of 14, seven animals were sham operated and seven were gonadectomised. Following surgery, all animals were housed in normal plastic cages in groups of 2-3 (with same surgical procedure) and allowed free access to standard chow diet and tap water to drink. Animals were weighed once a week following the procedure, upto 18 weeks post-surgery. Thereafter, animals were

culled, various measurements recorded and blood and organs taken for analysis. The second experiment included 28 SLOB males, 26 normal males, 23 SLOB females and 24 normal females. Each group was divided into 4 groups of 5-7 rats. These four groups consisted of animals (a)sham operated, (b)sham operated/oestrogen treated, (c)gonadectomised, (d) gonadectomised /oestrogen treated. Oestrogen was prepared using oestradiol-3-benzoate (Sigma) in sesame oil as the vehicle. After assessing previous literature (Boado *et al.*, 1983; Suzuki *et al.*, 1985) a dose of 30μg injected in sesame oil every 5 days was chosen. To deliver oestrogen in a total volume of 50μl, a stock solution of 600μg/ml was prepared by dissolving oestrogen in ethanol amounting to 10% of the total volume of sesame oil. All animals were weighed every 5 days and those receiving oestrogen injected at the same time. Two representative rats from each non-treated group were injected with vehicle alone throughout the study, the remainder were not injected due to time limitations. Results from these two rats were not different from the remainder non-treated animals in the same group, therefore data from all animals was pooled within each group.

5.5.1 Effect of ovariectomy in SLOB and normal female rats

At 10 weeks of age SLOB females weighed less than age-matched normal females. Following ovariectomy, both SLOB and normal rats gained more weight than intact rats (**Figure 5.17(a)**), as has previously been shown for normal rats by other researchers (Goulding *et al.*, 1987, Wronski *et al.*, 1989). However, the weight gain was much more rapid in SLOB animals than normal animals to the extent that mean bodyweight in SLOB females exceeded that of intact normal females after about 8 weeks post-surgery. Although intact SLOB females were lighter than intact normal females they gained the same amount of weight over the 11 weeks following surgery (Δwt SLOB 45.5±5.3g vs Δwt normal 48.4±3.8g). However, ovariectomy caused SLOB females to gain much more weight than normal females (**Figure 5.17(b)** Δwt SLOB 128±7.7g vs Δwt in normal 89±4.3g, $p<0.001$). This gain in weight was reflected in fat pad weights (**Figure 5.18(a),(b)**) and plasma leptin levels (**Figure 5.18c)**) 18 weeks post surgery. Both ovarian fat pad weight and supra-renal fat pad weight showed the greatest increase in ovariectomised SLOB rats, likewise plasma

(a) Bodyweight chart



(b) Bodyweight gain

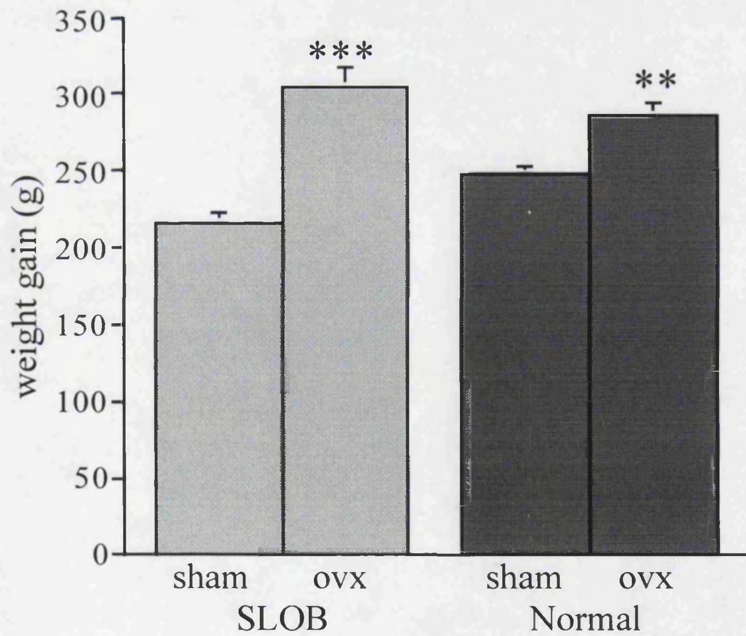


Figure 5.17 Body weights in female SLOB and normal, sham and ovx animals. (a) Bodyweight change over 11 weeks post sham or ovariectomy in 10 week old female rats. (b) Bodyweight gain after 11 weeks. (n=7, $^{**}p<0.01$, $^{***}p<0.001$).
■ SLOB sham ● SLOB ovx ■ Normal sham ● Normal ovx

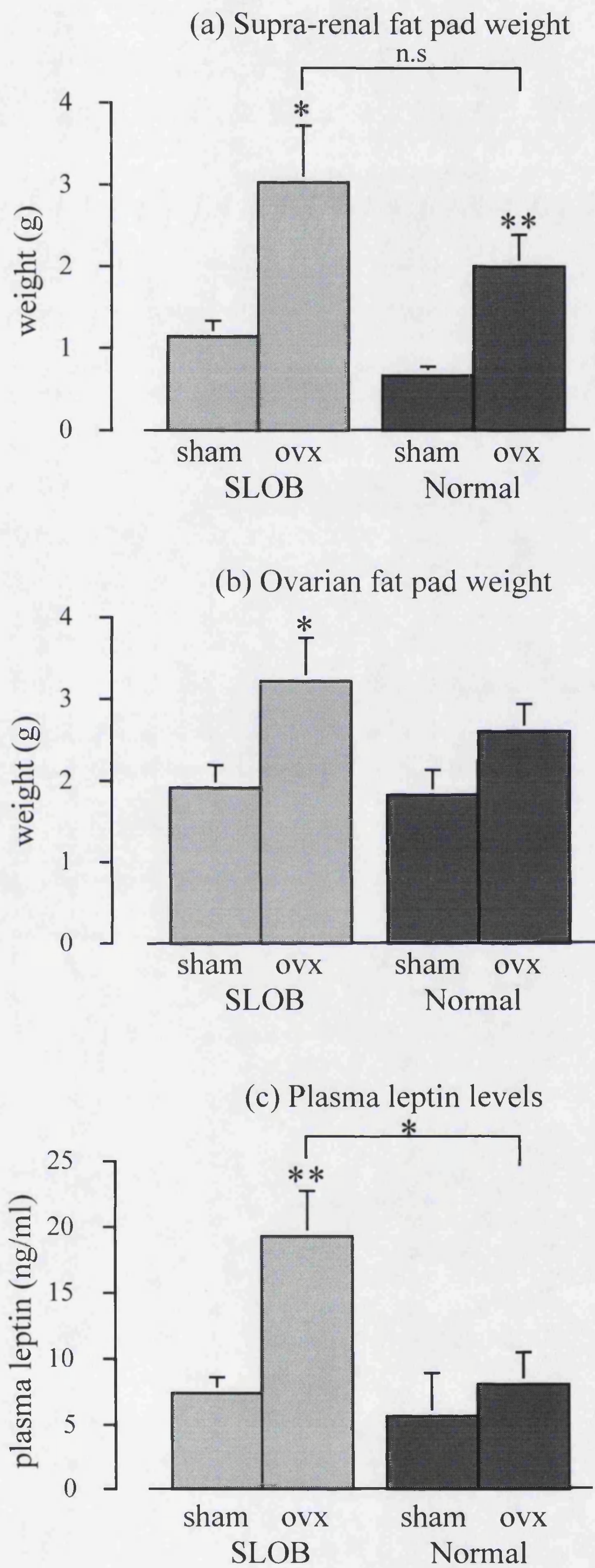


Figure 5.18 Effect of ovariectomy on fat pad weight and plasma leptin levels. Measurements taken after 18 weeks post surgery (n=7 *p<0.05, **p<0.01)

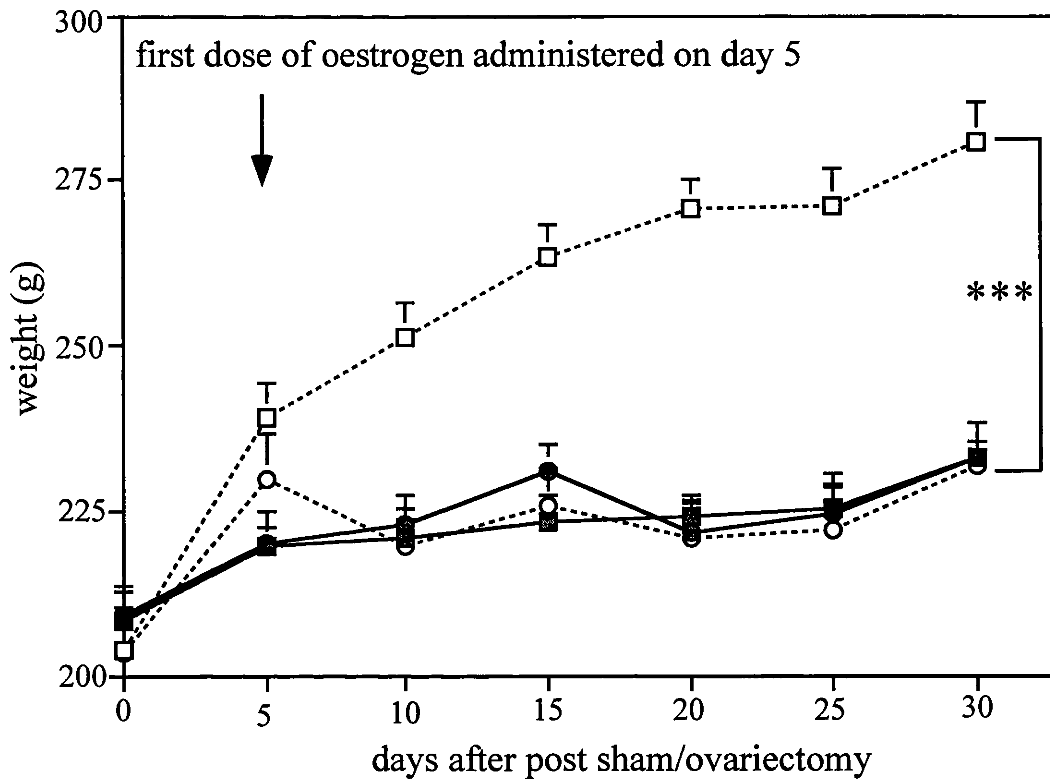
leptin levels were highest in this group. Thus it appeared ovariectomy uncovered a severe obesity phenotype in young SLOB female rats.

In order to test whether the effect of ovariectomy in SLOB rats could be reversed, the above experiment was repeated and included groups of animals, sham and ovariectomised (ovx) that were also treated with oestrogen. As before ovariectomy caused an increase in weight gain in both SLOB and normal rats. Oestrogen treatment of ovariectomised rats reduced their body weight to the same level as that of intact rats for both SLOB and normal groups, whereas the body weight of intact rats treated with oestrogen showed only a non-significant trend to decrease in weight compared with the non-treated rats (**Figure 5.19**). Likewise oestrogen administration to ovx animals returned plasma leptins and fat pad weights towards normal levels, and again the largest increase in fat pad weight and plasma leptin levels were observed in ovariectomised SLOB females (**Figure 5.20**) confirming the results of the first ovariectomy experiment. As the ovarian fat pad is not representative of a peripheral fat pad, I decided to measure the axillary fat depot in this study.

5.5.2 Alterations in hypothalamic neuropeptides in OVX and E_2 treated female rats

Many neuropeptide mRNA expression levels are altered in ovariectomised and oestrogen treated female rats, many of these changes being associated with alterations in bodyweight and energy balance (Haas and George, 1989; Bonovera *et al.,* 1994; Baskin *et al.,* 1995; Bennett *et al.,* 1998). Recently, attention has been focused on the possibility that the anorectic effects of oestrogen may be mediated by NPY (Bonovera *et al.,* 1994). In my experiments, ovariectomy in SLOB and normal females caused a reduction in hypothalamic arcuate NPY mRNA expression (**Figure 5.21**). These results were expected as ovariectomy causes an increase in bodyweight, adipose depot weight and plasma leptin levels, all associated with a fall in NPY mRNA expression (Gehlert, 1999). Administration of oestrogen to either sham operated or ovx rats caused an increase in arcuate NPY mRNA when compared to intact groups, also previously reported (Bonavera *et al.*, 1994).

(a) Ovariectomy and oestrogen treatment in NORMAL females



(b) Ovariectomy and oestrogen treatment in SLOB females

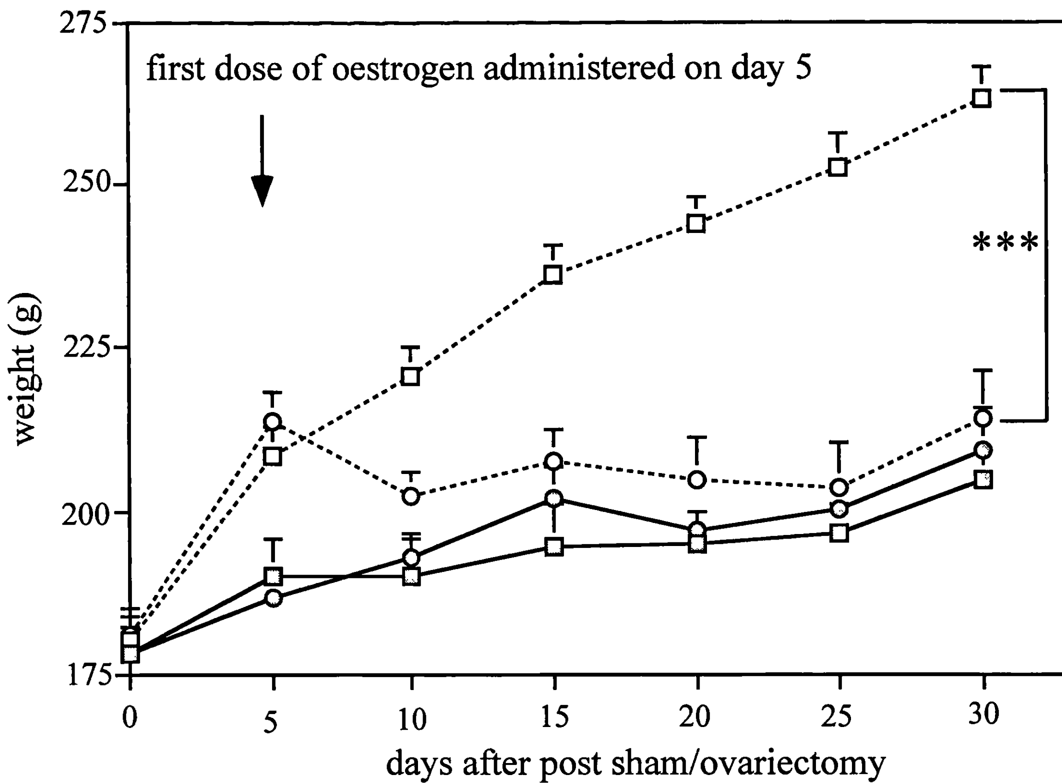


Figure 5.19 Ovariectomy and oestrogen treatment in SLOB and normal females. (a) Normal rats, (b) SLOB rats. Treated animals were injected every 5 days with 30µg of oestrogen (n=6-7). In each graph: filled squares = sham, filled circles = sham/E, open squares = ovx, open circles = ovx/E (***p<0.001).

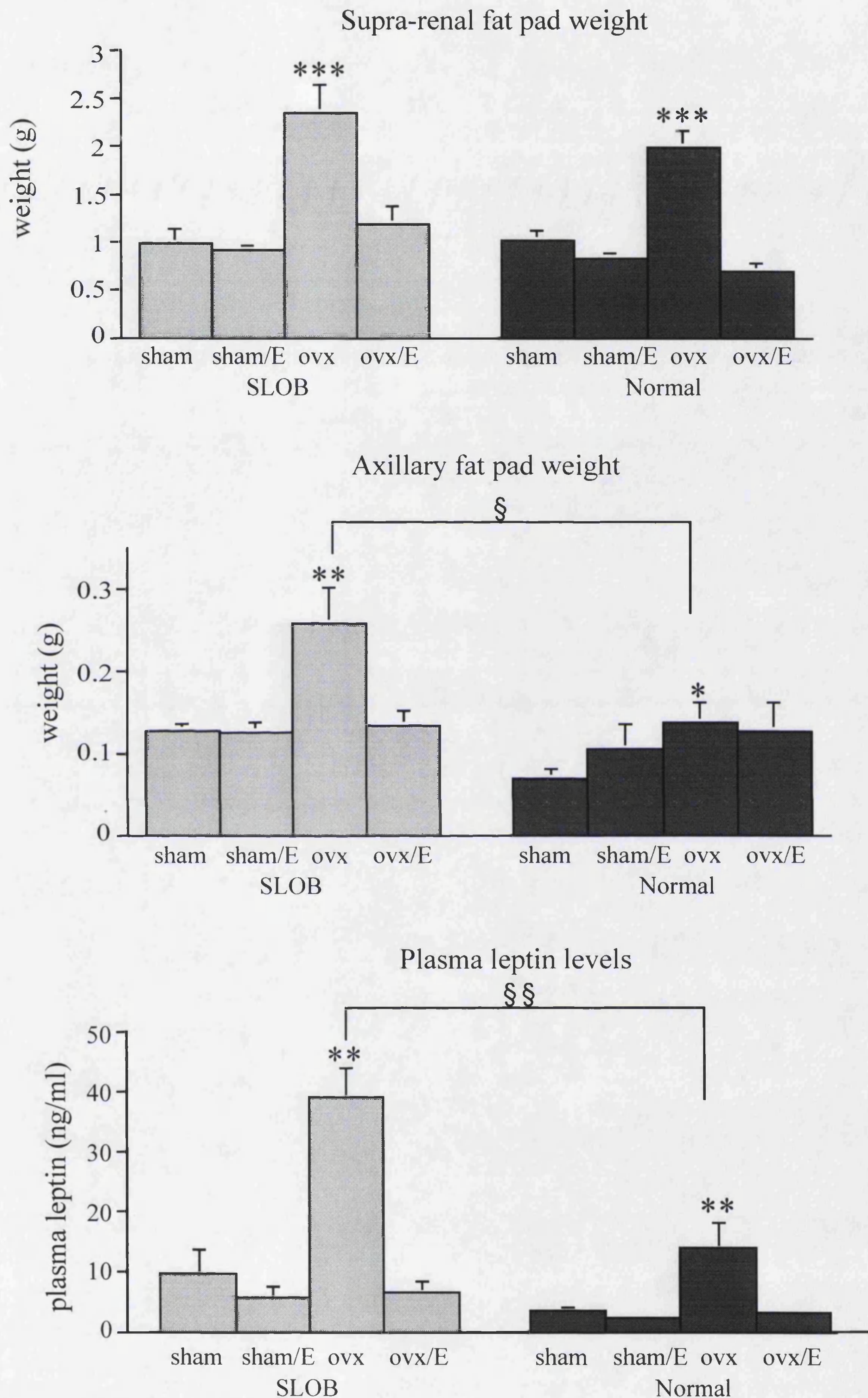
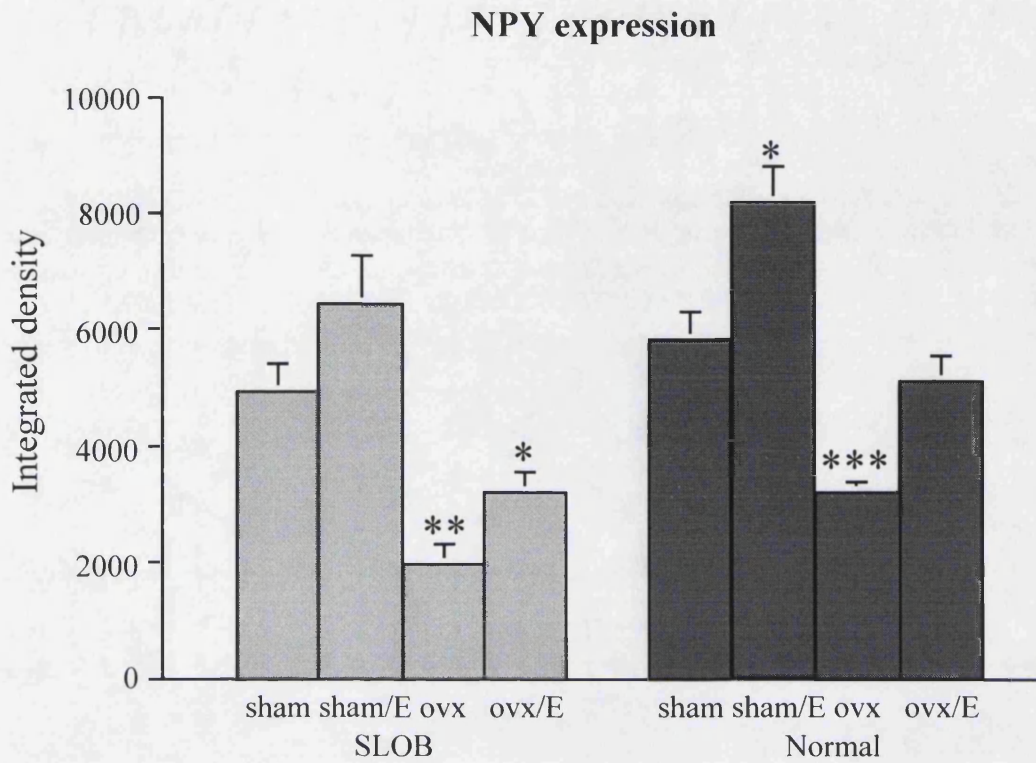


Figure 5.20 Effect of ovariectomy and oestrogen treatment in SLOB and normal female rats. Measurements taken in animals after 5 weeks post surgery/treatment (n=6-7, */§p<0.05, **/§§p<0.01, ***p<0.001).



