The Interaction of Diet and adipose Glucocorticoid Action in Metabolic Syndrome

Tak Yung Man

Master of Philosophy

The University of Edinburgh

March 2010

Declaration

I declare that this thesis has been composed entirely by myself on the basis of work carried out by myself except where otherwise acknowledged. Also no part of this work has been submitted for any other degree or professional qualification.

Tak Yung Man

Acknowledgements:

I would like to thank Karen Chapmen, Nicholas Morton and Jonathan Seckl for the opportunity to work on this project which has proved an interesting and enjoyable experience. All have been very supportive and a special thanks to Karen for her time and patience during the stressful 'writing period', and for her motivation and guidance throughout the years.

Thanks also to all the staff in the Endocrine Unit for the advice and assistance throughout the years. A special thanks to Lynne Ramage who's help and guidance in teaching me various techniques proved invaluable throughout the project. Val Kelly for her generous help in creating some of the plasmids and guiding me through the transfections technique.

I was also glad to have the opportunity to work alongside Elaine Marshall who helped me laugh through the many ups and downs.

A special thank you to Julie Nixon/Zoi Michailidou for their friendship and sound advice...not forgetting the numerous shopping/caffeine fixes.

My newlywed husband Andrew, for his love & support and fulfilling all cooking duties during the hectic 'writing period', although not always successfully. Looking forward to the many more years of practice.

Dedicated with love to my parents.

Abstract

Metabolic syndrome, a combination of co-associated features including visceral obesity, dyslipidemia, hypertension, insulin resistance and increased risk of cardiovascular disease, has reached epidemic levels. Recent evidence has indicated a pathogenic role for elevated glucocorticoid (GC) levels within adipose tissue specifically, in obesity. Levels of the glucocorticoid regenerating enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD-1), which converts inactive GC into active forms within cells, are elevated in adipose tissues of obese humans. Transgenic overexpression of 11β-HSD1 in adipose tissue recapitulates metabolic syndrome in mice, including visceral obesity, perhaps because glucocorticoid receptor (GR) levels are higher in visceral than subcutaneous adipose tissue. Therefore, increased adipose GC action, especially in visceral adipose tissue, might explain the striking similarities between idiopathic metabolic syndrome and rare Cushing's syndrome of plasma GC excess.

One of the major contributors to obesity and metabolic syndrome is consumption of excessive amounts of dietary fat. Specifically, saturated fats are associated with obesity and insulin resistance whereas unsaturated fats are reported to ameliorate some aspects of metabolic disease. Interestingly, chronic high fat feeding in mice decreases adipose 11β-HSD1 expression, usually without changing expression in liver. The reduced glucocorticoid regeneration in adipose tissue may ameliorate metabolic disease. This study investigated the effect of diets enriched in saturated or unsaturated fats on adipose tissue glucocorticoid action, focusing on 11β-HSD1 and glucocorticoid receptor expression.

Two studies were carried out, one with pair-feeding (to control low-fat diet) and one with *ab libitum* feeding. In both studies, a diet high in saturated fat (stearate) resulted in significant weight loss whereas a diet consisting of 58% mixed fats resulted in significant weight gain. In the *ab libitium* study (but not in pair-fed animals) both mono-unsaturated (oleic) and poly-unsaturated (safflower) fat-enriched diets induced weight gain and hyperinsulinaemia and lowered levels of adipose11β-HSD1 when compared to control diet. These data suggest that diets high in saturated fats elevate adipose tissue and liver 11β-HSD1 levels which might contribute detrimentally to, or

offset, any improvements in the systemic metabolic profile. Conversely, unsaturated fats cause an adipose-specific down-regulation of 11β-HSD1.

In addition, genetic evidence from human and animal studies has shown that regulation of glucocorticoid production and receptor density may be an important factor determining visceral adiposity. The role of adipocyte GR levels in determining visceral adiposity was investigated. Two types of transgenic mice were generated with altered GR levels in adipocytes using the adipocyte-specific aP2 promoter; one with a "sense" rat GR cDNA (2 independent lines; D- and B-FSG) and one with an "anti-sense" rat GR-cDNA (GR-5). Initial characterisation of D- and B-FSG mice showed expression of rat GR mRNA in both female and male transgenic mice with D-FSG showing a higher copy number than B-FSG. In D-FSG transgenic mice, female mice of the F_1 generation had greater adipose tissue mass than non-transgenic littermates. A 6-month high fat study was then carried out on line D-FSG. However, no differences in body weight, food intake, adipose tissue weight or blood pressure were found in either males or females. Further investigations of the levels of rat GR mRNA expression within various adipose tissues showed variable transgene expression between different generations of B- and D-FSG lines and even between adipose tissues within the same mouse. In contrast, following 6-months high fat diet, female mice under-expressing GR within adipose tissues (GR-5) showed a significant reduction in body weight and subcutaneous adipose tissue weight compared to their non-transgenic littermates. These data suggest that adipose GR density is an important determinant of visceral adiposity, in a sex-specific manner.

Collectively, the data in this thesis support a role for GR density and GC metabolism in adipose tissue as important determinants of adiposity in mice. Drugs that manipulate these targets are of therapeutic interest.