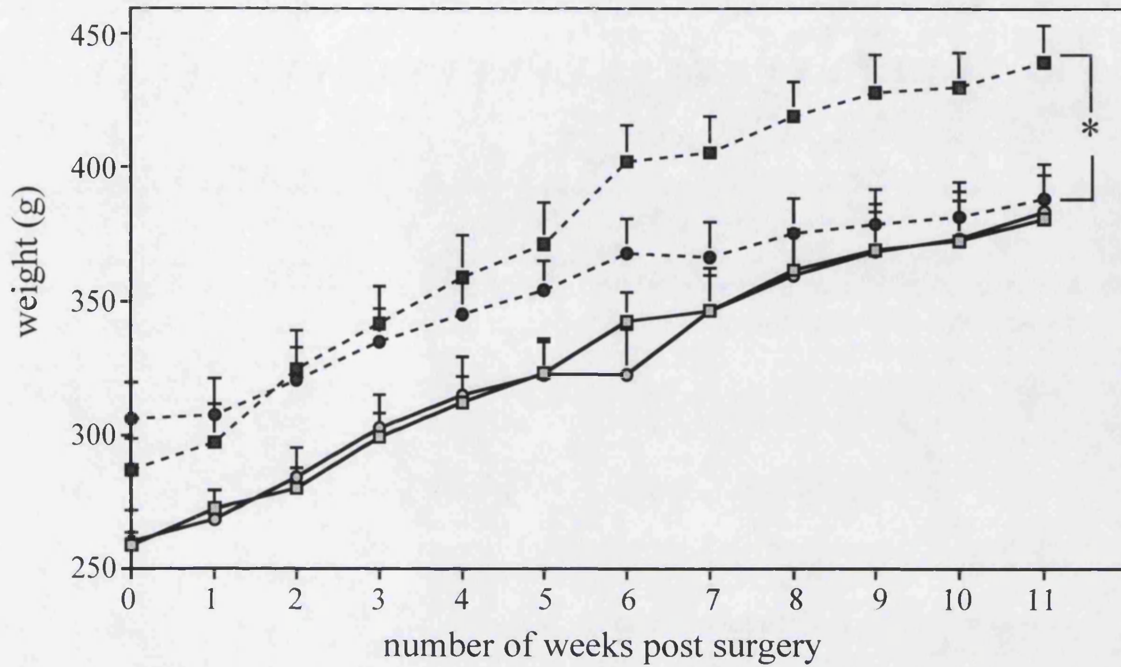
<u>Figure 5.21</u> Arcuate NPY mRNA expression. NPY mRNA levels measured by *in situ* hybridisation after 5 weeks post surgery/treatment. Significances shown when compared with sham operated group (n=6-7, *p<0.05, **p<0.01, ***p<0.001).

In situ analysis of CRF mRNA expression in the PVN of SLOB and normal rat brains showed no significant differences between ovx and oestrogen treated groups. However, SLOB females, regardless of treatment all had significantly lower CRF mRNA expression levels when compared to normal animals. Thus like old SLOB male rats, young SLOB females have reduced CRF mRNA levels.

5.5.3 Effect of gonadectomy in SLOB males

Due to the sex-specific phenotype, I decided to castrate young SLOB males and observe any changes in the development of the obesity phenotype. 11 weeks post-surgery, gonadectomy caused a significant reduction in weight gain in normal male rats compared to sham operated normal rats, whereas there was no difference in weight gain between gonadectomised and sham operated SLOB males (**Figure 5.22**). A loss in bodyweight following gonadectomy has previously been reported (Scow *et al.,* 1952; Kakolowski *et al.,* 1968) and usually ascribed as a result of lack of testosterone (Jansson *et al.,* 1983). Testosterone promotes lean muscle mass (Vermeulen *et al.,* 1999; Sheffield *et al.,* 2000); a reduction in testosterone would therefore cause a reduction in lean muscle mass and a subsequent decrease in body weight. However, it appears that a lack of testosterone had no effect on weight gain in SLOB males as they continued to gain weight at the same rate as sham operated SLOB males. **Figure 5.23(a)** shows 18 weeks post surgery plasma leptin levels were unaltered in sham or gnx SLOB male rats and were still significantly higher than normal rats. Thus, in terms of plasma leptin levels, obesity is still evident in these animals. However, in normal animals, gnx animals had decreased leptin levels compared to sham operated animals. Supra-renal fat pad weights were not altered by gonadectomy in either SLOB or normal animals. A significant difference was observed in testicular fat pad weight (**Figure 5.23(b), (c)**), but this result may be unreliable as it may have been affected by the surgical procedure and potential damage to the vasculature. Therefore the loss of weight in normal rats is probably due mostly to a reduction in lean body mass.

(a) Bodyweight chart



(b) Bodyweight change

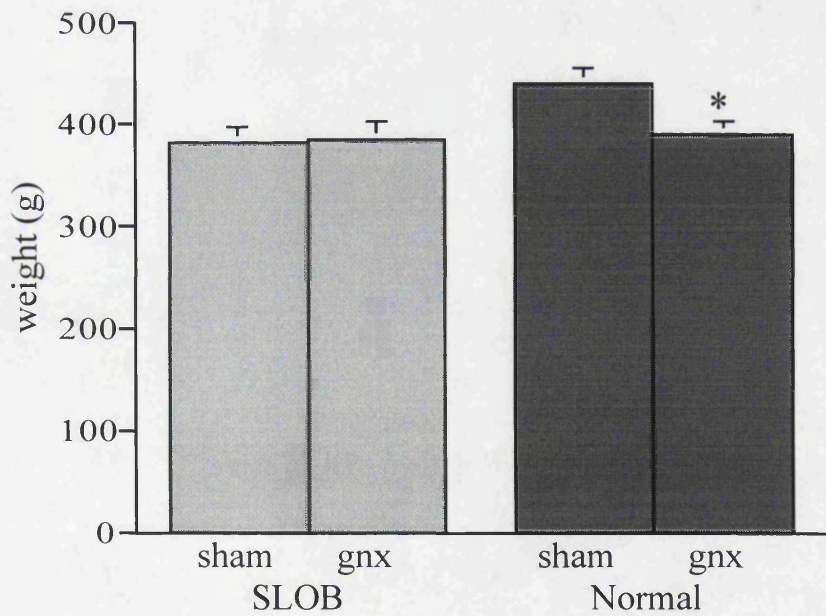


Figure 5.22 Effect of gonadectomy in male rats (a) Weekly bodyweights post surgery. (b) Bodyweight change after 11 weeks post surgery (n=7, *p<0.05).
□ SLOB sham, ○ SLOB gnx, ■ normal sham, ● normal gnx.

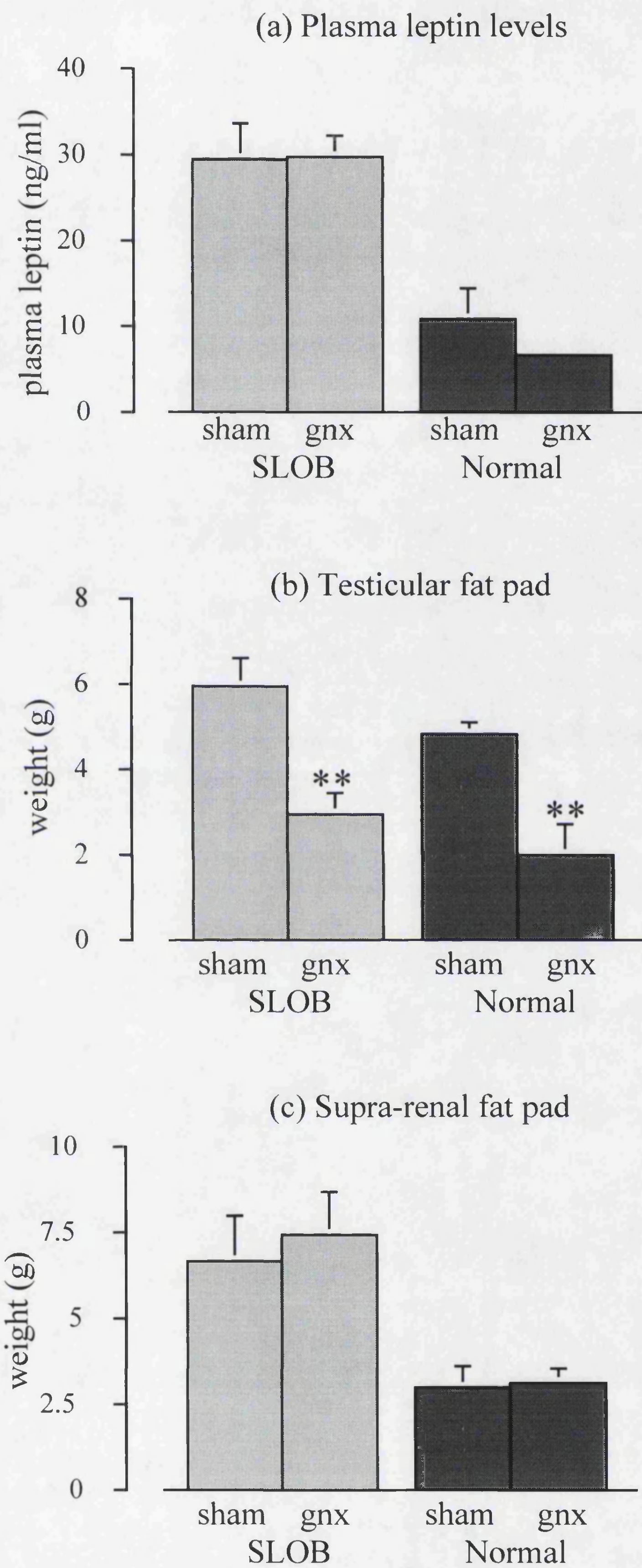
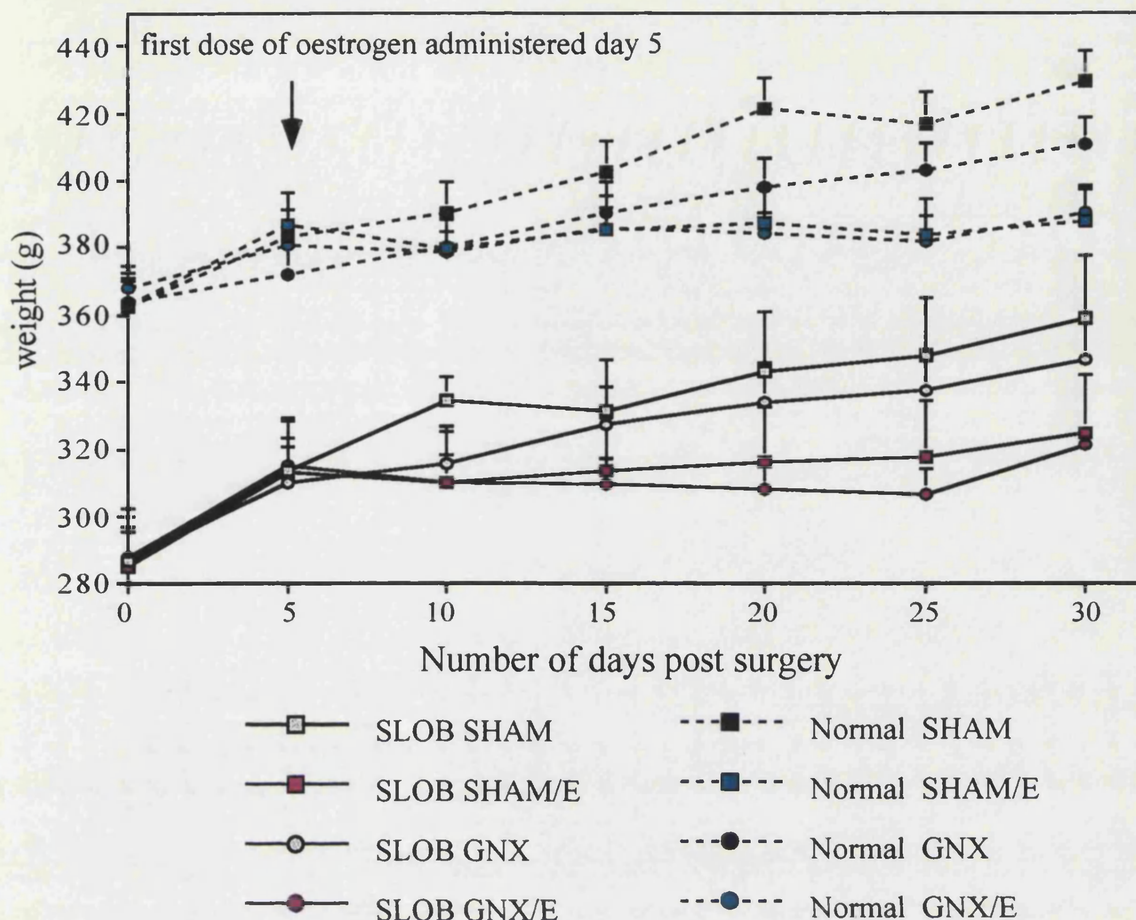


Figure 5.23 Effect of gonadectomy on plasma leptin levels and fat pad weights. Measurements taken in animals 18 weeks post surgery (n=7, **p<0.01).

5.5.4 Effect of oestrogen treatment in SLOB males

As I established that oestrogen had a protective role in development of the obesity phenotype in SLOB females, I was also interested to test the effect of oestrogen treatment in SLOB males. **Figure 5.24(a)** shows bodyweight data during 30 days post surgery/treatment in male rats. In all cases oestrogen caused a reduction in weight gain **(Figure 5.24(b))**. As before the effect of gonadectomy on bodyweight was more pronounced in normal rats than in SLOB rats. Oestrogen treatment in both SLOB and normal rats caused a similar reduction in bodyweight. Supra renal fat pad weights showed that obesity was still evident in terms of this parameter in all SLOB animals (sham: SLOB 4.3±0.5g vs normal 2.8±0.3g $p<0.03$, sham/E_2: SLOB 4.1±0.5g vs normal 2.1±0.2g $p<0.007$, gnx: SLOB 5.3±0.6g vs 2.9±0.1g $p<0.03$, gnx/E_2: SLOB 4.3±0.3g vs normal 2.3±0.3g $p<0.002$), thus oestrogen treatment during this study did not abolish obesity in SLOB males. However, this interpretation needs some caution as oestrogen is known to have a potent effect on males causing a reduction in food intake and body weight (Dubuc, 1976c) and unfortunately treatment in some animals eventually induced signs of discomfort and stress and therefore I terminated the study after 5 weeks.



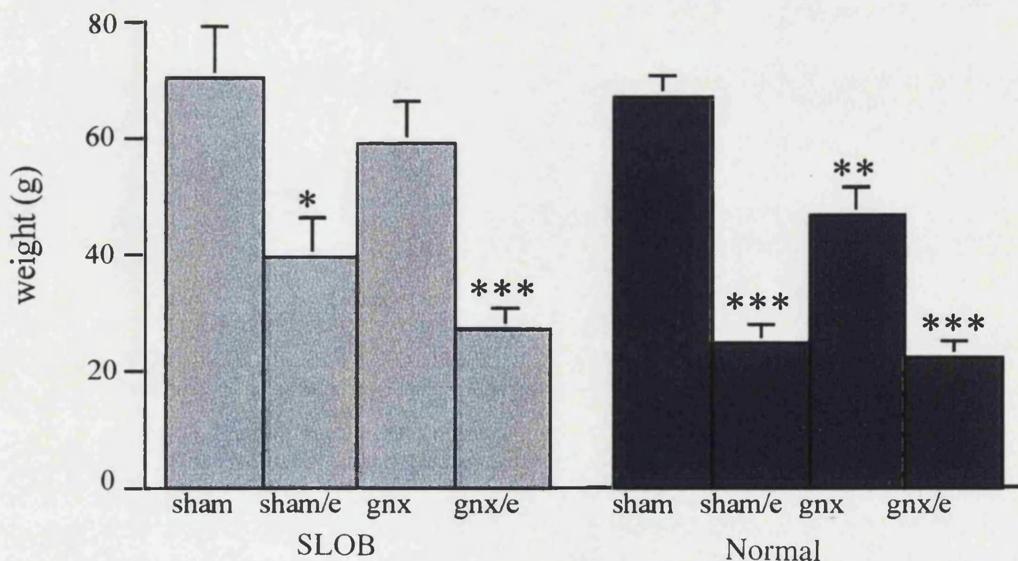


Figure 5.24 Gonadectomy and oestrogen treatment in male rats. Treated animals were injected with 30μg of oestrogen every 5 days. (a) Bodyweights taken every 5 days for 5 weeks post surgery (b) overall bodyweight after 5 weeks post surgery/treatment, significances shown for when compared to sham operated group (n=7, *p<0.05, **p<0.01, ***p<0.001).

5.6 Discussion

In this chapter I have demonstrated that, GH treatment does not abolish the obesity phenotype in GH-deficient SLOB rats, severe obesity is induced in young SLOB rats by high fat feeding, food restriction causes a similar weight loss in both SLOB and normal rats, ovariectomy induces a severe obesity phenotype in young SLOB females, reversed by oestrogen treatment, and SLOB males are still obese after gonadectomy and/or oestrogen treatment. In the following sections I will discuss these findings related to those observed in other rodent obesity models and review if the manipulations in this chapter aid in elucidating the cause of the SLOB phenotype.

5.6.1 Growth hormone–deficiency: what role does this play in the SLOB phenotype?

Ikeda *et al.,* (1994) generated transgenic rats expressing hGH that developed severe obesity. Unlike SLOB rats, obesity in these animals extended to both peripheral and central adipose stores rather than being confined to the abdomen, obesity occurred in both sexes at the same time, and these rats were also diabetic. Whereas in SLOB rats hGH was inserted under the control of the vasopressin promoter and is expressed in the hypothalamus, Ikeda *et al.*, used a chimeric gene comprising murine whey acidic protein (a mammary gland specific protein secreted into the milk) and hGH to produce their transgenics. Two founder rats were generated, integrating different copy numbers of the transgene. One line of rats displayed relatively high levels of serum hGH, extended body length, and increased weight gain. The other line had lower levels of hGH though still quite high compared to normal inter-pulse GH levels. They had normal body length but were severely obese. Both lines of rats exhibited reduced levels of endogenous GH secretion. The obese strain was studied and these rats were found to develop severe obesity with age and plasma glucose, insulin, triglyceride, and FFA levels in male transgenics significantly exceeded those in non-transgenic littermates (Ikeda *et al.* 1998). No effect on male sterility was observed, however female rats of the reduced hGH line were sterile (Ikeda *et al.,* 1997). Although some aspects of the phenotype are clearly different from those seen in

SLOB rats, I wondered whether the expression of hGH in SLOB animals had a role in modifying some aspects of the SLOB obesity phenotype. The pulsatile treatment of the transgenics generated by Ikeda with hGH noticeably reduced supra-renal and testicular fat pad weights and restored normal weight gain (Ikeda *et al.,* 1996). Therefore the cause of obesity in these rats was concluded to be a lack of GH. Was this the case for SLOB rats?

In chapter 3, I showed that compared to SLOB males the more GH-deficient *dwarf* male does not develop obesity at an old age (1 year). Furthermore, my data in this chapter showed that high fat fed *dwarf* males did not gain bodyweight to the extent of that seen in SLOB males, although they did exhibit elevated plasma leptin levels and supra-renal fat pad weights. However, these animals did not show an obesity phenotype and respond as normal rats do with a decrease in cumulative food intake when challenged with a high fat diet. This is also reflected in a decrease in NPY mRNA levels as will be discussed later. Therefore these data enabled me to conclude that the effects of high fat feeding in SLOB rats were probably not due to GH-deficiency as the *dwarf* rat did not develop severe obesity. Nevertheless, to strengthen the fact that GH-deficiency is most likely not the primary cause of SLOB obesity, I treated SLOB males with GH. As treatment with GH does not alter supra-renal or testicular fat pad weight or reduce plasma leptin levels in SLOB rats, little effect is observed on the obesity phenotype. I therefore conclude that GH-deficiency alone cannot be responsible for the SLOB phenotype. GH-deficiency in these rats did however alter some aspects of their weight gain, reflected in an increase in some individual organ weights following GH treatment, otherwise smaller in untreated SLOB animals. In normal animals this dose of GH had no effect on weight gain, fat pad weight, plasma leptin levels or organ weight, this was probably because animals used in this experiment were 20 weeks old, having passed their dynamic growth phase. Had the experiment been carried out in younger animals a more pronounced GH effect on growth of bodyweight would have been observed in normal animals. Nevertheless, it is clear that GH treatment of relatively obese SLOB rats does not reverse this phenotype. However, it is still possible that GH deficiency in SLOB rats has a role in deteriorating or ameliorating certain aspects of their phenotype, for example their fat distribution and adipose lipolytic activity.

5.6.2 Effect of high fat feeding in SLOB rats

Early studies by Mayer *et al.,* (1951) showed that *ob/ob* mice with established obesity tend to choose more fat than lean controls when allowed to select caloric sources. Such studies have yet to be carried out in SLOB rats and thus it is not yet known whether this is also the case for SLOB rats. However, my studies have shown that when allowed free access to a diet higher in fat, SLOB animals consume more food than normal animals on a similar diet. Although *ob/ob* mice prefer a diet higher in fat than normal mice, *ob/ob* mice can develop a degree of obesity even in the complete absence of dietary fat from the time of weaning (Mayer *et al.,* 1951). When compared to lean mice similarly restricted, this is true in both the relative sense (increased percentage of body fat) and in the absolute sense (increased total body fat). In comparison, SLOB males normally fed a low fat diet consisting of just 4% fat also develop severe obesity. However, analysis of whether obesity develops in the complete absence of dietary fat, is still to be determined in SLOB rats. Conversely, when allowed free access to a high fat diet from the time of weaning, obesity occurs more readily in *ob/ob* mice (Genuth 1976) as I also found in SLOB males and females, with high fat feeding starting at 100 days of age. Thus both the *ob/ob* mouse and SLOB rats respond similarly to the challenge of a high fat diet. On a low fat diet both these animal models are genetically predisposed to develop obesity and on a higher fat diet animals are predisposed to rapidly develop obesity.

My cumulative food intake data in SLOB males on a high fat diet is informative; high fat fed SLOB males continued to consume almost as much food as chow fed SLOB males, whereas a significant decrease was observed in high fat fed normal and *dwarf* rats. The food intake in high-fat fed SLOB males was also significantly greater than that in high fat fed normal males. Thus, young SLOB males who have an incipient obesity phenotype, when challenged with a high fat diet, induces severe obesity comparable to that seen in old SLOB males and is reflected in increased food intake, increased weight gain, elevated fat pad weights, and elevated plasma leptin levels. This apparent lack of reducing food intake suggests SLOB rats may be leptin resistant as is suspected in the obese human population (Schwartz *et al.,* 1996c; Kennedy *et al.,* 1997). Although on a low fat diet, SLOB rats appear to have normal food intake,

when challenged with a high fat diet resulting in a further elevation of plasma leptin levels, they are unable to respond to this increase in leptin and thus do not reduce their food consumption.

In females the effects of high fat feeding are more pronounced in both normal and *dwarf* rats as well as SLOB rats when compared to male rats. The marked effects on bodyweight gain and fat pad weight in *dwarf* females confirmed the results of Clark *et al.,* (1996) who also showed obesity could be induced by high fat feeding female *dwarf* rats much more readily than in males. What is interesting again is that despite elevated fat pad mass and plasma leptin levels in *dwarf* females they do significantly reduce their cumulative food intake whereas only a non-significant reduction was observed in SLOB and normal females. As Δbodyweight gain is greatest in SLOB females, as in young SLOB males, high fat feeding also induced a severe obesity phenotype in young SLOB female rats. It is possible that on a low fat diet (4% fat) SLOB females are protected from developing obesity and even with elevated plasma leptin levels at a young age, the onset of severe obesity is delayed to a much later age than that seen in SLOB males. However, when challenged with a diet of higher fat content, young SLOB females are unable to resist diet-induced obesity and thus like young SLOB males become obese, and perhaps leptin resistant. As *dwarf* females on high fat diet also show some obesity phenotype and *dwarf* males do not, there is a possibility that both GH-deficiency and sex-specificity have a role in the development or protection from severe obesity in SLOB females. At least two explanations are possible: sex-steroid differences and/or the sexual dimorphic pattern of GH secretion (Tannenbaum *et al,* 1976; Edén, 1979; Clark *et al.,* 1987). GH treatment in SLOB female rats has not yet been carried out, however, the role of oestrogens are discussed in 5.6.5.

Gender differences in feeding high-fat diets have also been found in *Zucker* rats. Male *Zucker* rats on a low-fat content diet (12.3kcal% fat) develop diabetes, the process being accelerated as a function of dietary fat-content, whereas only diets of high fat-content (48 kcal% fat) induce the development of non-insulin dependent diabetes mellitus (NIDDM) in obese *Zucker* females (Corsetti et al., 2000). Both this study and my results indicate significant differences between *Zucker* males and females and SLOB males and females in development of diabetes (*Zucker* rats) and

obesity (SLOB rats) as a function of dietary fat-content. From these observations it is reasonable to conclude that a low fat diet is enough to induce obesity in *Zucker* and SLOB males but a diet higher in fat content is required to induce obesity in *Zucker* and SLOB females. Studies on cold-acclimatised *Zucker* rats have also indicated that feeding high-fat diets can specifically increase thermogenesis in brown adipose tissue (Rothwell *et al.,* 1983), an organ now regarded as the major site of heat production by nonshivering mechanisms in small mammals. Further studies on high fat feeding SLOB rats have yet to be carried out to establish the mechanisms underlying the results of high fat feeding so far shown in this chapter. I have largely concentrated on calorie intake; differences in diet-induced thermogenesis, lipolysis stimulation and fat oxidation have all been proposed in order to explain adiposity increase induced by high fat feeding, these studies are clearly warranted in SLOB rats.

5.6.3 Effect of high fat feeding on neuropeptide analysis

Some limited investigations on hypothalamic CRF, *tub* and NPY mRNA expression were conducted in these high fat-feeding experiments. Note that I was only able to obtain results from the male study as yet. As I have found in some aspects male and female rats respond differently in response to a high fat diet, especially *dwarf* rats, these hypothalamic neuropeptides should in the future also be analysed in high fat fed female rats.

CRF mRNA expression was found to be decreased in the PVN of all high fat fed groups. Previously it has been reported that CRF mRNA expression is reduced in lean rats following food deprivation (Brady *et al.,* 1990), but is increased in *Zucker* rats or *ob/ob* mice (Timofeeva *et al.,* 1997; Huang *et al.,* 1998). Conversely, reports assessing CRF mRNA concentration in response to high fat feeding are rare. In chapter 4, I showed that CRF mRNA levels are significantly reduced in chow fed SLOB males compared with normal males, however the chow fed animals in this study did not show the same result. Animals in this study were 128 days of age at the time of culling and collecting brains, in the previous CRF analysis, brains were from animals culled at 365 days of age. The difference in CRF mRNA expression in SLOB males may therefore be attributable to differences in age of animals or severity

of the phenotype. The age/phenotype-dependence seems likely as in young high-fat fed SLOBs I have induced the phenotype seen in old chow-fed SLOBs and likewise both these groups show the same reduced CRF mRNA levels.

This age difference was not seen in arcuate NPY mRNA expression. NPY levels were reduced in 128 day old chow-fed SLOB males as in older chow-fed SLOB males. The reduction in NPY mRNA expression in high fat fed normal and *dwarf* rats could account for the reduction in cumulative food intake. As NPY is a potent stimulator of food intake, and as leptin reduces NPY levels (Gehlert and Heiman, 1997; Gehlert, 1999), in high fat fed normal and *dwarf* rats plasma leptins are elevated, arcuate NPY expression reduced and consequently food intake reduced. However, in chow fed SLOB males, already exhibiting elevated leptins, reduced NPY levels, and a normal food intake; the challenge of a high fat diet although increasing plasma leptin levels further, has no effect on NPY expression or food intake. These results indicate that normal and *dwarf* males are obesity resistant because a high fat diet produces a compensatory anti-obesity effect in them which favours increased energy expenditure and decreased energy storage. However, in SLOB rats elevated plasma leptin levels have no effect on the already blunted arcuate NPY mRNA levels and thus cumulative food intake remains high, again suggesting the presence of leptin resistance in high fat fed SLOB rats. This demonstrates that impaired hypothalamic compensation in response to a high fat diet in SLOB rats is associated with increased weight gain and obesity. Similar results have also been observed in other rodent obesity models. C57BL/6J mice like SLOB rats develop obesity when fed a high fat diet and show no reduction in hypothalamic NPY mRNA levels (Bergen *et al.,* 1999). Whereas, in obesity resistant A/J mice like normal and *dwarf* males, high fat feeding does not cause obesity and causes reduced NPY mRNA levels (Bergen *et al.*, 1999).

As expression levels of *tub* mRNA in the paraventricular and dorsomedial nuclei were only altered in high fat fed *dwarf* rats and not in high fat fed SLOB or normal rats, suggests *tub* expression may in part be regulated by GH. Although the SLOB rat is also GH-deficient, it is not as deficient as the *dwarf* rat. Also, SLOB rats are not GH-deficient centrally, that is, in the brain they actually exhibit excess GH from hGH from the transgene, but peripherally they display GH-deficiency. Whereas *dwarf* rats, are both centrally and peripherally GH-deficient, and this central deficiency may be

involved in *tub* mRNA expression. Little is known about *tub* function as yet, until more data is available and until further studies assessing *tub* mRNA expression in the *dwarf* rat are conducted, the nature of these results remain unresolved.

5.6.4 Effect of food restriction in SLOB males

As mentioned earlier, long-term food restriction has little effect on body composition in mature *ob/ob* mice (Alonzo *et al.*, 1955; Dubuc *et al.*, 1976b) and although skeletal and lean growth is severely limited, mice continue to exhibit high rates of fat deposition (Dubuc *et al.*, 1983). Also in the *Zucker* rat prevention of hyperphagia throughout life by continuous food restriction has no effect on production of the complete obese phenotype (Cleary et al., 1980). Both these rodent models of obesity exhibit severe hyperphagia, which is not evident in obese SLOB males. Food restriction in SLOB rats may therefore have caused a different result to that in *ob/ob* and *Zucker* rats. Food restriction in obese SLOB males was found to cause a significant decrease in bodyweight accompanied by a decrease in plasma leptin levels and fat pad weight. *Ad libitum* SLOB animals also lost weight initially but this effect was not significant by the end of the study. As discussed earlier, the loss in weight initially observed in SLOB rats may have been due to reduced mobility. Obese SLOB rats have larger abdominal circumferences compared to normal rats (abdominal circumference in male 2 year old rats; SLOB 348±13.8mm vs normal 281.7±9mm $p<0.003$). Thus these animals when confined to a small area such as a metabolic cage may exhibit reduced freedom of mobility and increased housing stress. Although this experiment was only carried out once, my results do indicate that adult food restriction in SLOB rats, does cause a significant reduction in bodyweight and body composition. The results for SLOB rats therefore resemble those seen when humans are food restricted (Danforth, 1985). In terms of body composition and adipose tissue depot, the greatest loss was observed in the supra–renal fat pad of food restricted SLOB rats, whereas normal animals showed a significant loss in all three adipose tissues depots measured. As plasma leptin levels were significantly reduced in food restricted SLOB animals this suggested the central supra-renal fat depot as a large contributory factor to circulating leptin levels. It would therefore appear that, SLOB rats utilise energy from their major adipose store, the supra-renal fat pad, and this is

reflected in a severe reduction in bodyweight, leptin levels and supra-renal fat mass. It would be interesting to see if the effect of food restriction in SLOB rats also extends to the hypothalamus. That is, with reduced leptin and reduced obesity, does NPY mRNA expression increase in SLOB rats; this analysis remains to be performed.

5.6.5 Effect of gonadectomy and oestrogen treatment

Following ovariectomy, both SLOB and normal rats gained more weight than intact rats as has previously been shown for normal rats by other researchers (Goulding *et al.*, 1987, Wronski *et al.*, 1989). However, what was notable was that ovx SLOB females gained twice as much weight as ovx normal females which was also reflected in fat pad weight and plasma leptin levels. Thus ovariectomy in young (10 wks) SLOB females induced a severe obesity phenotype, which was otherwise prevented from developing until a much later age (after 1 year) by the presence of the ovaries. In order to assess whether it was the ovarian oestrogen that had a protective role in the development of the severe obese phenotype in SLOB females, I tested whether the weight gain in ovx SLOB females could be prevented by the concomitant treatment with oestrogen. In this second shorter experiment, I also measured fat pads and plasma leptin levels. As this experiment was only conducted for 5 weeks as opposed to 11 weeks as before, the effect of ovariectomy on weight gain in SLOB females was not yet greater than that in normal females. The reason for this shorter duration was because I was only interested in reversing the effect of ovariectomy using oestrogen whilst minimising the stress from repeated oestrogen injections. However, even after 5 weeks, plasma leptin levels were significantly greater in ovx SLOB females compared to ovx normal females. In this second experiment, I decided to try to measure another fat pad, the axillary fat pad, instead of the ovarian fat pad as was first measured. This was because the ovarian fat pad is located in the abdominal region alongside the supra-renal fat pad, I wanted to assess the development of obesity in terms of fat pad weight in an anatomical location other than abdomen. For this reason I chose the axillary fat pad as it was relatively easy to dissect out. Although supra-renal fat pad weights in ovx SLOB females by the end of 5 weeks were not yet greater than ovx normal females, ovariectomy did cause a significant increase in the axillary fat pad of SLOB females compared to normal females. Thus, unlike in SLOB males

where obesity is apparent most predominantly in the abdominal supra-renal adipose depot, results from this study showed obesity in SLOB females was evident in both central and peripheral adipose stores. Ovariectomy and oestrogen treatment have also been carried out in lean and obese *Zucker* rats (Gale and Van Itallie, 1979). In this study, ovariectomy caused overeating and similar weight gains in both genotypes, however subsequent oestrogen treatment completely reversed ovarian obesity in lean animals but failed to alter food intake or weight gain in the fatty rats. Therefore, unlike in ovx SLOB females where oestrogen replacement reduces weight gain, obese *Zucker* rats continue to gain weight.

I did not record food intake in this study but registration of food intake in other studies show ovariectomised rats have an increased food consumption when allowed to feed *ad libitum* (Matsumoto *et al.*, 1985). This and a more pulsated GH secretion pattern which is also observed (Gevers *et al.*, 1995; Gevers *et al.*, 1998) would explain the increased bodyweights in ovx females. However, even with controlled food intake ovariectomised rats gain more weight than control rats (Izawa *et al.*, 1985; Wronski *et al.*, 1988; Wronski *et al.*, 1989). This observation indicates that ovariectomised rats have better food efficiency or lower physical/metabolic energy consumable than control rats. Further studies assessing food intake in ovx SLOB and normal rats are necessary and would indicate if gain in weight in ovx SLOB females is purely as a result of hyperphagia. However, analyses of neuropeptides involved in feeding behaviour may give some clues to food intake in ovx SLOB females, these are discussed here.

Administration of oestrogen to either sham operated or ovx rats caused an increase in arcuate NPY mRNA expression when compared to intact groups, as has previously been reported (Bonavera *et al.*, 1994). Ovariectomy was found to cause a significant decrease in NPY mRNA levels, in both SLOB and normal females. This result corresponds to elevated plasma leptin levels, which decreases arcuate NPY mRNA expression (Schwartz *et al.*, 1996a). Subsequently, this should cause a decrease in food intake, and thus does not agree with the results of Matsumoto *et al.*, (1985). These results together with the finding that NPY neurones in the arcuate possess oestrogen receptors (Sar *et al.*, 1990), suggest that oestrogen could have direct effects

on NPY biosynthesis in the arcuate nucleus and subsequently may contribute to the induction of a severe obese phenotype in young SLOB females and additionally the sex-specific phenotype. Although analysis of the number of oestrogen receptors in male and female adipose tissue has been assessed (Gray and Wade, 1980), gender differences in oestrogen receptor concentration in the rat arcuate nucleus have not yet been reported. However, Scott *et al.,* (2000) recently reported gender differences in oestrogen receptor α and β (ERα and ERβ) mRNA in the hypothalamus of sheep, where they found an increased ERα mRNA expression in the arcuate nucleus of male sheep.

Previously, oestrogen and ovariectomy have been shown to cause a decrease in CRF synthesis in the median eminence of normal rats (Haas *et al.,* 1989). I did not observe any differences in hypothalamic paraventricular CRF mRNA expression in ovariectomised or oestrogen treated animals compared to intact or untreated animals. This is in concordance with other reports. Redei *et al.,* (1994) measured CRF mRNA expression in the PVN of mature ovx and oestrogen treated rats and found no effects. In addition, Broad *et al.,* (1995) assessed CRF mRNA expression in oestrogen treated and progesterone treated ewes, and again found no changes. However, as in SLOB males I did find that CRF mRNA expression in the PVN was significantly reduced in intact SLOB females compared to normal intact females ($p<0.001$). Therefore, PVN CRF mRNA expression in both male and female rats shows a similar reduction, and unlike NPY, this CRF mRNA expression is independent of oestrogen. It is interesting to note females used to measure CRF mRNA expression in this study were approximately 105 days of age. Previously, I discussed that CRF mRNA expression in SLOB male rats 128 days of age was not significantly reduced but was reduced in male rats of 365 days of age. Therefore, unlike in SLOB males, CRF mRNA expression is already reduced in young SLOB females, why this is the case remains to be determined.

In addition to NPY and CRF, I also measured *tub* mRNA expression in the paraventricular and supraoptic nuclei of intact and ovariectomised, oestrogen treated and untreated, normal and SLOB females. No differences were found between any of the SLOB groups or normal groups. This is the first time *tub* mRNA expression has

been assessed in ovx and oestrogen treated females in any species. Since significant differences were not found, these data show *tub* mRNA expression is not affected by ovariectomy or oestrogen treatment in SLOB and normal females used in this study. However, in chapter 4 I reported a sex-specific *tub* mRNA expression in normal rats in the paraventricular nucleus, my results in this chapter suggest oestrogen may not be responsible for this difference.

As ovariectomy caused a significant increase in bodyweight gain in SLOB females and this effect was prevented by the administration of oestrogen, I decided to assess the effects of gonadectomy and oestrogen treatment in male SLOB rats. Gonadectomy had little effect on the obesity phenotype in SLOB males; they were able to sustain their weight gain, whereas normal males showed a significant loss in weight by the end of the study. Already gonadectomy is known to inhibit gain in weight and longitudinal growth in male rats and this inhibition is overcome by administration of testosterone (Scow, 1952; Jansson *et al.,* 1983). Oestrogen treatment caused a similar effect in both normal and SLOB males. SLOB males lost weight and showed reduced fat pad weights and plasma leptin levels, however all these effects were also observed in normal rats. Oestrogen is known to reduce food intake and inhibit growth in male rats (Dubuc, 1976c), unfortunately I did not measure food intake in these animals. However, I concluded that oestrogen treatment in SLOB males did not have an obesity prevention effect independent of the effect seen in normal rats. Therefore although oestrogen protects SLOB females from developing obesity, it did not prevent obesity in SLOB males in this study. In order to strengthen this argument, it will be necessary to carry out the long-term oestrogen treatment of SLOB males from weaning, this could be achieved by choosing a much lower dose of oestrogen. Alternatively, sex-reversal experiments in both male and female SLOB rats from birth and then analysing the development of an obese phenotype would also indicate what role sex steroids have in the SLOB phenotype.

5.7 Summary

GH treatment in the GH-deficient male SLOB rat does not abolish the obese phenotype and thus GH deficiency is not the cause of obesity in these animals. High fat feeding in young male and female SLOB rats induces an obesity phenotype resembling that of old SLOB rats. High fat feeding in SLOB males has no effect on arcuate NPY mRNA levels, and may contribute to the acceleration of the obese phenotype. Food restriction in SLOB rats causes them to loose weight rapidly at the same rate as food restricted normal rats. Severe obesity is induced in young ovariectomised female rats and is prevented by oestrogen treatment. Arcuate NPY mRNA analysis in these animals show ovariectomy causes a reduction in mRNA expression. Gonadectomy does not prevent obesity in SLOB males and oestrogen treatment has a similar effect to that in normal males.

This chapter has shown various manipulation induce a severe obesity phenotype in both male and female young SLOB rats. Some studies have also indicated clues to the nature of the sex-specific phenotype, but the mechanisms for these are far from known. In the next chapter I present data on genetic analyses, and further characterisation and manipulation of another line of transgenic rats (JP59) generated with the same transgene construct as is present in SLOB rats.

Chapter 6

Genetic Studies and Analysis of the SLOB Transgene

6.1 Introduction

Epidemiological studies indicate that 30-70% of the variation in bodyweight in humans is determined by genetic factors (Stunkard *et al.,* 1990; Carmelli *et al.*, 1994; Barsh *et al.,* 2000), and several studies have used whole-genome scanning methods to localise the common predisposing genes (York and Hansen, 1998; Levine and Billington, 1998). Monogenic mouse obesity models have been an important resource in studies of obesity and have led to the suggestion of several candidate genes for evaluation in human populations (Echwald, 1999). Both quantitative trait locus (QTL) mapping and positional cloning techniques have been successfully applied to the search for these murine obesity genes (Fisler and Warden, 1997). Rapid recent progress in this area has been aided by detailed knowledge of the correspondence between mouse and human genomic maps. However, although molecular cloning of mutations such as obese (lep^{ob}), diabetes ($Lepr^{ob}$), fat (Cpe^{fat}), agouti yellow (A^{y}), and tubby (*tub*) have identified novel genes or metabolic pathways involved in the development of the obese phenotype, and corresponding cases have been found in the human population, these are exceedingly rare (Carlsson *et al.,* 1997; Farooqi and O'Rahilly, 2000). Thus the search for genes involved in the complex obesity syndrome continues.

As the SLOB rat is a transgenic model of obesity and the phenotype is invariably associated with the presence of the transgene, I have some certainty that the presence of this altered genome should ultimately lead to the abnormality primarily responsible for its unique phenotype. When a transgenic model is generated and an unexpected phenotype results, there are two principle reasons that could explain the outcome. First, the inserted transgene in whole or in part, may have disrupted or otherwise affected the function of genes in the surrounding host DNA into which the insert has integrated. As the percentage of coding DNA comprising genes is very low, the chances of this happening are rare, but it is still a possibility. Thus, the unexpected phenotype reflects the alteration of host DNA, and need not necessarily be related to any expressed product from genes present in the transgene DNA. Second, it is possible that known or unknown transgene products are responsible for the

phenotype. The construction of transgene DNA fragments frequently involves minor alterations in flanking sequences (e.g the removal or insertion of restriction sites) designed to aid manipulation of the DNA fragments during the construction of the intended transgene. However, such innocuous alterations may have major functional consequences if they are introduced in DNA sequences that are later recognised to form part of additional genes present in the transgene. These mutated products from genes lying unrecognised in the transgene, may then be responsible for the development of the unexpected phenotype. In this case it is necessary to analyse the uncharacterised sequence of the transgene which provides a direct means of discovering such unrecognised genes. Of course it is also possible that unexpected phenotypes arise as a result of a combination of both insert and host DNA sequences at the site of insertion.

Work on the SLOB rat within the Robinson lab involves the efforts of several scientists. Dr Paul Le Tissier at the NIMR is an expert in positional cloning and has carried out genetic analysis for both the possibilities discussed above. That is, studies towards the identification of the site of integration of the SLOB transgene construct (cVO14) in the rat genome, and a full characterisation of the cosmid insert. This led him to the identification of a novel gene *5'OT-EST*. In order to understand work carried out on this gene, I will briefly summarise his findings in section 6.2. This will explain the background to my expression studies of *5'OT-EST*, physiological characterisation of a new transgenic mouse line and another line of transgenic rats (JP59) who also exhibit the same transgene as SLOB rats.

6.2 Identification of the novel gene *5'OT-EST*

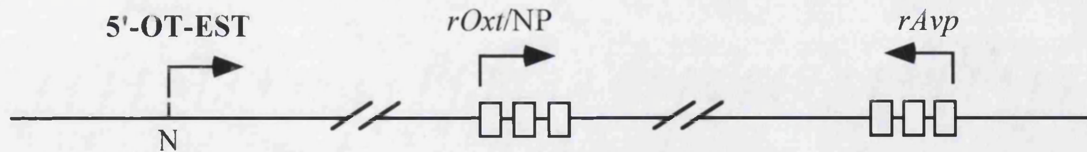
The original construct made by Wells (1997) used to generate SLOB rats was further characterised by Dr Le Tissier. He identified a novel gene present in the construct which he called *5'OT-EST* because its location lay 5'of the oxytocin (OT) gene in rat DNA and in the transgene, and because partial matches to DNA sequences homologous to this gene were found in expressed sequence tag (EST) databases from rat, mouse and human DNA sources. This gene in normal rat DNA was found to

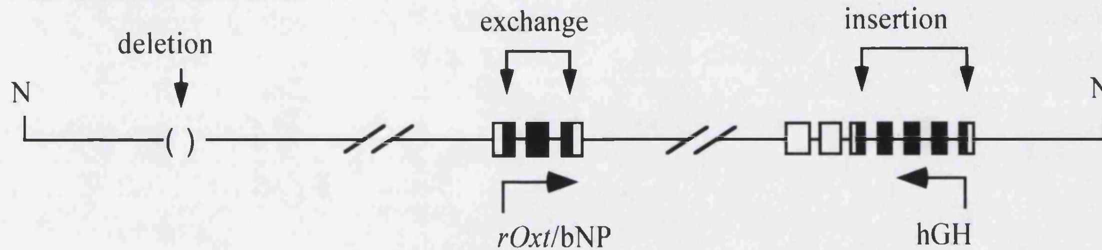
consist of four exons (w-z) encoding a 200aa residue protein. Complete sequencing of the transgene construct revealed no other novel genes.

During the construction of the SLOB construct, a *Not*I site was present in the construct far upstream of the OT gene that was removed to facilitate excision of the construct as a *Not*I fragment for microinjection. Unknown at the time, this *Not*I site was actually present in exon w of *5'OT-EST*. Dr Le Tissier discovered that removal of this site had caused a 412bp deletion downstream of the *Not*I site, and had removed part of exon w, intron 1, all of exon x and part of intron 2 (**Figure 6.1**). Therefore the transgene construct now contained a truncated version of the novel gene, *5'OT-EST*. Transgenic SLOB animals therefore had additional copies of a mutated form of *5'OT-EST* predicted to produce a peptide of only 41aa. Furthermore, as SLOB rats have at least four copies of the transgene, assuming these are expressed, then rats could be expressing from at least four copies of this truncated *5'OT-EST*. Mouse and human homologs of *5'OT-EST* (**Figure 6.2**) were deduced by reconstruction from sequence matches in the EST database and confirmed by sequencing of RT-PCR products.

6.3 Expression pattern of 5'OT-EST

With the sequence of this novel gene available, specific riboprobes were generated from these sequences by Dr Paul Le Tissier, and I used these to investigate the expression of both endogenous *5'OT-EST* and truncated *5'OT-EST* present only in transgenic SLOB animals. *In situ* hybridisation of hypothalamus showed specific localisation in PVN and SON (**Figure 6.3(a, b)**). This localisation is interesting and may imply that *5'OT-EST* shares common regulatory sequences with the other genes in the VP/OT locus (Young, 1992). Preliminary studies also suggested *5'OT-EST* and hGH from the transgene were co-localised in VP cells in the hypothalamus (courtesy of Dr Pamela Bennett, NIMR; Dr Helen Christian, Oxford University). *In situ* hybridisation also revealed *5'OT-EST* was expressed in the testes, probably in sertoli cells (**Figure 6.3(c, d)**). Oligonucleotide probes specific either for exon x or for the novel exon w' confirmed independently that both normal and truncated *5'OT-EST* transcripts were expressed in a similar pattern in SLOB rats (courtesy of Dr Pamela Bennett). A ribonuclease protection assay (RPA) detected variable levels of *5'OT-*





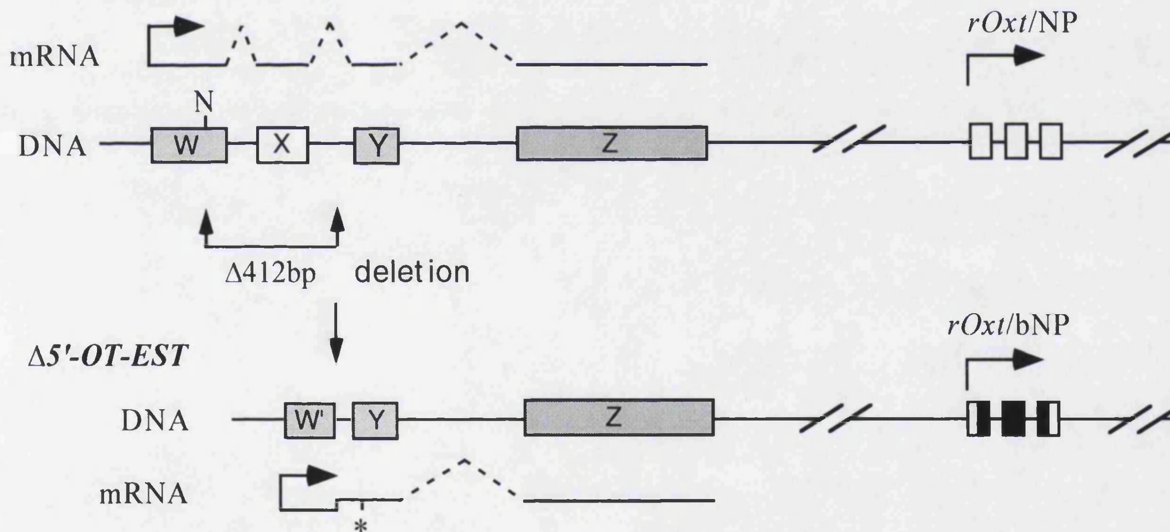


Figure 6.1 Organisation and structure of *5'OT-EST*, *Oxt* and *Avp* genes in the rat, and modifications introduced in the construction of cosmid cVO14 used to generate SLOB rats. Exons are indicated by boxes. *5'OT-EST* has four exons, w,x,y and z, during construction of the cVO14 construct, the removal of a *Not*I site (N) within this gene resulted in truncation (* in-frame stop codon).

(a) Rat, mouse and human *5'OT-EST* homologs

```
Rat    MLRALNRLAARPGGQPPTLLLLPVRGRKTRHDPPAKSKVGRVKMPPAVDPAELFVLTERY
Mouse  ---------Q---DR---P-----------------------QT--------F-------
Human  -----S--G-GT-CR-RAP-V--A---------L----IE--N---------F---M---

Rat    RQYRETVRALRREFTLEVRGKLHEARAGVLAERKAQEAIREHQELMAWNREENRRLQELR
Mouse  G----------L----D--R----------------Q--T--R-------D----M----
Human  QH--Q------M--VS--QR-V-------------LKDAA--R------QA-----H---

Rat    IARLQLEAQAQELRQAEVQAQRAQEEQAWVQLKEQEVLKLQEEAKNFITRENLEARIEEA
Mouse  ------------VQK--A-R----------------------------------------
Human  ----RQ--RE--Q---LE--RK-E-V---A-R--R---Q----V------------V---

Rat    LDSPKSYNWAVTKEGQVVRN          100%
Mouse  --------------------           90%
Human  ---R-N----ITR--L---PQRRDS      70%
```

(b) Amino acid sequence of truncated *5'OT-EST*

```
ATGTTGCGGGCTTTGAACCGCCTGGCCGCGCGGCCCGGGGGCCAGCCCCCAACCCTGCTCCTTCTGCCC
MetLeuArgAlaLeuAsnArgLeuAlaAlaArgProGlyGlyGlnProProThrLeuLeuLeuLeuPro

GTGCGCGGCCcacggccccgctcattctcggctcctttttcctcgcagGATAGCTAGGTTGCAGCTCGA
ValArgGlyProArgProArgSerPheSerAlaProPheSerSerGlnAspSer*
```

Figure 6.2 (a) Predicted amino acid sequences of *5'OT-EST* in rat, mouse and human. Percentage homology with the rat are shown. (b) Truncated region of *5'OT-EST* in cVO14 and predicted amino acid sequence of its product. Upper case indicates original exon sequences. The novel exon is predicted to code for the first 25 amino acids of the normal sequence followed by a novel 16 amino acid sequence. Translation is terminated by nucleotides 7-9 of the original exon Y which now form an in-frame stop codon (*).

EST expression in many other tissues including heart, lung, liver, muscle, kidney, spleen, gut, adipose tissue, and adrenal glands. I produced RNA extracts for these tissues which were then used in the RPA assay conducted by Dr Le Tissier. **Figure 6.4** shows nuclease protection on heart, lung and testis from normal and SLOB male rats. In addition, Dr Le Tissier also carried out RT-PCR and sequencing which confirmed endogenous *5'OT-EST* transcripts were present in testicular RNA from normal rats, and both the endogenous and the predicted truncated mRNA sequences were present in SLOB rat testes. Dr Babis Magoulas generated an antibody for *5'OT-EST* which was used for Western blotting. He confirmed *5'OT-EST* protein expression was also detected in a wide range of tissues including hypothalamus, heart, testes, kidney and liver.

The hypothalamic expression of *5'OT-EST* prompted me to speculate whether this gene might be involved in the regulation of feeding behaviour. In order to assess this I carried out *in situ* hybridisation on brains from two groups of normal Wistar males. One group had been fed a normal chow (4% fat) diet and the other group fed a high fat (60%) diet (n=6). Animals were 6 months old at the start of the experiment which was conducted for 4 weeks after which brains were collected for *in situ* analysis. **Figure 6.5** shows high fat feeding caused a significant increase in *5'OT-EST* mRNA expression in the PVN and a similar change in the SON, but this did not reach statistical significance, probably due to a much larger variance. These results suggested that *5'OT-EST* might have a role in, or be affected by changes in food intake or metabolism. However, further studies have yet to be conducted. It is also not yet known if *5'OT-EST* is altered in response to stimuli affecting VP and OT, such as dehydration and suckling.

6.4 Do JP59's have an obesity phenotype?

I mentioned in chapter 2 (2.1.1) that when Wells (1997) originally generated transgenic rats expressing the hGH reporter gene, three (2 male and 1 female) transgenic founder rats were produced. One of these died, the male was used to generate the SLOB line and the remaining female was used to generate the JP59 line of rats. In her thesis, Wells (1997) also concluded that SLOB rats had at least four

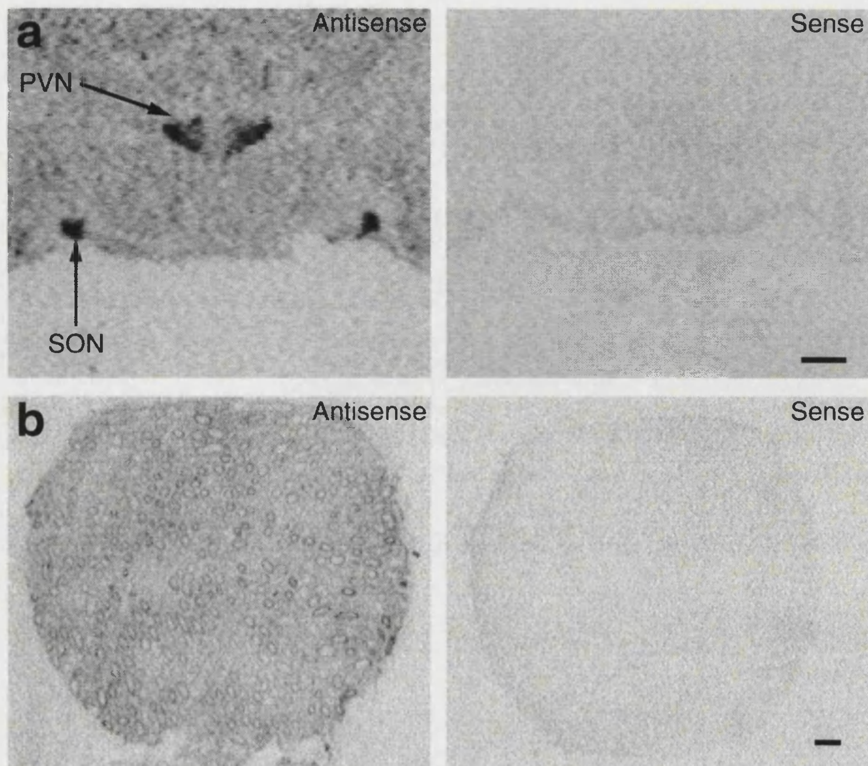


Figure 6.3 ***In situ*** **hybridisation analysis of *5'OT-EST* in normal rats.** *5'OT-EST* is expressed in (a) hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei, and in (b) testes. Riboprobe detects endogenous *5'OT-EST* expression, size bar represents 5mm.

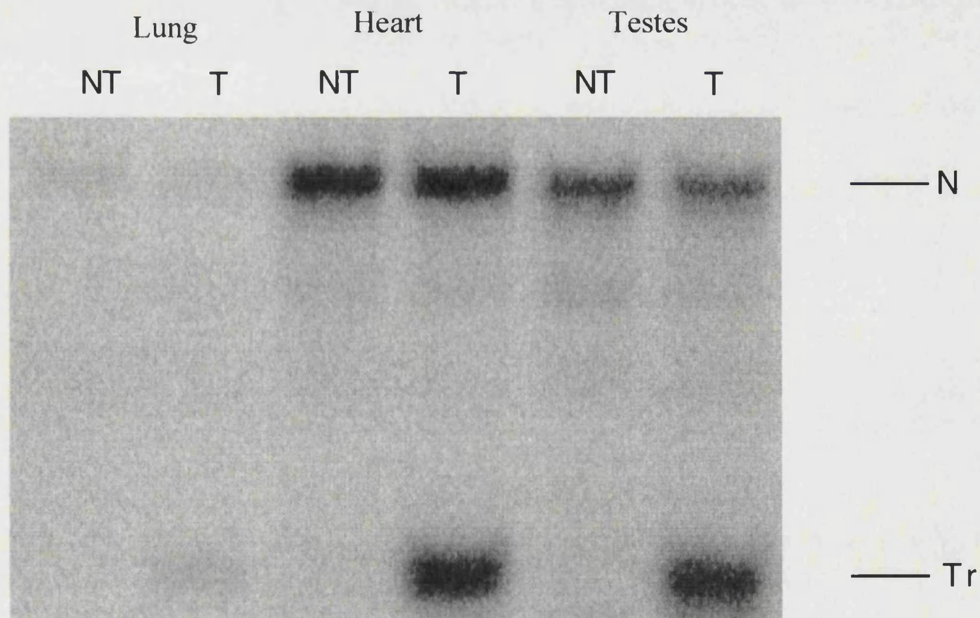


Figure 6.4 **Ribonuclease protection assay of RNA extracted from lung, heart and testis of normal (NT) and SLOB transgenic (T) rats.** The exon w probe used is predicted to yield protected fragments of 147 and 84nt from normal (N) and truncated (Tr) *5'OT-EST* transcripts respectively. Given the difference in size of protected fragments, the band intensities suggest a two -fold excess of transgene expression, consistent with four hemizygous transgene copies in SLOB rats.

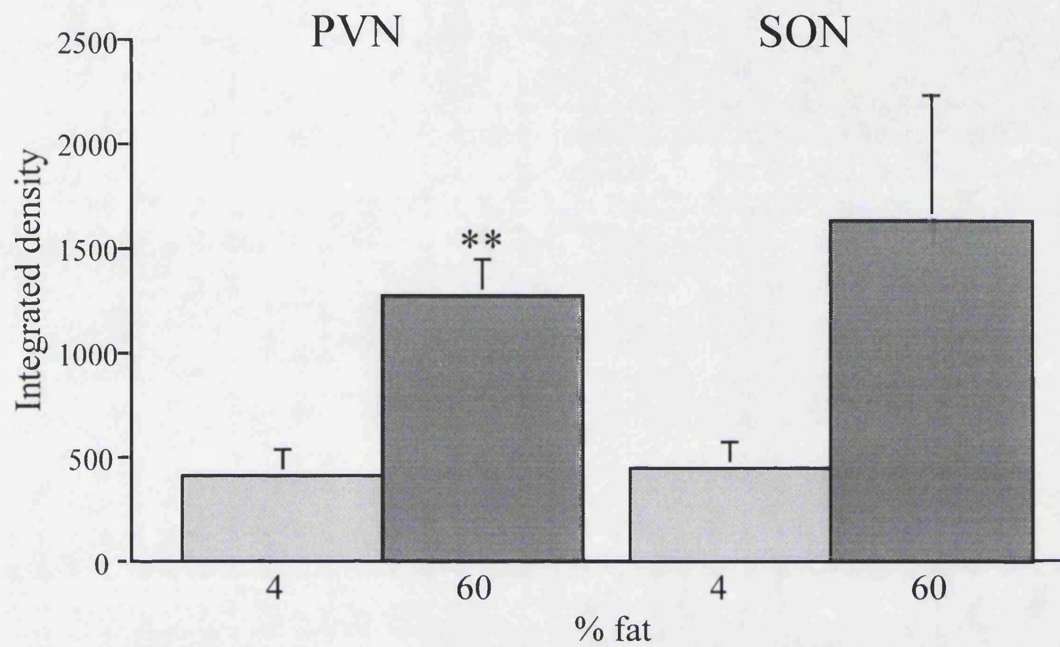


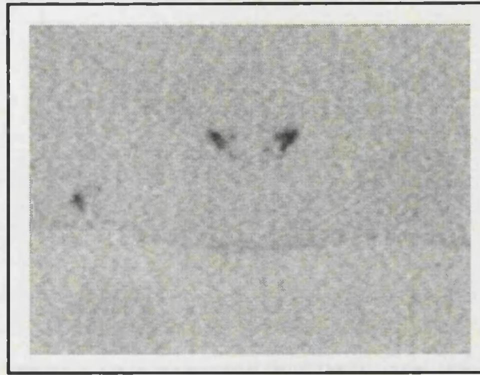
Figure 6.5 ***In situ*** **hybridisation analysis of** ***5'OT-EST.*** Normal Wistar males were fed chow (4% fat) or high fat (60%) diet for 4 weeks. Shown above is the mean integrated density of 5'OT-EST measured in 6 animals per group in the PVN and SON (**$p<0.01$).

copies of the transgene, whereas JP59 rats only had a single copy. Initial studies on JP59 rats carried out by Wells (1997) suggested no obvious obesity phenotype. Since the identification and analyses of the novel gene *5'OT-EST* and in view of the fact JP59 rats have only 1 copy of the transgene, I wondered whether generating a homozygous line of JP59 rats to double the copy number would reveal an obesity phenotype similar to that in SLOB rats. This was possible because unlike SLOB males, JP59 males are fertile at all ages. Presented here are my studies to investigate more closely whether hemizygous JP59 and recently generated homozygous JP59 rats show a SLOB phenotype. Such data would then reveal whether it is more likely the SLOB phenotype is due to transgene (including *5'OT-EST*) products or if it is more likely to be due to where the transgene construct has inserted into the SLOB rat genome, which is extremely unlikely to be the same in two independent transgenic lines.

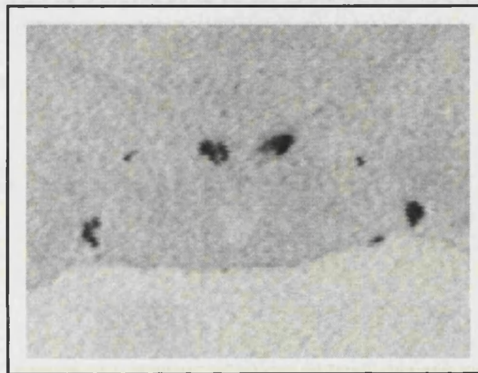
6.4.1 Copy number and hGH expression in homozygous JP59 rats

I used Southern blotting and phosphor-imaging techniques as described in chapter 2 (2.2.2) in order to identify homozygous pups. Homozygosity was also confirmed in these animals by backcrossing with wildtype Wistar mates. When a complete litter of transgenics, typed by PCR, was all hemizygous, homozygosity in the parent animal was confirmed. As well as using Southern blotting, to check the transgene had successfully incorporated into homozygous animals, I also carried out *in situ* hybridisation for hGH. Normally, hGH mRNA is expressed strongly in the PVN and SON of SLOB rats, in hemizygous JP59 rats the hGH expression is much lower than in SLOB animals but is still evident. As homozygous JP59 rats have double the copy number compared to hemizygous JP59 rats, I would therefore expect hGH expression to be increased in homozygotes, but still to be lower than in SLOB rats. **Figure 6.6** shows *in situ* hybridisation for hGH carried out on hemizygous JP59, homozygous JP59 and hemizygous SLOB brains. As expected hGH mRNA expression was highest in SLOB rats and lowest in hemizygous JP59 rats. Therefore I concluded copy number and transgene expression was increased in homozygous JP59 rats compared to hemizygous JP59 rats and therefore assumed *5'OT-EST* expression was also increased in homozygous JP59 rats.

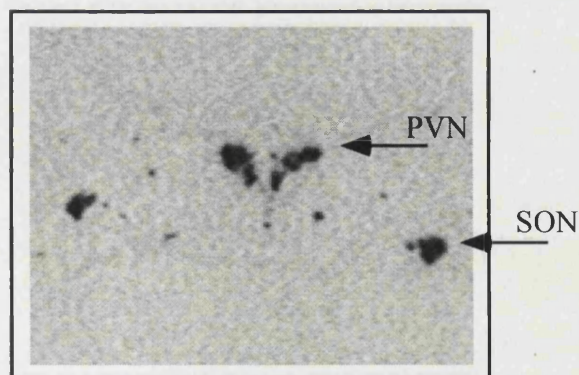
Hemizygous JP59



Homozygous JP59



Hemizygous SLOB



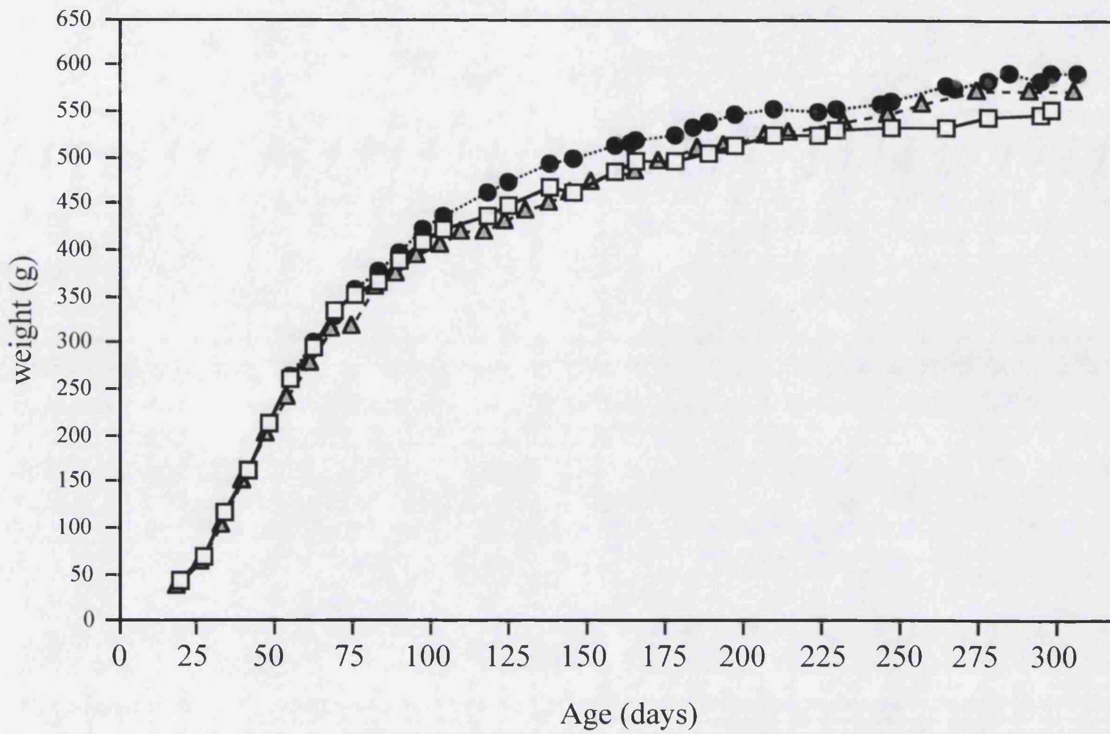
<u>Figure 6.6</u> *In situ* hybridisation for hGH. (a) Hemizygous JP59 rat, (b) homozygous JP59 rat, (c) hemizygous SLOB rat. Shown is hGH expression in the paraventricular (PVN) and supraoptic (SON) nuclei in brains from male rats, approximately 11 months of age.

6.4.2 Comparison of normal, hemizygous and homozygous JP59 rats

Hemizygous JP59 animals bear a single copy of the transgene and homozygous animals bear two copies, but still half the number in SLOB rats. If the result of the phenotype, as with hGH hypothalamic expression, was an effect of gene-dosage of the insert, a phenotype that might be mildly expressed in hemizygous JP59's should be more prominent in homozygous JP59's.

I studied groups of male and female normal, hemizygous JP59 and homozygous JP59 rats (normal male n=9, normal female n=9, hemizygous male n=7, hemizygous female n=7, homozygous male n=11, homozygous female n=9) for 10 months. During this time, these animals were weighed weekly from about 3-15 wks of age and then every 2-3 weeks thereafter. All animals were culled at 300 days of age, measurements recorded and blood taken for analysis. Growth curves were constructed for these animals and are shown in **Figure 6.7**. No significant differences were seen in growth in either male or female normal rats compared to hemizygous and homozygous JP59 rats. At 300 days of age the mean bodyweight in each group showed no difference between normal, hemizygous or homozygous animals (male: normal 552.5±18.3g, hemizygous 578±17.1g, homozygous 565.7±19.5g; female: normal 305.2±5.5g, hemizygous 321±7.7g, homozygous 315.3±7.8g). Thus in terms of overall bodyweight, up to 300 days of age, JP59 rats did not develop signs of overt obesity.

As previously found by Wells (1997), SLOB rats are GH-deficient, which she ascribed to a local negative feedback from transgene hGH in the hypothalamus. As a result, pituitary rGH levels are low in SLOB rats. Although my growth curves do not show JP59 rats are growth retarded, it is possible pituitary rGH levels may still be somewhat reduced in JP59 rats and if this is true may not be diminished enough to cause an overall retardation reflected in reduced bodyweight. If the result of establishing homozygosity acts as a gene-dosage effect of the hGH component of the transgene, I would also expect rGH levels might be reduced to a greater extent in homozygous animals compared to hemizygous animals. I found that rGH levels were



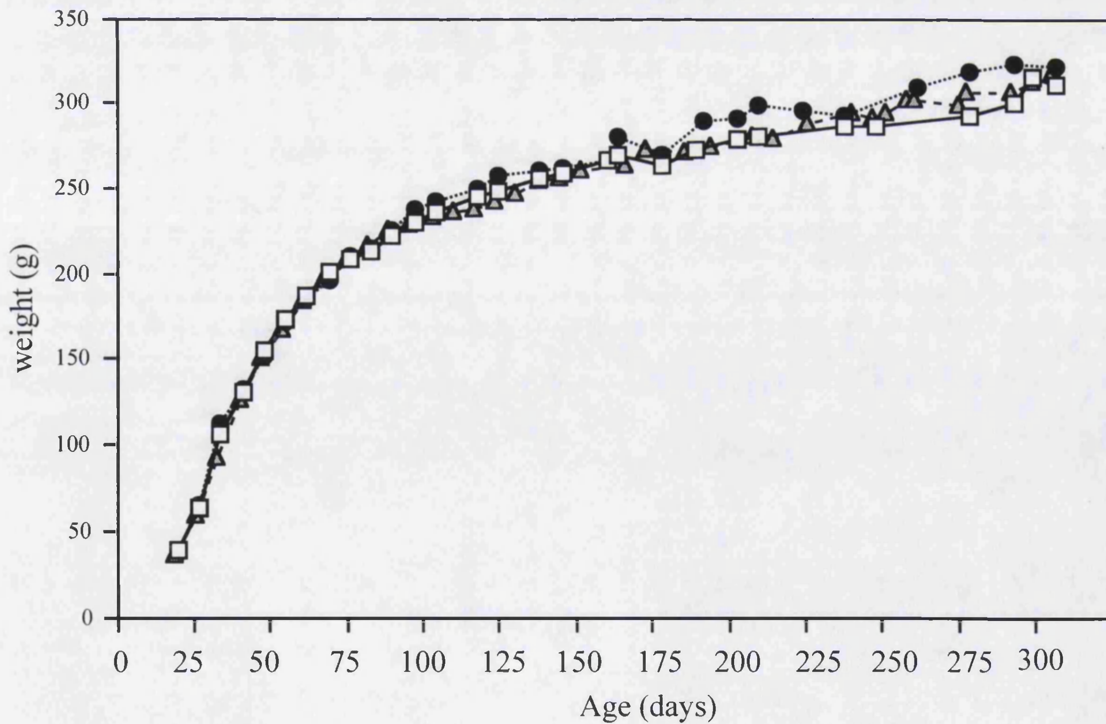


Figure 6.7 Growth curves for normal, hemizygous and homozygous JP59 male and female rats. Animals were weighed at least every 3 weeks from weaning to 42 wks of age (n=7-11). —□— Normal ····●···· JP59 Hemizygous - - -Δ- - - JP59 Homozygous

not significantly reduced in male hemizygous JP59 rats, but significantly so in homozygous male rats (**Figure 6.8(a)**). However, in females, rGH levels were significantly reduced in hemizygous animals but no further reduction was seen in homozygous animals (**Figure 6.8(b)**). These effects are much less than those seen in SLOB male and female rats as reported by Wells (1997).

The effect of increasing the magnitude of GH-deficiency in homozygous animals is also reflected in muscle weights. In both male and female homozygous JP59 rats, soleus muscle weight was significantly reduced compared to normal animals, but soleus muscle weight was not significantly reduced in either hemizygous male or female rats compared to normal animals (**Figure 6.9**). However, this mild GH-deficiency did not effect spleen weight in either male or female JP59 hemizygous or homozygous animals compared to normal animals.

From these results I concluded there did appear to be a gene dosage effect in JP59 rats related to the GH effects of the transgene. It would therefore be interesting to see whether an obesity phenotype might emerge in homozygous JP59 rats.

6.4.3 Do JP59's develop obesity?

Although bodyweight was unaltered in JP59 rats compared to normal rats, I found earlier that obesity markers such as plasma leptin levels and supra renal fat pad weights, are more sensitive than total body weight at assessing obesity. For example in chapter 3 I showed 80 day old SLOB male rats had reduced bodyweights, exhibited elevated plasma leptin levels and increased supra-renal fat pad weights. Similar measurements were therefore also taken in JP59 rats. Plasma leptin levels, supra renal fat pad weights and testicular/ovarian fat pad weights were measured in normal, hemizygous JP59 and homozygous JP59, male and female rats at 300 days of age (**Figure 6.10**). Plasma leptin levels were not significantly altered in male JP59 rats, but were significantly increased in female homozygous JP59 rats compared to normal rats. Supra-renal fat pad weight and ovarian fat pad weight were also significantly increased in female hemizygous and homozygous JP59 rats compared to normal females. As the ovarian fat pad is situated in the abdominal region, this increase

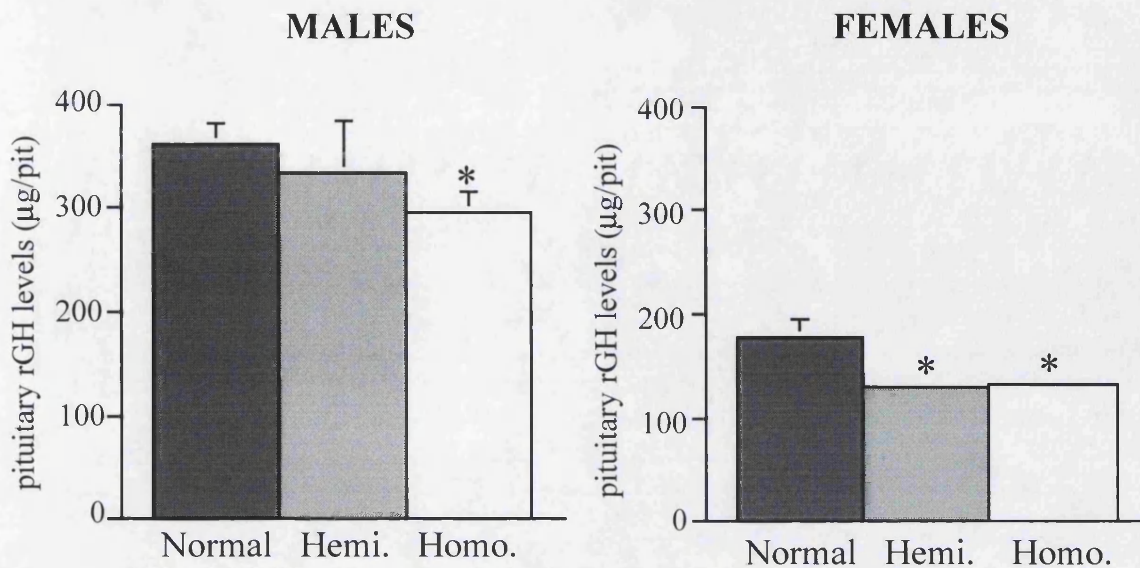


Figure 6.8 Pituitary rGH levels. rGH was analysed using radioimmunoassay, carried out on pituitaries taken from animals 300 days of age (n=7-11, *p<0.05).

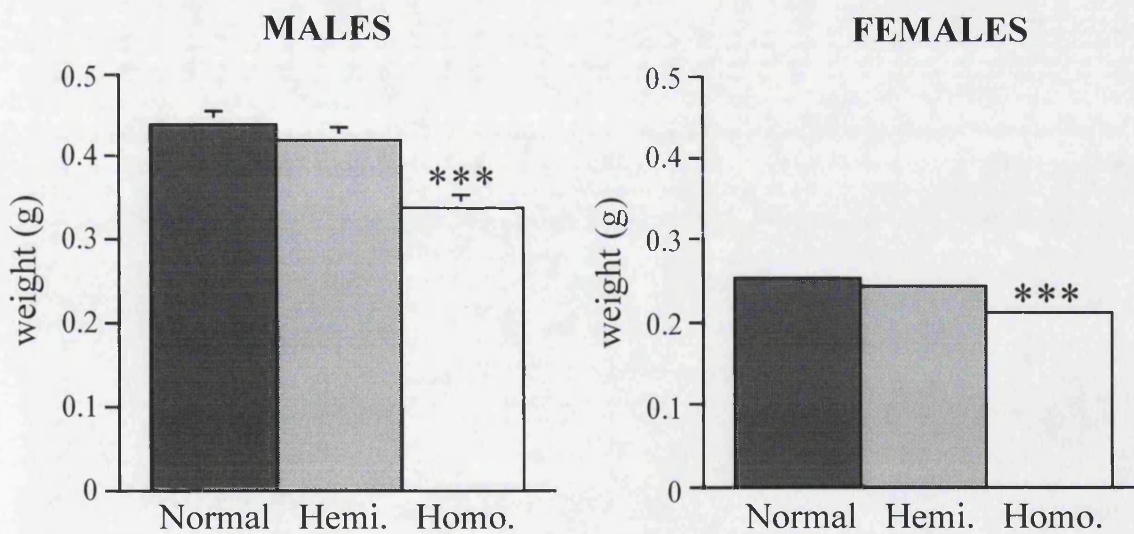
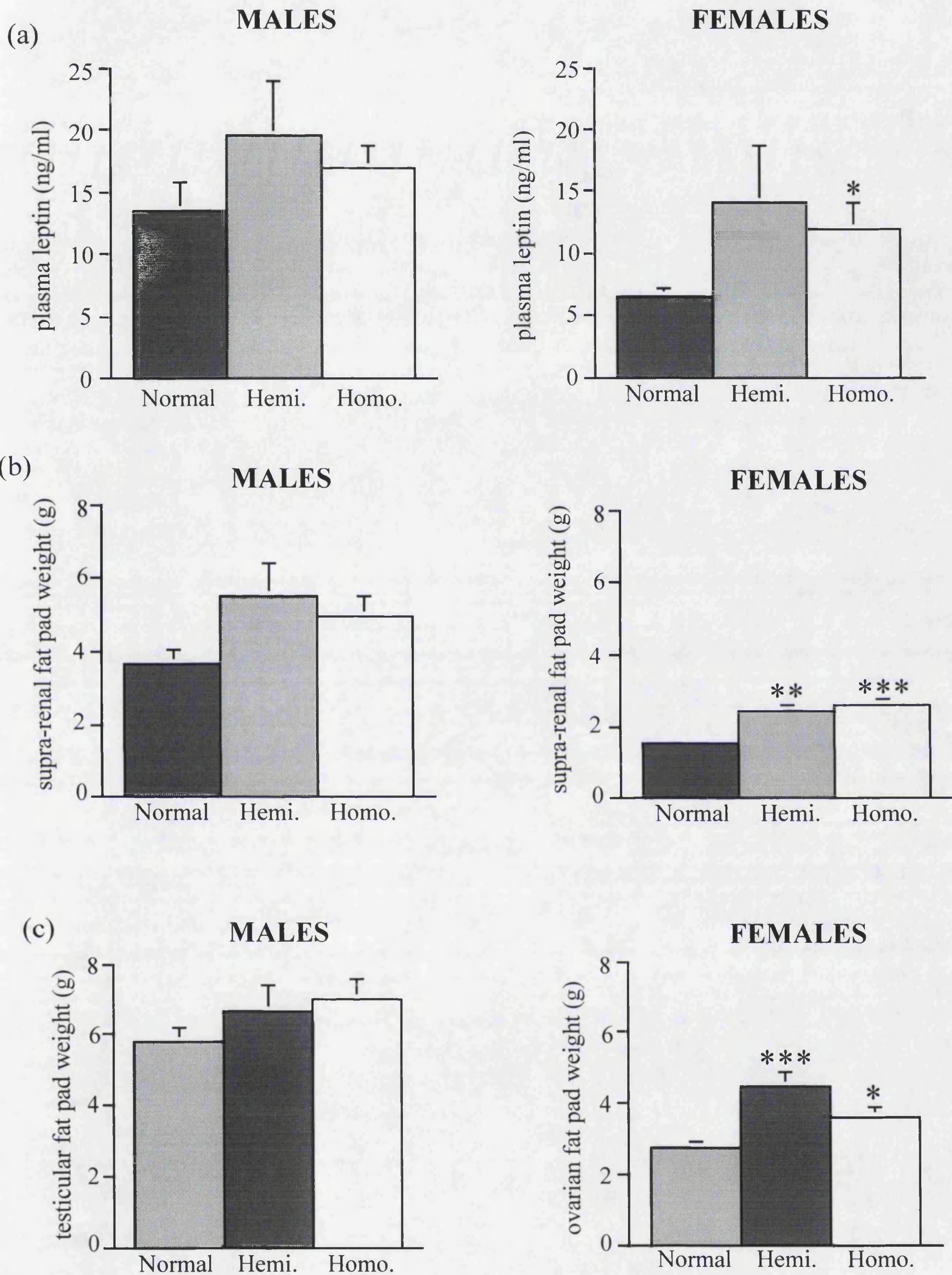


Figure 6.9 Soleus muscle weight. Measured in animals 300 days of age (n=7-11, ***p<0.001).



<u>Figure 6.10</u> Plasma leptin levels and fat pad weights. (a) Plasma leptin levels, (b) supra-renal fat pad weight, (c) testicular/ovarian fat pad weight. Measurements taken in animals at 300 days of age, significances are shown for hemizygous and homozygous animals compared with normal animals (n=7-11, *p<0.05, **p<0.01, ***p<0.001).

therefore contributes to emphasising visceral obesity. However, no significant differences were observed in any of the parameters measured in hemizygous or homozygous JP59 males compared to normal males. These results indicated that JP59 hemizygous and homozygous females were showing signs of obesity, whereas JP59 males were not. This was unlike SLOB rats, in which the obesity phenotype is predominant in males.

6.4.4 Effect of high-fat feeding male homozygous JP59 rats

In young SLOB males, high fat feeding (30% fat) rapidly induces an obesity phenotype like that seen in old SLOB males. I wanted to assess what effect high fat feeding would have in male homozygous JP59 rats and how this compares to the effects in SLOB males. I therefore decided to feed groups of young (100 day, n=6-7) SLOB, normal and homozygous JP59 male rats as previously described in chapter 5.

As expected, after 28 days on a high fat diet, all animals gained significantly more weight than chow (4% fat) fed controls (**Figure 6.11**). In the chow-fed groups, I found that JP59 rats gained significantly more weight than chow-fed normal animals, and as I found earlier, chow-fed SLOB rats did not gain significantly more weight than chow-fed normal animals. In addition, I also found high fat fed SLOB males gained significantly more weight than high fat fed normal males, but this was also true for JP59 males.

Food intake was also measured, and chow-fed JP59 rats consumed significantly more food over the 14 day food intake recording period, compared to chow fed SLOB and normal rats (**Figure 6.12**), which could explain the significant gain in weight seen in chow-fed JP59 rats. A change to a fat enriched diet should cause the rats to reduce the amount of grams consumed per day to compensate for higher calorie intake. This occurred in both JP59 and normal rats, but as reported in chapter 5, a reduction in food intake in SLOB rats was not observed. Although high fat fed JP59 males did reduce their food intake compared to chow fed JP59 males, they still consumed significantly more food than high fat fed normal and SLOB males did.

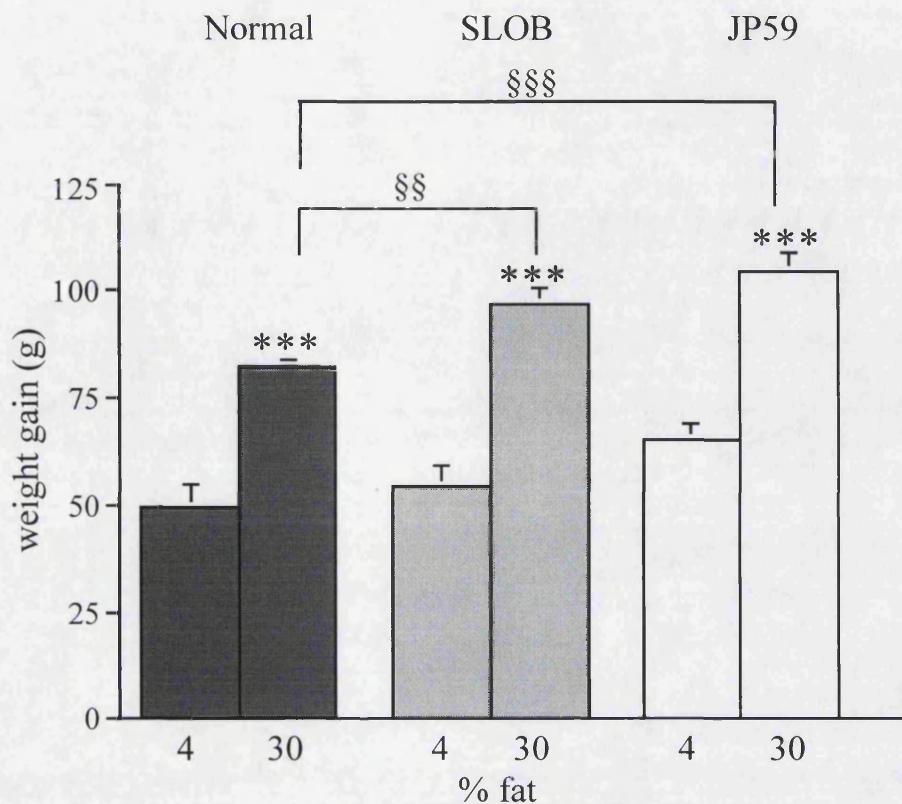


Figure 6.11 Bodyweight gain. Mean weight gain after 4 weeks on 4% or 30% fat diet in normal, SLOB and homozygous JP59 males. (n=6, §§p<0.01, ***/§§§p<0.001).

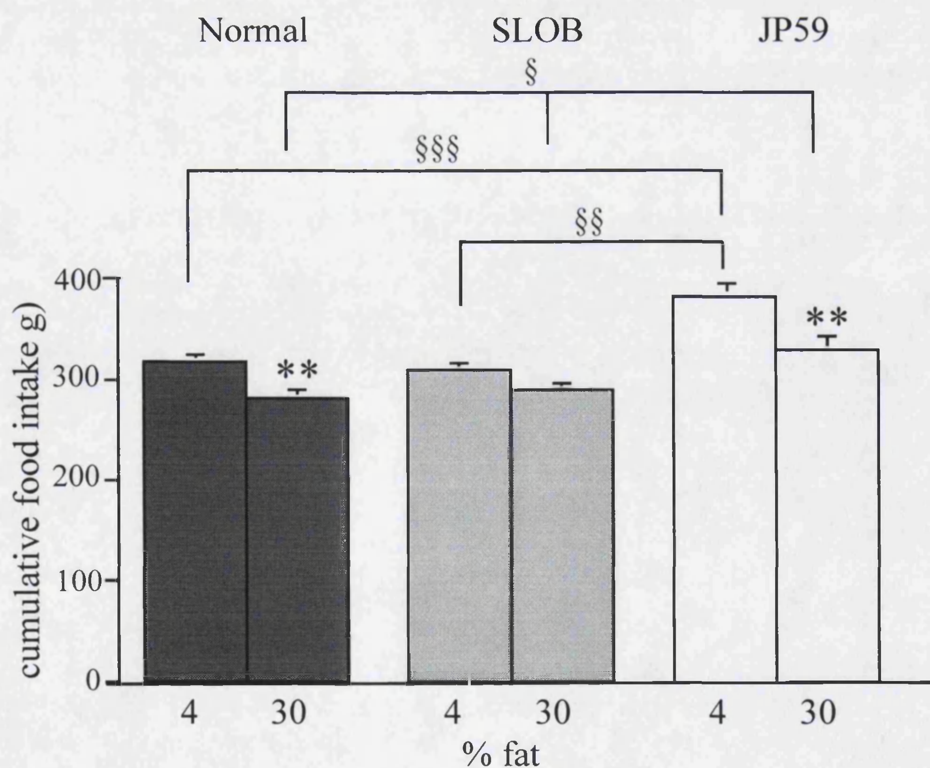


Figure 6.12 Cumulative food intake. Mean food intake over 14 days on 4% or 30% fat diet in normal, SLOB and homozygous JP59 males. (n=6, §p<0.05, §§/**p<0.01, ***/§§§p<0.001).

As expected, leptin levels increased in all high fat fed animals compared to control groups with the largest effect occurring in SLOB animals (**Figure 6.13**). Note, that the leptin rises were the same for high fat fed normal and homozygous JP59 rats. In the chow fed groups, SLOB animals exhibited the greatest plasma leptin levels, compared to normal and JP59 rats.

Analysis of adipose tissue weights suggested that an obesity phenotype could also be developing in male JP59 rats. High fat fed SLOB and JP59 males had significantly greater supra-renal fat pad weights compared to high fat fed normal animals (**Figure 6.14(a)**). However, testicular fat pad weights showed that high fat fed JP59 rats also had significantly greater fat pads compared to high fat fed normal and SLOB rats (**Figure 6.14(b)**). Thus, high fat feeding compared to normal rats, in SLOB rats causes a significant adiposity in the central supra renal fat pad, but in homozygous JP59 rats causes adiposity in both central (supra-renal) and peripheral (testicular) fat pads.

An interesting result I found in these animals was when I measured plasma IGF-I levels at the end of the study. As already shown (chapter 3), IGF-I levels are markedly reduced in SLOB males, probably due to GH deficiency. I showed earlier in this chapter that pituitary rGH levels were significantly reduced in homozygous JP59 males, but I found IGF-I levels were normal in chow fed JP59 males (**Figure 6.15**). I interpreted this to be due to the fact that pituitary rGH levels in JP59 rats are not as markedly reduced as in SLOB rats and enough is secreted to maintain IGF-I levels normal. Fat feeding had no effect on IGF-I levels in SLOB and normal animals, but was associated with a highly significant reduction in IGF-I in high fat fed JP59 rats compared to chow fed JP59 rats. Obesity is known to affect GH secretion as do free fatty acids (Williams *et al.,* 1984), so a simple explanation for this might be that mild GH reduction in JP59 rats is exacerbated by high fat feeding and results in reduced IGF-I levels. In this study, the normal animals fed a high fat diet did not become obese and IGF-I levels were unaltered. In SLOB animals, pituitary GH and plasma IGF-I are already significantly impaired and thus a high fat diet has no further effect.

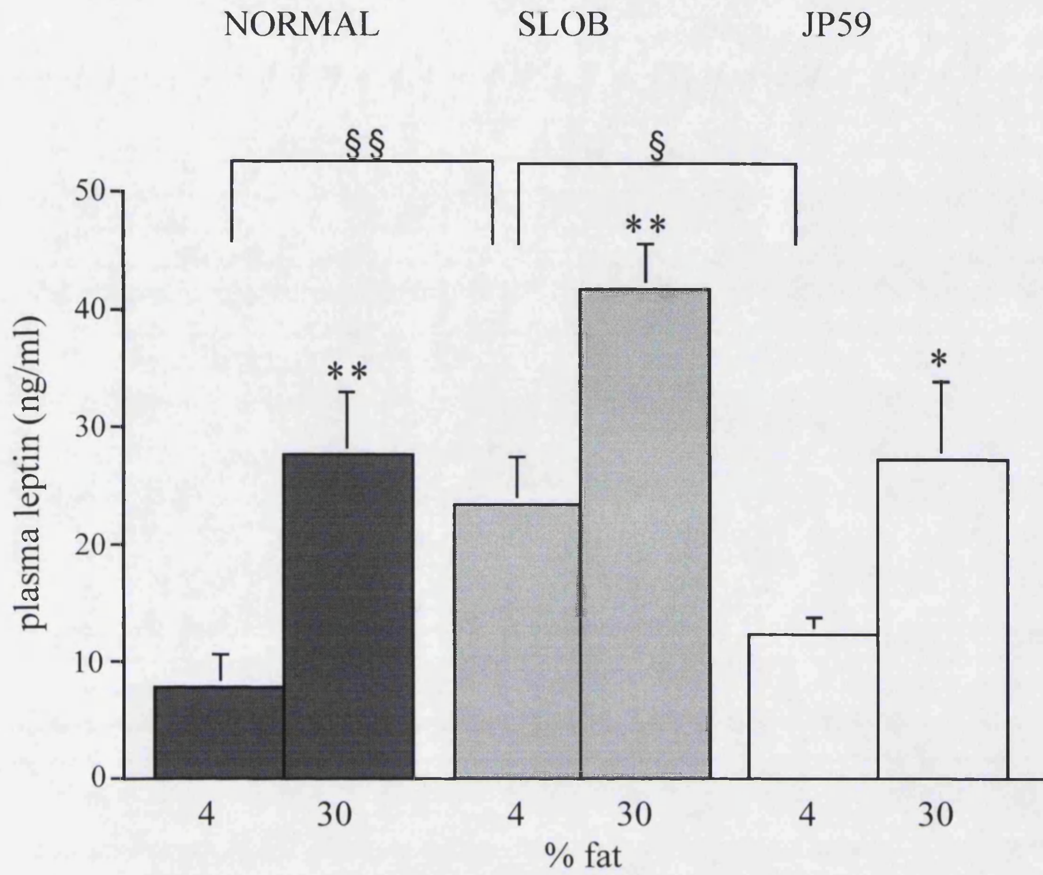
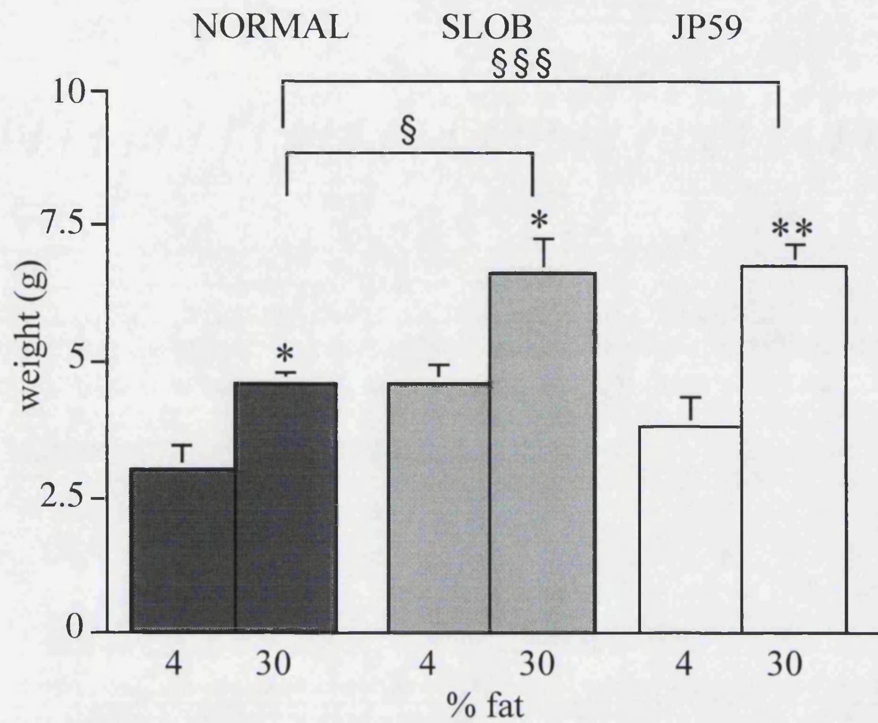


Figure 6.13 Plasma leptin levels. Measurements taken after 4 weeks on 4% or 30% fat diet in normal, SLOB and JP59 male rats. (n=6, *p<0.05, **p<0.01).



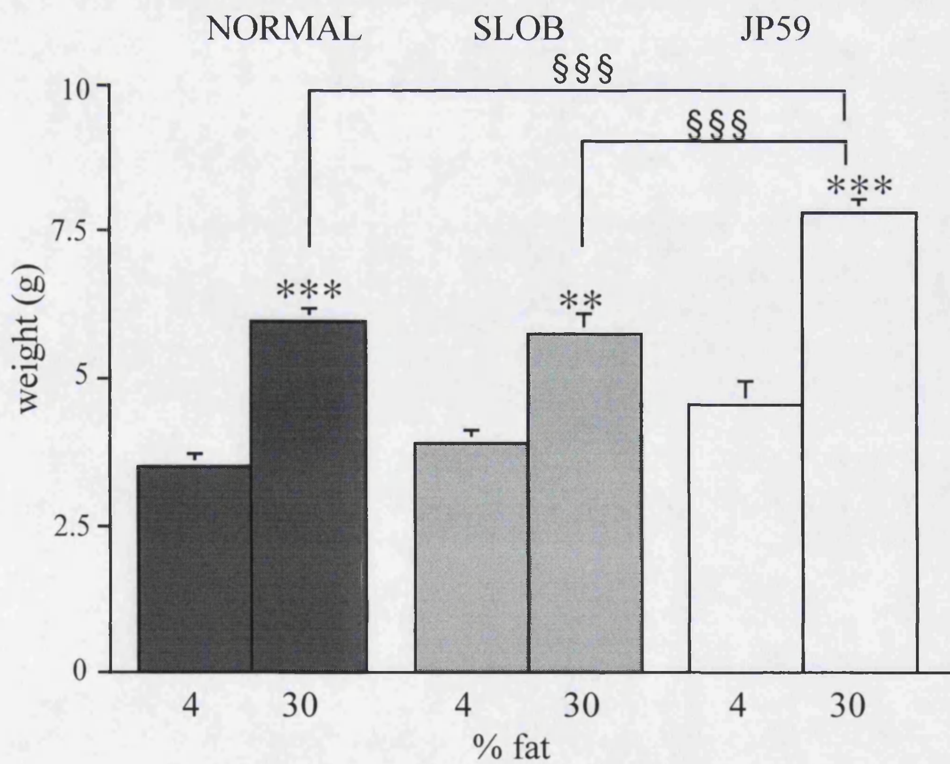


Figure 6.14 Fat pad weights. Fat pad measurements taken after 4 weeks on 4% or 30% fat diet. (n=6, */§p<0.05, **p<0.01***/§§§p<0.001).

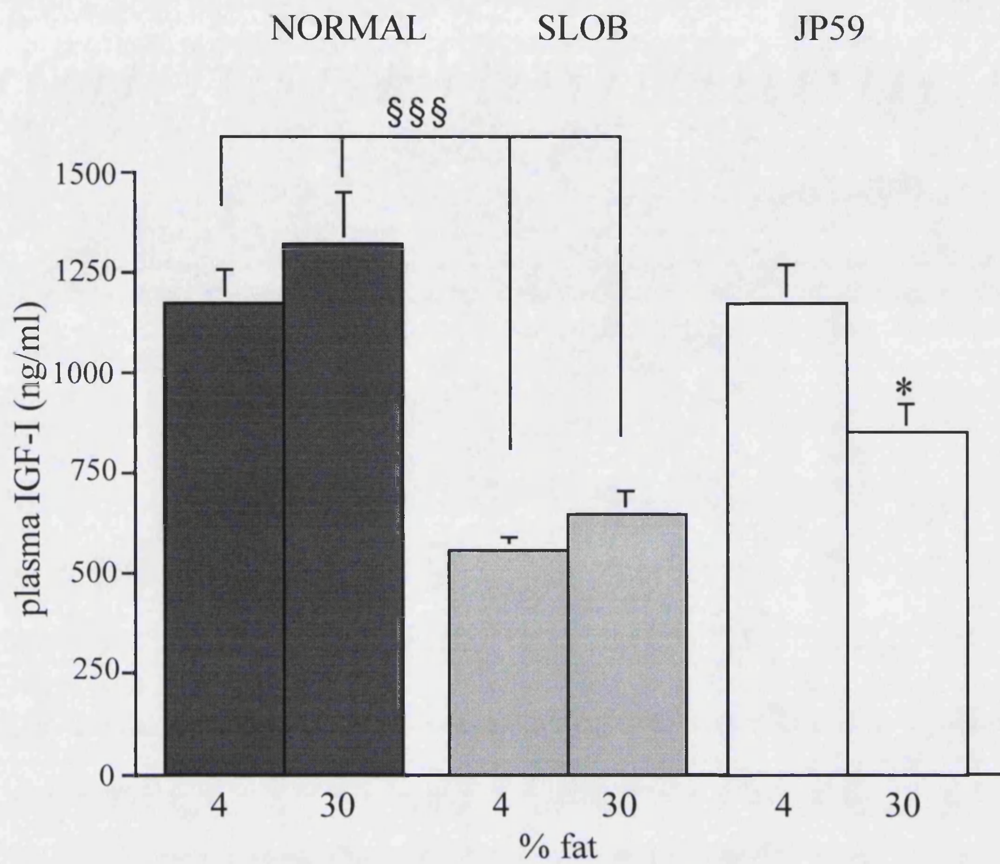


Figure 6.15 Plasma IGF-I levels. Measurements taken after 4 weeks on 4% or 30% fat diet in normal, SLOB and JP59 male rats using radioimmunoassay. (n=6, *p<0.05).

A significant reduction in pituitary rGH levels was also observed in high-fat fed JP59 rats compared to chow-fed JP59 rats (chow 818.7±106µg/pit vs fat 365.7±35µg/pit p<0.007). Whereas high-fat feeding in normal animals raised pituitary rGH levels (chow 376.4±70µg/pit vs fat 620±70µg/pit p<0.04) it had no effect in SLOB animals (chow 110.9±18µg/pit vs fat 131.8±12µg/pit p=0.34). Pituitary rGH levels in chow-fed JP59 rats were also greater than in chow or fat fed normal animals. Earlier I found homozygous JP59 male rats had reduced pituitary rGH levels compared to normal animals. However, there is a large age difference between the animals used to measure rGH in this experiment (128 days) and those used previously (300 days). It is likely that the fall in pituitary rGH levels in JP59 rats is therefore a progression of developing GH deficiency as was documented in SLOB rats (Wells, 1997).

6.5 Generation of transgenic mice exhibiting truncated *5'OT-EST*

Although I have showed evidence that *5'OT-EST* could be a contributory factor for the SLOB phenotype, the ultimate test for testing this hypothesis would be to generate transgenic animals harbouring only truncated *5'OT-EST*. This would enable me to study a phenotype without any effects caused as a result of the hGH transgene component and subsequent GH-deficiency. In addition, generating new transgenic animals would also alter the integration site of the transgene, as it is unlikely the transgene insert would incorporate into the same site. As generating transgenic mice is much easier than generating transgenic rats, a strong test was to try to produce SLOB mice, using an altered SLOB construct (**Figure 2.1**, generated by Dr Paul Le Tissier) consisting of half of the original SLOB rat construct, without the OT or VP transgenes. This was microinjected into fertilised oocytes and transferred into pseudopregnant recipients by Dr Kathleen Mathers (Biological Services). I identified three male founder mice, which were mated with normal C57BL/10 females to generate three transgenic lines of mice. Litters from all three lines generated male and female transgenic pups, and both sexes were subsequently found to be fertile. I carried out subsequent copy number and physiological studies on these lines of mice.

6.5.1 Copy number analysis

Since I believed copy number of truncated *5'OT-EST* might be an important factor in determining the magnitude of any obesity phenotype as already shown in SLOB and JP59 rats, attempts were made to determine copy number in these mice. A probe was generated spanning the entire length of *5'OT-EST*. Southern blotting and phosphorimaging techniques were used to reveal copy number in the three SLOB founder males. Two bands were visible in transgenic animals, the endogenous band as a 5.5kb fragment and the transgene band as a 2.0kb fragment (shorter due to deletion) (**Figure 2.4**). Because of the difficulties in quantifying copy number absolutely, I identified the three founders as low (LCM), medium (MCM) and high (HCM) copy number lines.

6.5.2 Expression of *5'OT-EST* in SLOB mice

I needed to be sure all three lines of SLOB mice were expressing the truncated *5'OT-EST*. In order to assess this, both RT-PCR and nuclease protection assays were performed. RT-PCR in HCM revealed endogenous and truncated *5'OT-EST* products in the heart, testes and spleen. *In situ* hybridisation using a probe which did not differentiate between endogenous and truncated *5'OT-EST* showed as in SLOB rats, *5'OT-EST* mRNA expression was detected in the testes and PVN and SON of the hypothalamus. Furthermore, a nuclease protection assay carried out by Dr Paul Le Tissier showed all three lines of mice were expressing both endogenous and truncated transcripts in the hypothalamus, heart and testes. From these results I was able to conclude that all three lines of SLOB mice were expressing truncated *5'OT-EST*.

6.5.3 Do SLOB mice show an obesity phenotype?

So far animal numbers have been limited due to constraints on animal housing space, however I have been able to make some physiological characterisation in the high copy line of mice compared to normal mice, these results are presented here.

125 day old male and female SLOB and normal mice were culled and fat pads excised (n=3). No significant differences were found between SLOB and normal mice in either bodyweight, supra renal fat pad weight or testicular/ovarian fat pad weight (**Figure 6.16**). Two major criticisms of these measurements are evident, first sample size is very small due to availability of animals, second I have only data for one age. In addition, these parameters should also be measured in the remainder medium and low copy number SLOB mouse lines. However, from the results presented here I concluded that the high copy number SLOB mice at 125 days of age did not show an obesity phenotype. Further analysis with greater sample size and also measuring plasma leptin levels are required before an accurate conclusion is made about all three lines of SLOB mice, such studies have yet to be conducted.

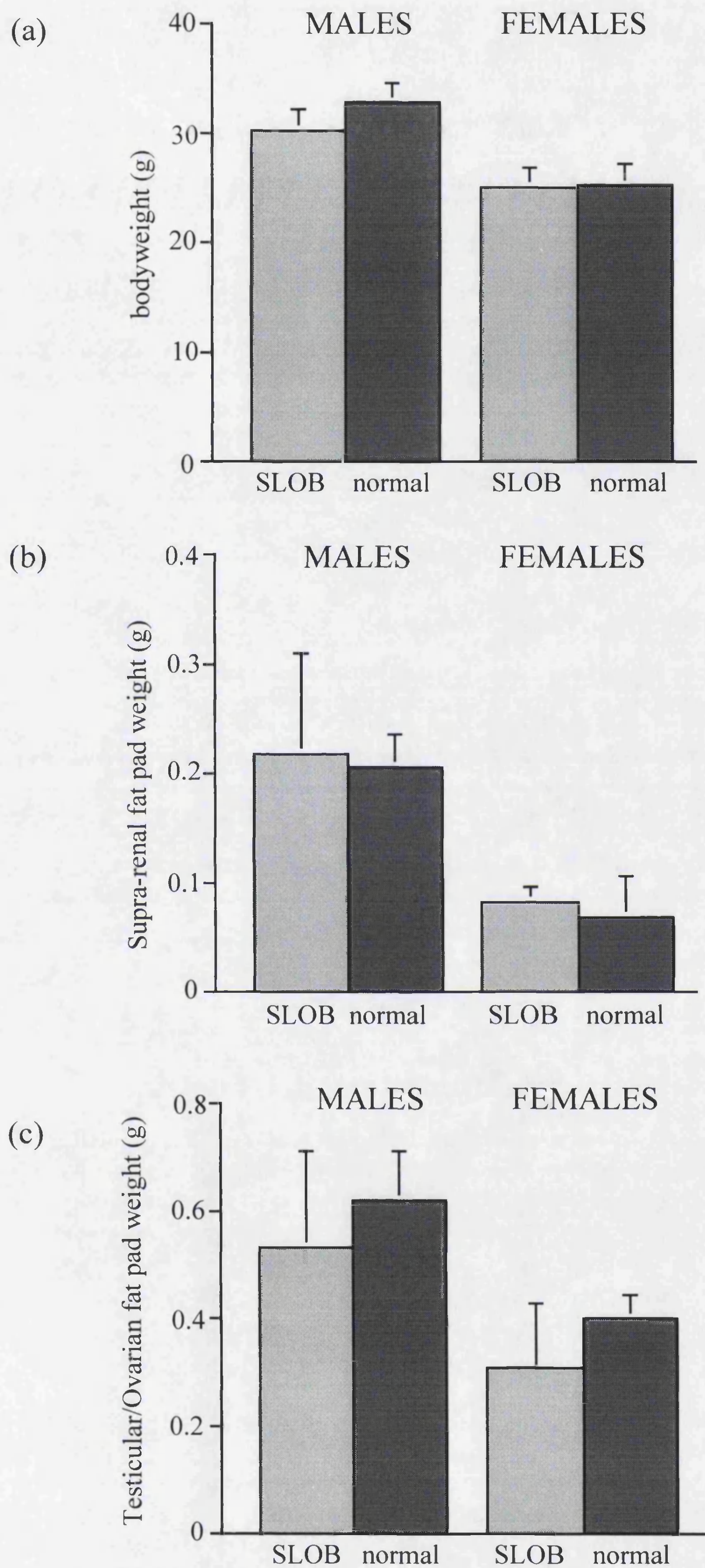


Figure 6.16 Measurements in SLOB mice. Measurements were taken in SLOB and normal mice at 125 days of age (a) bodyweight, (b) supra-renal fat pad weight, (c) testicular/ovarian fat pad weight (n=3).

6.6 Discussion

In this chapter I presented three main areas of data conducted to assess how the SLOB transgene construct has a role in the SLOB phenotype. These were expression studies on *5'OT-EST*, analyses of the phenotype of JP59 rats, and analyses of the phenotype of SLOB mice. Here I discuss how these studies aid in assessing the underlying cause of the SLOB rat phenotype.

6.6.1 *5'OT-EST* expression studies

Hypothalamic *5'OT-EST* mRNA expression was found to be elevated in the PVN of high fat fed normal Wistar males. Although such studies have only been carried out once, this result indicated that *5'OT-EST* was regulated by macronutrient and food intake and thus may well have a role in feeding behaviour. Once a reliable probe for use in *in situ* hybridisation has been produced, which only detects truncated *5'OT-EST*, expression studies in chow and high fat fed SLOB rats can be carried out. Currently the expression levels from the oligonucleotide probe generated for this purpose are low and not easily quantifiable. The hypothalamic and testes expression pattern is similar to the expression pattern of the *tub* gene (Noben-Trauth *et al.,* 1996). In addition, both *tubby* males (Coleman and Eicher, 1990) and SLOB males are infertile after about 12 weeks of age, thus testes expression of *tub* and *5'OT-EST* genes may have a role in reproductive function. Together with the fact that *tubby* mice exhibit relatively late onset obesity as in the SLOB rat, these data also show *5'OT-EST* and *tub* may have some common regulatory and functional properties. Unfortunately, little is known at present on how *tub* causes obesity (Noben-Trauth *et al.,* 1996; Kleyn *et al.,* 1996; Stubdal *et al.,* 2000).

Both nuclease protection and Western blotting showed *5'OT-EST* mRNA and protein were expressed in a number of tissues, of which the expression in the heart was very strong. At present I do not know the relevance of this expression, but as the heart is an important organ implicated in obesity and coronary health status (Larsson *et al.,* 1984; Kovacs *et al.,* 1996) this expression is interesting.

6.6.2 *5'OT-EST* function

So far I have not carried out studies to functionally characterise *5'OT-EST*, however, Dr Le Tissier has carried out database searches which give information about a possible role for *5'OT-EST*; his findings are summarised here. Conventional BLAST searches revealed no homology of the predicted protein product of *5'OT-EST* to any known protein. However, application of a more sophisticated search algorithm generated by Taylor (1998) which searches for similarities between overall structure identified similarities to apolipoprotein E in its alpha helical domains but without an apparent LDL-receptor binding domain. As ApoE is centrally involved in lipid metabolism and transport (Mahley, 1988), it is possible that *5'OT-EST* may play some role in the cellular handling of lipid. It is also possible, the abnormal levels of apoE found in female SLOB rats which I presented in chapter 3, may be related to this novel gene and the sex-specific SLOB phenotype.

In addition, Dr Le Tissier's studies have also identified that protein tyrosine phosphatase receptor (*Ptpra*), a gene which has been implicated in the control of insulin sensitivity (Ren *et al.,* 1998) lies immediately upstream of *5'OT-EST*, but *Ptpra* sequences are not present in the SLOB construct. Mice deficient in protein tyrosine phosphatase-1B (*Ptp-IB*) gene have increased tissue specific insulin sensitivity, increased energy expenditure and decreased adiposity (Elchebly *et al.*, 1999; Klaman *et al.,* 2000). These results indicate that *Ptp-IB* has a role in modulating both insulin sensitivity and fuel metabolism. Physical linkage showed *5'OT-EST*, *Ptpra*, VP and OT all mapped to the distal region of mouse chromosome 2. Both *5'OT-EST* and *Ptpra* were also found to lie within 0.21 cM of *mahogany* (*mg*), a gene implicated in suppression of obesity (Nagle *et al.*, 1999, Gunn *et al.*, 1999). These findings therefore suggested that one or more of these three genes may contribute to Mob5 (Peters *et al.*, 1998), a quantitative trait locus (QTL) for obesity which maps to this region of mouse chromosome 2. Such a tight linkage complicates genetic dissection of their relative contribution(s) to obesity in mouse as well as in humans, and separate studies of the potential role of each of these gene products in human obesity are necessary.

6.6.3 Do JP59 rats show a phenotype similar to that in SLOB rats

If the SLOB rat phenotype is a result of components of the transgene and because JP59 rats have the same transgene, I would expect the phenotype in JP59 rats to be similar to that in SLOB rats. However, as mentioned earlier SLOB rats have at least double the transgene copy number than homozygous JP59 rats. Thus any effect observed in SLOB rats should be reduced in JP59 rats. This was already shown in terms of hGH hypothalamic mRNA expression by Wells (1997) and shown again in this chapter; SLOB rats have higher hGH mRNA expression compared to JP59 rats.

Breeding to homozygosity had no effect on overall growth in male or female JP59 rats, but did have an effect on pituitary rGH levels and soleus muscle weight. Thus the effect of increased transgene copy number was increased GH-deficiency, but not to the extent of that seen in SLOB rats or to the extent of affecting overall bodyweight. Analyses of whether an obesity phenotype was present showed male JP59 rats did not have significantly elevated plasma leptin levels nor increased fat pad weights. However, homozygous females did have significantly elevated leptin levels and increased fat pad weights. Even in the hemizygous female JP59 rats, fat pad weights already weighed more than in normal females. Fertility in both male and female homozygous JP59 rats was not impaired as is in SLOB males.

These data suggested that a lesser, slightly different, but still significant visceral obesity phenotype was present in female JP59 rats. The importance of this observation was that it suggested that the obesity phenotype simply manifests in SLOB rats to a greater extent than in JP59 rats and is due to some component of the insert and probably not entirely due to an insertional effect of the transgene. However, it did not identify which component of the transgene insert was responsible for this effect, nor did it explain why obesity is evident in JP59 females but not in males.

To further investigate if JP59 rats had a similar phenotype to SLOB rats, I challenged young (100 days) homozygous male JP59 rats to a high fat diet. Despite homozygous male JP59's on a normal diet not showing an obesity phenotype, on a high fat diet

these animals did develop obesity. As was found in the high fat feeding experiment shown in chapter 5, SLOB males when challenged with a high fat diet developed obesity and exhibited significantly greater plasma leptin levels, supra-renal fat pad weights and bodyweight gain compared to normal animals and in addition they do not reduce their food intake. High fat fed JP59 males exhibited significantly greater fat pad weights and bodyweight gain compared to normal animals and did reduce their food intake when on a high fat diet. However, despite increased fat pad weight, plasma leptin levels were not significantly greater in high fat fed JP59 rats than in high fat fed normal rats. With high fat fed JP59 rats, both the central and peripheral fat pad weights were increased, whereas in high fat fed SLOB rats only the central fat pad was significantly greater than in high fat fed normal rats. My results also showed whether on chow or high fat diets, JP59 rats, consumed more food than SLOB or normal rats. Why this occurs is unclear and will require further investigation.

From my results I was able to determine that despite an increased food intake in chow fed JP59 males, they are able to resist the development of obesity. However when challenged with a fat enriched diet they rapidly develop obesity in both central and peripheral adipose depots, unlike the phenotype of SLOB rats, who selectively accumulate fat in the central adipose depots.

6.6.4 Studies on SLOB mice

So far my experiments carried out on SLOB mice have been very limited, however preliminary studies, showed that SLOB mice did not have an obesity or infertility phenotype as in SLOB rats. This analysis however, does not necessarily mean that truncated *5'OT-EST* is not a component of the underlying cause of the SLOB phenotype. The reasons for this statement are; first, I have already established copy number as a highly contributory factor of the obesity phenotype as depicted in the extent of the phenotype in SLOB rats compared to JP59 rats. Although we have generated 3 lines of transgenic SLOB mice which I described as low (LCM), medium (MCM) and high (HCM) copy lines, copy number, even in the HCM line may still not be comparable to that in SLOB rats. Second, it is possible that both the hGH/VP component of the transgene as well as truncated *5'OT-EST* are required for

development of an obesity phenotype. This could be analysed by generating transgenic mice exhibiting just hGH/VP and then crossing these with the existing *5'OT-EST* SLOB mice and analysing whether the resulting offspring develop obesity and/or infertility. Third, species variation may also mean *5'OT-EST* in mice is not regulated as in rats and therefore mice do not develop an obesity phenotype with truncated *5'OT-EST*.

The data presented in this chapter on *5'OT-EST* location, expression, homology to apoE and my studies on JP59 rats, suggest that this gene remains a good candidate for contributing to this phenotype. The simplest interpretation of my results is that truncated *5'OT-EST* has only a partial role in the SLOB phenotype, and that obesity also results from an insertional mutagenic effect in this line. Further studies in characterising both the nature of *5'OT-EST*'s targets, and the site of insertion in SLOB rats are underway.

6.7 Summary

Genetic analysis of the transgene construct revealed a novel gene which we termed *5'OT-EST*. This gene is expressed in specific hypothalamic nuclei involved in bodyweight regulation, and expression is altered in normal high fat fed animals. In SLOB rats, *5'OT-EST* is truncated, and SLOB rats exhibit at least four copies of this truncated gene. The *5'OT-EST* gene codes for a protein which has some structural similarities to apolipoprotein E, a protein involved in lipid transport.

Chow fed homozygous JP59 female rats show an obesity phenotype whereas homozygous male rats do not show a similar phenotype until challenged with a fat enriched diet. High fat fed JP59 males develop obesity in both central and peripheral stores, whereas high fat fed SLOB males accumulate fat only in central depots.

Generation of transgenic mice expressing truncated *5'OT-EST* as yet has not revealed an obesity phenotype in these mice, however studies on these mice are ongoing. At this point, it is still not possible therefore to distinguish between insertional effects and transgene effects of this dominant phenotype in SLOB rats.

Chapter 7

General Discussion and Concluding Remarks

7.1 A transgenic approach to the study of obesity

Over the past 10 years most of the naturally occurring single-gene mutations resulting in obesity in rodents have been cloned (Spiegelman and Flier, 1996; Chua and Leibel, 1997; Levine and Billington, 1998; York and Hansen, 1998). These mutations include *agouti*, *ob/ob*, *db/db*, *fat/fat* and *Zucker*, which have been extensively studied in an effort to understand the physiological and biochemical basis for their obese phenotype. In all of these models, the phenotype preceded the elucidation of the genotype, which in the case of the *ob/ob* mouse took over 40 years. Research in the obesity field dramatically increased since the discovery of the *ob* gene and its product, leptin (Zhang *et al,* 1994; Friedman and Halaas, 1998). Much work on these models has focused on the identification of the molecular mediators and regulatory pathways for feeding and bodyweight regulation (Elmquist *et al.,* 1998, 1999; Flier and Maratos-Flier, 1998; Woods *et al.,* 1998b; Kalra *et al.,* 1999; Inui *et al.,* 1999). Such data has generated renewed interest in the genetic framework of energy homeostasis. Transgenic technology has also advanced in recent years. This procedure which permits the introduction of genes into the germ line of mice or rats, and homologous recombinant gene knockouts, which allows elimination of endogenous gene expression, are powerful tools for exploring the complex pathogenesis of obesity. Such genetic manipulations have generated additional models allowing the study of individual components of obesity syndromes (Levine and Billington, 1998; York and Hansen, 1998; Inui, 2000). As well as those models in which alterations in energy homeostasis are expected, some models were created for reasons other than obesity, but unexpectedly these transgenic models may display obesity as part of their phenotype. This is as occurred with the SLOB rat; originally generated to investigate the hormones released from the posterior pituitary gland by the insertion of double reporter genes. However, as evident by the work presented in this thesis, now joins the long list of rodent obesity models, though still relatively unusual, being autosomal dominant, in rats not mice, and with unusual obesity characteristics.

7.2 Characterisation of the SLOB rat phenotype

In this thesis I have presented data on this transgenic autosomal dominant model of late-onset predominantly male-specific visceral obesity. These characteristics are commonly found in the obese human population (Bjontorp, 1992; Kuczmarski *et al.*, 1994) and apart from the SLOB rat, no other rodent obesity model characterised so far exhibits all three of these factors together. It is important to firstly point out that an unusual but very useful characteristic of SLOB rats is that they exhibit an autosomal dominant phenotype. Apart from the *agouti* mouse, all other rodent obesity models described in Chapter 1 are autosomal recessive models (Weigle and Kuijper, 1996). In the future it will therefore be possible to breed SLOB (females/young males) rats with other rodent models such as the *Zucker* and *dwarf* rat. Such new strains of rat should provide greater scope and understanding of the SLOB phenotype.

Why is it that SLOB males selectively accumulate adipose tissue in the abdomen? In chapter 3, I discussed the significance of visceral fat and its role in cardiovascular disease and obesity (Larsson *et al.*, 1984; Banerji, 1997); this is further explored here. Several differences concerning innervation and blood supply in the adipose tissue microenvironment have been reported depending upon anatomical location (West *et al.*, 1989; Santos *et al.*, 1991; Tavernier *et al.*, 1995) which may influence where genetically obese rodents store fat. *Zucker* obese rats, show a 21-fold higher subcutaneous adipose tissue, 9-fold higher retroperitoneal adipose tissue and 7-fold higher mesenteric adipose tissue compared with lean rats (Hashimoto *et al.*, 1996). In this same study, and the study by Portillo *et al.*, (1999) subcutaneous adipose tissue was also shown to be less sensitive to lipolytic agents than epididymal (testicular) and perirenal (supra-renal) adipose tissues in young obese and lean *Zucker* rats. In the severely obese SLOB rat, the largest accumulation of fat selectively occurs in the supra-renal adipose depot, a unique trait to this model and one that is not seen in any of the previously characterised rodent obesity models. As high fat feeding in SLOB males rapidly caused the selective accumulation of fat in the supra-renal adipose depot and conversely food restriction in SLOB males selectively reduced supra-renal fat pad weight, my studies show metabolic manipulations predominantly effect central adipose (supra-renal) stores rather than peripheral (testicular) stores in SLOB rats.

Portillo *et al.,* (1999) tested the effect of high fat diet on lipolysis in isolated adipocytes from the visceral and subcutaneous white adipose tissue from Wistar rats. A high amount of fat (60%) in the diet induced an increase in adipose tissue, which was accompanied by a reduction of β-adrenergic agonist induced lipolysis in central adipose tissue, but this reduction was not seen in subcutaneous adipose tissue, indicating a different pattern of response between these two anatomical locations. As lipolytic activity in low fat diet SLOB rats is reduced in both central and peripheral regions and in view of the fact that high fat fed SLOB rats exhibit increased abdominal obesity; it would be interesting to measure lipolytic activity in high fat fed SLOB animals and assess what further impairment is observed in both central and peripheral adipose depots.

An increase in adipose tissue is generally attributable to both hyperplasia and hypertrophy (Bray and York, 1979); thus it is unusual that SLOB rats show only hyperplasia. However, whether increased fat cell number in the supra renal fat pad of SLOB rats is the cause of SLOB obesity or a consequence of another effect such as increased VLDL secretion and increased central adipose LPL activity, remains to be elucidated.

As glucose homeostasis is normal in SLOB males and because of the lipid abnormalities also reported in these animals, makes this model an attractive model for exclusively investigating obesity and dyslipidemia. This is an important characteristic of SLOB rats compared to other rodent obesity models, many of which show diabetes and obesity as aspects of their phenotype. In addition, it means drugs which are often used to treat both insulin resistance and obesity, can be used in SLOB rats to determine the specific effects of these drugs on obesity distinct from insulin sensitisation. Such drugs include the PPARγ agonists thiazolidinediones, used to treat patients with insulin resistance (Olefsky, 2000). I have not assessed diabetic status in female SLOB rats. If glucose homeostasis were normal in female rats, would obesity still be delayed in young SLOB females if they were made diabetic? Such a test would make this model even more attractive for study of obesity and diabetes and the role of oestrogen in protecting females from developing obesity, in the presence or

absence of diabetes, also highlighted as a problem in some women after the menopause (Colombel and Charbonnel, 1997).

7.3 A sexually dimorphic phenotype

Much remains to be done to decipher the nature of the sexually dimorphic SLOB phenotype, in terms of obesity development and reproductive status. Food availability regulates reproductive function in mammals (Wade *et al.,* 1996). For instance, fasting impedes puberty, reduces gonadotropin secretion, disrupts ovulation, and decreases steroid induced reproductive behaviours (I'Anson *et al.,* 1991; Kalra and Kalra, 1996; Wade *et al.,* 1996). Although the link between undernutrition and reproductive dysfunction is well established, infertility accompanying obesity has received less attention despite the identification of numerous mammalian species with obesity and infertility as part of their phenotype (Kalra and Kalra, 1996). For example, in rats, diet induced obesity is associated with fewer total oestrous cycles and diminished fertility (Bray, 1992). Also, leptin treatment restores the sterility defect in *ob/ob* mice (Chehab *et al.,* 1996) and leptin treatment in humans with defects in leptin or leptin signalling also improves reproductive status (Farooqi *et al.,* 1998). In addition, obesity in middle aged men is also often associated with infertility (Bray, 1997; Norman and Clark, 1998). Therefore, it is interesting that severely obese SLOB males are also infertile. All preliminary studies have not revealed any indications as to why obese SLOB males are infertile. However, studies have shown that like in *fat* and *tub* males (Coleman and Eicher, 1990), SLOB males are able to breed at a very young age. It is possible the novel gene *5'OT-EST* present in a truncated form in SLOB rats and expressed in the testes, may have a role in reproductive function. It is interesting that the *tub* gene, mutated in *tubby* mice (Kleyn *et al.,* 1996), is also expressed in the testes (Noben-Trauth *et al.,* 1996). Like *5'OT-EST*, the function of *tub* remains unresolved. However, the predicted amino acid product of the *tub* gene shows homology to a mouse testis-specific phosphodiesterase (Noben-Trauth *et al.,* 1996). A role for phosphodiesterases in obesity is not known, but in view of this result further analysis may reveal additional functions of these enzymes. There are also a class of *tub* like proteins (TULPs), expressed in the retina and thought to be candidates for occular diseases (North *et al.,*

1997) as *tubby* mice exhibit retinal degeneration as well as obesity (Ohlemiller *et al.*, 1991). Further analysis of TULPs may also suggest a possible function for *tub*.

In this thesis I have focused on the obesity aspect of the SLOB phenotype. Future studies should explore in more detail the infertility aspect of SLOB males, and whether the expression of *5'OT-EST* in the testes has any relevance to this infertility. In chapter 6, I indicated that a sophisticated search algorithm generated by Taylor (1998) which searches for similarities between overall tertiary protein structure, identified similarities between *5'OT-EST* and apolipoprotein E in its alpha helical domains but without an apparent LDL-receptor binding domain. This data also indicated *5'OT-EST* had no apparent motifs and therefore suggested it was a cytoplasmic cellular protein. As well as apoE having a role in lipid transport (Mahley, 1988), *in situ* hybridisation and northern blot analysis have shown apoE mRNA in the epithelium and interstitum of the testis and epididymis (Law *et al.*, 1997). The exact function of apoE in spermatogenesis and sperm maturation remains to be elucidated. However, as apolipoproteins are involved in the cellular handling of lipids, it is speculated they are also involved in the removal of lipids during the formation of sperm (Sirtori, 1998). This data demonstrates that if the function of *5'OT-EST* is similar to that of apoE, it may also be involved in both lipid transport and male reproductive status.

Cardiovascular disease is more prevalent in men and post-menopausal women, than in pre-menopausal women or post-menopausal women treated with oestrogen replacement therapy (Sullivan *et al.*, 1988; Barret-Connor and Bush, 1991; Stampfer *et al.*, 1991). These observations suggest a cardio-protective effect of oestrogen. Cardiovascular disease status has not yet been assessed in either male or female SLOB rats, however, both sexes have reduced longevity compared to normal animals (SLOB males reduced by ~6 months, SLOB females reduced by ~1 month). Although oestrogen therapy in clinical trials has yet to be conducted, the protective action of oestrogen has been demonstrated in numerous animal studies (Kushwaha and Hazzard 1981; Adams *et al.*, 1990; Holm *et al.*, 1995; Sulistiyani *et al.*, 1995). The cardio-protective effect of oestrogen has been explained by its ability to lower plasma cholesterol (Walsh et al., 1991), and it also has a direct effect on arterial walls (Bush et al., 1987; Holm et al., 1997). As mentioned, the very late-onset of severe

obesity in SLOB females seems to be comparable to that seen in post-menopausal women (Colombel and Charbonnel, 1997), thus experiments conducted in chapter 5 suggest the SLOB female as a good model for study of post-menopausal human obesity. The exact mechanism of how oestrogen delays the onset of obesity in SLOB females is not known, however a recent study examined one possible mechanism for the oestrogen-dependent inhibition of obesity. Homma *et al.,* (2000) identified a unique oestrogen response element on the lipoprotein lipase promoter. Subsequently they concluded that oestrogen suppressed transcription of the lipoprotein lipase gene in 3T3-L1 adipocytes stably expressing the estrogen receptor. As LPL activity is sexually dimorphic (Galan *et al.,* 1994), and in view of the changes observed in LPL activity in SLOB rats, it is possible both LPL activity and oestrogen have a role in the onset of obesity in SLOB male and female rats.

So far, I have yet to determine whether long term treatment with oestrogen in SLOB males abolishes the obesity phenotype. Neonatal sex-reversal of both male and female SLOB rats may demonstrate the role of gonadal steroids in the development of the SLOB phenotype and whether obesity is prevented in SLOB males and perhaps accelerated in SLOB females.

As well as oestrogen and *5'OT-EST* having a possible role in male specific obesity and infertility in SLOB rats, lipid abnormalities may also contribute to these factors. For example, targeted disruption of hormone-sensitive lipase (HSL) results in male sterility and adipocyte hypertrophy in mice although these mice do not develop obesity (Osuga *et al.,* 2000). Hormone sensitive lipase is an enzyme involved in mediating the hydrolysis of triglycerides in adipose stores and the hydrolysis of cholesterol esters in the adrenals, ovaries and testes (Strålfors, *et al.,* 1987). Although $HSL^{-/-}$ mice do not develop obesity, it is possible that an alteration in HSL activity may contribute to SLOB male infertility and together with other abnormalities, an overall obesity and infertility phenotype is observed. So far I have not measured HSL in SLOB rats, this is now underway in collaboration with Fredrik Frick and Prof. Staffan Edén (University of Gôteborg, Sweden).

Oestrogen receptor β mRNA has been identified in oxytocin and vasopressin neurones of the rat supraoptic and paraventricular nuclei (Hrabovszky *et al.,* 1998).

These data suggest that oestrogen could directly regulate gene(s) in oxytocin and vasopressin neurones. *In situ* hybridisation carried out by Dr Pamela Bennett and Dr Sara Wells showed *5'OT-EST* is co-localised in vasopressin neurones. In addition, electron microscopy studies carried out by Dr Helen Christian (Oxford University) proposed that hGH from the transgene is also co-localised in vasopressin neurones. Thus, it is possible oestrogen may be regulating the hGH/VP and *5'OT-EST* component of the SLOB transgene which may in turn be affecting the sex specific obesity and fertility phenotype observed. Measuring hGH and *5'OT-EST* mRNA expression in the brains of ovariectomised and oestrogen treated rats might indicate if such a relationship is apparent.

7.4 Alterations in energy balance

One of the most interesting characteristics distinguishing SLOB rats from previously characterised rodent obesity models is the fact that despite severe obesity, obese SLOB males are not hyperphagic. As discussed in chapter 3 small increases in energy intake over a long period of time can still cause obesity to develop (Hervey, 1969; Bray and York, 1979: Bray, 1992); however, SLOB rats do not appear to consume more food than normal animals. Therefore, obesity in these animals must ultimately result from an abnormality in energy expenditure.

Adaptive thermogenesis refers to the ability to dissipate energy as heat following exposure to cold or excessive overeating and is largely determined by the proton permeability of the inner mitochondrial membrane (Garlid *et al.,* 1998). In this thesis, my studies on energy homeostasis have mainly focused on energy intake. It is clear in view of the fact that obese SLOB rats are not hyperphagic, it will be important to study energy expenditure in these animals. For example the function of mitochondrial uncoupling proteins (UCPs) that are involved in adaptive thermogenesis have been the focus of much recent study (Ricquier *et al.,* 1999; Lowell and Spiegelman. 2000). Studies show that UCP2 is upregulated more efficiently in obesity resistant A/J mice, than in obesity prone C57BL/6J mice (Surwit *et al.,* 1998). In addition, mice overexpressing human UCP-3 in skeletal muscle are found to be hyperphagic but not obese (Clapham *et al.,* 2000). UCP2 and UCP3 may therefore be important regulators

of diet-induced thermogenesis and modulators of their activity may provide potential treatments of obesity. However, the relevance of these proteins in human obesity, remains to be determined. Measurement of these proteins in the SLOB rat may reveal disturbances in the maintenance of thermogenesis and thus provide important clues to energy homeostasis.

High-fat feeding causes a significant reduction in food intake in the diet-induced obesity resistant A/J mouse, but has no change in the obesity susceptible C57BL/6J mouse (Bergen *et al.,* 1999). These results together with those I found in the SLOB rat suggest that a high-fat diet produces compensatory changes in normal rats or A/J mice resistant to diet-induced obesity but not in SLOB rats or C57BL/6J mice susceptible to diet-induced obesity. Such studies therefore aid in our understanding of the mechanisms which predispose some individuals to become obese on a particular diet, whereas other individuals on the same diet do not become obese. In addition, by feeding SLOB rats a diet more representative of a western human diet, meant this model was more representative of the human obese syndrome.

High fat diets decrease the number of insulin receptors in liver, skeletal muscle and adipose tissue, decrease glucose uptake into skeletal muscle and adipose tissue, and decrease hepatic glycolysis and glycogen synthesis (Anderson *et al.*, 1985). Thus, differences in diet induced thermogenesis (Gong *et al.,* 1990), lipolysis stimulation (Vernon, 1992) and fat oxidation (Schutz *et al.,* 1989) have been proposed in order to explain adiposity increase induced by high fat feeding. It has also been reported that sympathetic activity in adipose tissue is diminished after high fat feeding and that this effect could contribute to reduced lipolytic activity (Matsuo *et al.,* 1995). In addition, it is suggested high-fat diets may influence hypothalamic-pituitary-adrenal (HPA) activity, elevating adrenal glucocorticoid production (Hulsmann *et al.,* 1978; Kolterman *et al.,* 1979; Brindley *et al.,* 1981). Subsequently, elevated glucocorticoids antagonise most actions of insulin and result in increased basal and glucose-stimulated insulin levels and pancreatic β–cell hyperplasia (Lenzen *et al.*, 1984; Martin-Sanz *et al.*, 1990). Glucocorticoids also decrease levels of lipoprotein lipase, which controls the hydrolysis of VLDL and thus exaggerates hypertriglyceridemia (Taylor *et al.*, 1988). My analyses of insulin, glucose, corticosterone, LPL activity and VLDL secretion were all carried out in the low fat fed SLOB rat. It would be

interesting to repeat these experiments in high fat fed SLOB rats, to be more comparable to the average human diet. However, abnormalities are already observed in the low fat fed SLOB rat in LPL activity and VLDL secretion but with normal plasma insulin, glucose and corticosterone levels.

Food restriction in SLOB and normal male rats caused a similar decrease in bodyweight as is also seen in dieting obese humans (Danforth, 1985) and unlike that seen in *ob/ob* and *db/db* mice (Dubuc *et al.,* 1983). That is, food restriction in *ob/ob* and *db/db* mice does not reduce obesity. The difference seen between *ob/ob* mice and SLOB rats may in part be due to the chronically elevated adrenal cortical hormones found in *ob/ob* mice and as already mentioned these agents increase lipogenesis in conjunction with protein catabolism (Bray *et al.,* 1979). While food restriction has no effect on obesity in *ob/ob* mice, adrenalectomy has been shown to reverse hyperphagia and the high rates of weight gain in these mice (Solomon and Mayer, 1973; Solomon *et al.,* 1977). Adrenalectomy also reduces hyperphagia, body weight gain, carcass fat content and lipoprotein lipase activity in *Zucker* rats (Yukimura *et al.,* 1978; Stern *et al.,* 1983). SLOB rats exhibit an abnormality in their HPA axis and that is significantly decreased hypothalamic CRF mRNA expression, a result not observed in other rodent obesity models. It would therefore be interesting to observe the effects of adrenalectomy in SLOB rats despite the fact they have apparently normal corticosterone levels.

7.5 Changes in the hypothalamus: how these relate to the SLOB phenotype

Leptin inhibits NPY gene expression in the arcuate nucleus, which causes a reduction in food intake (Schwartz *et al.,* 1996b; Gehlert and Heiman, 1997). As plasma leptin levels are elevated in SLOB rats and this may well be the cause of reduced arcuate NPY mRNA expression levels, indicates that food intake in SLOB rats should also be reduced. It is possible that in fact food intake is reduced in SLOB rats and that this is observed as a normal non-hyperphagic state. When fed a high fat diet, NPY mRNA levels in SLOB males remained low and likewise food intake was not altered. This result shows that although elevated plasma leptin levels in chow fed SLOB males are

perhaps enough to reduce NPY levels in the arcuate nucleus, a further increase in leptin levels has no effect on the already blunted NPY system. This indicates, when challenged with a high fat diet, SLOB rats may be functionally resistant to leptin. Obesity is associated with a decreased leptin transport across the blood brain barrier (Burguera *et al.,* 2000). Leptin transport across the blood brain barrier in SLOB rats may be diminished in high fat fed animals, and whilst a relatively small increase in plasma leptin is detected by the hypothalamus, a larger increase may not be able to signal in this already saturated system. This may also be the case in humans; on a low fat or calorie restricted diet these individuals are able to loose weight, but when they return to a normal food intake they rapidly gain weight (Rosenbaum *et al.,* 1988; Wadden *et al.,* 1993). In this case, their leptin levels increase again and they are no longer able to sustain their weight reduction, due to perhaps a leptin resistance.

Interactions between NPY, leptin, and lipid mobilisation in adipose tissue have also been investigated. Leptin is found to increase lipolysis and NPY found to decrease lipolysis (Martínez *et al.,* 2000). This was the first evidence suggesting that NPY and leptin may interact in a homeostatic loop to regulate body-fat mass and energy balance not only at the level of the central nervous system, but also directly at the adipocyte level. In view of the fact that SLOB rats have elevated plasma leptin levels and reduced hypothalamic NPY mRNA levels, one would expect an increase in lipolysis, however, in the two adipose stores tested lipolytic activity was decreased. This demonstrates that although the study of Martinez *et al.,* shows a direct relationship between NPY, leptin and lipolytic activity, this relationship is not exclusive and other pathways may exist, that could compensate or even produce opposing effects.

Leptin regulates GH secretion; the administration of leptin antiserum leads to a decrease in spontaneous GH secretion and administration of leptin to food deprived rats leads to a reversal of the inhibitory effect of fasting on plasma GH levels (Carro *et al.,* 1997; Tannenbaum *et al.,* 1998). Carro *et al.,* (1998) showed leptin-induced GH secretion is suppressed by NPY. Thus, it is possible that NPY mediates the effects of leptin on GH secretion. Leptin and NPY could act through parallel pathways to alter GH release with NPY overcoming the stimulatory effect exerted by leptin on plasma GH levels. Although reduced rat GH levels in SLOB rats are

thought to be the result of local hypothalamic negative feedback from hGH in the SLOB transgene, it is possible in view of the relationship between leptin, GH and NPY, that GH levels are also influenced by leptin and NPY levels in SLOB rats.

In this thesis I have presented for the first time data on hypothalamic nuclei specific mRNA expression of the *tub* gene, demonstrating a sex-specific expression in normal rats and alterations in both SLOB and the severely GH-deficient *dwarf* rat. As the function of the *tub* protein remains unresolved (Noben-Trauth *et al.*, 1996; Stubdal *et al.*, 2000), it is difficult to ascertain what these results mean. However, my data indicate that *tub* may have a role in sexual dimorphism, GH regulation and obesity. Further studies are warranted which may aid in elucidating the function of the *tub* protein.

Hypothalamic CRF mRNA expression in *Zucker* rats (Pesonen *et al.*, 1992), *tubby* mice (Guan *et al.*, 1998) and *ob/ob* mice (Jan and Romsos, 1998) is normal. However, a reduced expression is observed in food restricted lean rats (Brady *et al.*, 1990) but is increased in food deprived *Zucker* and *ob/ob* animals (Timofeeva *et al.*, 1997; Huang *et al.*, 1998). Thus, the reduced CRF mRNA expression in the paraventricular nucleus of SLOB rats is similar to that of food deprived normal rats. It would therefore be interesting to measure CRF mRNA levels in food deprived SLOB rats. As discussed in chapter 4, a relationship also exists between CRF, SS and GH secretion (Rivier and Vale, 1985), therefore it is also possible that CRF expression in SLOB rats may be altered in an attempt to increase endogenous GH secretion. Measuring CRF mRNA expression in *dwarf* rats as well as in the Transgenic Growth Retarded (Tgr) rat, two models of GH deficiency, may indicate if such a role is present.

7.6 A cause for the SLOB phenotype?

Data presented in Chapter 6 has generated some ideas as to the potential underlying genetic cause of the SLOB phenotype. JP59 rats who have the same transgene insert as SLOB rats do exhibit an obesity phenotype although this phenotype is not the same as that seen in SLOB rats. Studies on the expression and regulation of *5'OT-EST*,

although so far have been few, demonstrate this gene as having a potential role in energy homeostasis and reproductive status. In view of these findings and those in SLOB mice, I feel it is most probable that the SLOB phenotype arises due to a combination of various factors rather than a single cause. These factors are GH deficiency, expression of truncated *5'OT-EST*, and the site of integration of the SLOB transgene. Although I showed in chapters 3 and 5, experiments that indicate isolated GH-deficiency is most likely not the cause of SLOB obesity, GH-deficiency may still contribute to modifying certain aspects of the SLOB phenotype such as fat distribution and lipolytic activity. The fact that JP59 rats do show some obesity phenotype indicate that products from the transgene including *5'OT-EST* are responsible for the similar aspects of the phenotype seen in both SLOB and JP59 rats. The fact that the JP59 obesity phenotype is dissimilar from the SLOB phenotype in some aspects indicates that the number of copies of the transgene or the site of integration of the transgene in SLOB rats may also be contributing to the SLOB phenotype. Therefore, it is clear that identifying the exact cause of the SLOB obesity phenotype will be an arduous one. As I was successful in breeding from very young SLOB male and female rats and thus generating potential homozygous pups, it may now be possible to breed the SLOB line to homozygosity, as was done with the JP59 line of rats. If a gene-dosage effect of the transgene is responsible for how the phenotype progresses, it would be interesting to see if homozygous male and female SLOB rats develop obesity to a much larger extent than hemizygous SLOB rats. This result would strengthen the idea of products from the transgene and site of integration as contributory factors for the cause of the SLOB phenotype. Other approaches are to generate further transgenics, and to investigate the function of *5'OT-EST* protein when transfected into cells.

Monogenic mouse models have been important in suggesting candidate genes for evaluation in human populations (Weigle and Kuijper, 1996). However, the fact that only few cases of human obesity caused by a single gene disorder have been identified, suggests that most cases of obesity in the human population arise from polygenic disorders, and thus explains the great difficulty in genetically segregating the obese syndrome. As described in Chapter 1, only a few individuals have been found to have a mutation in the gene coding for either leptin or the leptin receptor (Montague *et al.,* 1997b; Clement *et al.,* 1998; Strobel *et al.,* 1998). The *agouti*

model which implicates the hypothalamic MC4 receptor in bodyweight regulation, has led to the identification of a frameshift mutation in MC4-R leading to a dominant form of morbid obesity (Ollman *et al.*, 1997; Vaisse *et al.*, 1998). Mutations have also been found in individuals with extreme early-onset obesity, in POMC, the precursor for the natural ligand of MC4-R (Krude *et al.*, 1998). In addition, a missense mutation in the prohormone processing enzyme carboxypeptidase E which causes obesity in the *fat/fat* mouse, and mutations in the related human peptidase PC1 have been described (Naggert *et al.*, 1995; Jackson *et al.*, 1997). Mutations in the human homologue of *tub* have yet to be reported but recent studies have shown that the *tub* family member *tulp1* carries mutations leading to autosomal recessive retinitis pigmentosa, consistent with the photoreceptor degeneration described in *tub* mice (Noben-Trauth *et al.*, 1996; Hagstrom *et al.*, 1998). Although these mutations have aided in understanding the biology of human obesity, cumulatively these mutations are rare and do not explain genetic predisposition to obesity observed in most populations. The SLOB rat seems to offer a model in which a complex genetic insertion event may cause a collection of abnormalities which result in an overall obesity and infertility phenotype. Therefore this model is more representative in this respect in terms of the human obese population.

7.7 Final Summary

Few rat models of obesity exist. Apart from the *Zucker* rat, most characterised rodent models of obesity are mouse models (Weigle and Kuijper, 1996). Rat models allow greater physiological manipulation, and therefore the SLOB rat will in the future provide advantages over the existing mouse obesity models. The SLOB rat is also the first rodent obesity model to show late onset male specific visceral obesity, without diabetes. Therefore this model serves as a great tool for testing a variety of compounds to exclusively treat visceral obesity.

When I started my PhD., I aimed to address the nature of the sexually dimorphic phenotype in SLOB rats, characterise SLOB physiology in terms of lipid biochemistry, diabetic status and hypothalamic neuropeptide analysis, and finally to try to explore the underlying genetic cause of the SLOB phenotype. My studies showed that obesity could be induced in ovariectomised female SLOB rats and reversed by oestrogen. Although obese male rats are infertile, very young male rats could be mated and therefore a homozygous SLOB rat line may now be possible. As well as ovariectomy, high fat feeding also induced an obesity phenotype in young SLOB females, as was also evident in high fat fed young SLOB males. Lipid biochemistry analysis revealed a number of abnormalities including increased VLDL secretion, reduced lipolytic activity and increased supra-renal LPL activity. Hypothalamic neuropeptide analysis showed changes in NPY, CRF and *tub* mRNA expression. Genetic studies leading to the identification of *5'OT-EST* and my expression studies of this gene and further physiological analysis of JP59 rats and SLOB mice, implicate this gene as a candidate for SLOB etiology. These studies have provided a basis for significant further study into the function of *5'OT-EST*.

Whatever the underlying genetic cause of the SLOB phenotype this thesis has demonstrated that the SLOB rat is a unique model of obesity that will be valuable in evaluating future pharmacological and dietary strategies aimed at reducing and preventing the accumulation of visceral obesity.

Acknowledgements

This thesis would not have been possible without the help of many people at the NIMR. First, I would like to thank my supervisor Iain Robinson, for his continued support and encouragement throughout my PhD, and for putting up with my angry foot stamping moments. A huge thanks also to Paul Le Tissier for being such a wizard at Molecular Biology and being such an excellent other half of the SLOB team. Thanks to Danielle Carmignac for her excellent surgical skills, the use of her computer on many occasions (we finally have our own iMAC!) and for keeping me sane in times of stress and frustration. The man with all the answers really is Keith Fairhall, thanks Keith for everything, you're one in a million. Where would the SLOBs be without Emma Sparks, thanks Emma for being my sums lady, singing animal house companion and for processing all those hundreds of SLOB tails. Thanks also to Pamela Bennett for her expertise in *in situ*. Fredrik Frick, the swedish blonde, thanks for showing me that the biochemistry of lard can be fun! Marta Fuente, the spanish princess, thanks a million for all your help with the *in situs*, we had some laughs, also thanks for all your encouragement whilst I was writing my thesis. I am also grateful to Marie Caulfield and Dave Whitcutt in Biological Services for doing such an excellent job maintaining the SLOB rat line. Thanks also to Kathleen Mathers, lady of transgenic animals, for generating transgenic SLOB mice and for your help with sperm analysis in SLOB rats. Thanks to my second supervisor Pete Hindmarsh, for reminding me of the clinical aspect of my project and why the hard slog will be worth it in the end. Finally, thanks to other members of the Neurophysiology and UCL team, my family and friends for their support and for having faith in me, and especially to Dean who has been an absolute superstar!

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