Table of Content

List of Figures

List of Tables

Lists of Abbreviations

11β-HSD	11β-hydroxysteroid dehydrogenase
11-DHC	11-dehydrocorticosterone
aP2	Adipocyte fatty acid binding protein
ACTH	Adrenocorticotropic hormone
AF	Activating function
AU	Arbitrary unit
BAT	Brown Adipose tissue
BSA	Bovine serum albumin
BMI	Body Mass Index
b.p.	basepair
BW	Body weight
CIP	Calf intestinal phosphatase
Cort	Corticosterone
CNS	Central nerve system
CRD	Cortisone reductase deficiency
CRH	Corticotrophin releasing hormone
depc	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
Epi	Epididymal
ER	Endoplasmic reticulum
G-6-pase	Glucose-6-phosphate
Gon	Gonadal
GC	Glucocorticoid
GR	Glucocorticoid receptor
GREs	Glucocorticoid response elements
GTT	Glucose tolerance test
H6PDH	Hexose-6-phosphate dehydrogenas

HF	High fat
HPA	Hypothalamic-pituitary-adrenal
Hsp	Heat shock protein
IRS	Insulin receptor substrate
ITT	Insulin tolerance test
IL-	Interleukin
LF	Low fat
LPS	Lipopolysaccharide
LXR	Liver X receptor
MCP-1	Monocyte chemotactic protein-1
Mes	Mesenteric
MMTV (LTR)	Mouse mammary tunour virus (long terminal repeat)
mRNA	messenger RNA
MR	Mineralocorticoid receptor
NAP+	nicotinamide adenine dinucleotide
NEFA	Non-esterified fatty acid
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Non-tg	Non-transgenic
PAI-I	Plasminogen activator inhibitor type 1
PCR	Polymerase chain reaction
Per	Perirenal
PEPCK	Phosphoenolpyruvate carboxykinase
PNMT	phenylethanolamine-N-methyl transferase
PPAR	Peroisome proliferator-activated receptor
RNA	Ribonucleic acid
RPA	RNase protection assay
PKB	Protein kinase B
RT	Room temperature
RP	Retroperitoneal
Sc	Subcutaneous
SPA	Scintillation proximity assay
SREBP	Sterol-regulatory element-binding protein

TAT	Tyrosine aminotransferase
TEMED	N,N,N',N'-Tetramethyl-1-,2-diaminomethane
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
TG	Triglyceride
TLR	Toll-like receptor
TZD	Thiazolidinediones
Tg	transgenic
WAT	White adipose tissue

1 Introduction

Obesity occurs when energy intake into the body exceeds energy expenditure, resulting in undesirable weight gain. The severity of obesity is indicated by body mass index (BMI), with BMI of 25 and above classified as overweight and over 30 as obese (James, Rigby et al. 2006). Obesity, especially visceral obesity (central, android, intra-abdominal fat), has been associated with various health complications including hypertension, type 2 diabetes, dyslipidemia, insulin resistance and an increased risk of cardiovascular disease. Together, these risk factors are collectively referred to as the Metabolic Syndrome (reviewed in (Reaven 2006). In the last 20 years, obesity has reached epidemic proportions in western countries and is increasingly prevalent in developing countries. In the UK, >35% of the population is currently estimated to be clinically obese (Rennie and Jebb 2005).

1.1 Contribution of diet to obesity, metabolic syndrome and insulin resistance

Imbalanced energy intake (e.g. high intake of energy dense foods rich in simple sugars and high in fat) and energy expenditure (e.g. lack of exercise) results in obesity, with fat accumulation mainly in adipose tissue. These changes usually result in reduced body tissue sensitivity toward insulin and the development of insulin resistance, with a strong association between increased adiposity and insulin resistance (Fujioka, Matsuzawa et al. 1987). The obesity epidemic observed in modern society has occurred so rapidly that environmental factors must be to blame. One recognized contributory factor in this process has been the widespread increase in the availability and consumption of highly palatable diets, high in fat, sugar and salt. Whilst there are rare genetic conditions that cause severe and largely uncontrollable food intake behaviour in humans (O'Rahilly et al., 2002), it is believed that these *obesogenic* foods short-circuit the natural satiety mechanisms found in normal people and thus contribute to the more common and idiopathic forms of obesity (Friedman 2000; Dallman, la Fleur et al. 2004; Morton, Cummings et al. 2006). Furthermore, certain types of fatty acids within the diet have a more profound effect, not only on normal satiety mechanisms, but also on the direct metabolic systems that control the physiological processes that handle and store

the excess nutrients in the body (Friedman 2000; Dallman, la Fleur et al. 2004; Morton, Cummings et al. 2006).

1.1.1 Inflammation and obesity

Increasingly, evidence is accumulating to show that in obesity, adipose tissue is hypoxic and "inflamed" (Hotamisligil 2006; Hotamisligil and Erbay 2008). The hallmarks of this chronic inflammatory state are increased macrophage content within adipose tissue in obesity and an increase in pro-inflammatory macrophage markers (Hotamisligil 2006; Hotamisligil and Erbay 2008). Adipocytes within adipose tissue act not only as energy stores for excess nutrients but also function as endocrine cells to regulate energy intake and expenditure. Adipocytes synthesize and secrete hormones as well as various inflammatory cytokines (adipokines) including Tumor necrosis factor-α (TNFα), Interleukin-6 (IL-6), leptin, monocyte chemotactic protein-1 (MCP-1) and plasminogen activator inhibitor type 1 (PAI-1) (reviewed in (Wellen and Hotamisligil 2005). During obesity, increased adipocyte volume and number is associated with elevated release of free fatty acids, which in excess can cause lipotoxicity within tissues (Wei, Wang et al. 2006). This results in activation of inflammatory signaling pathways such as Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (see below) as well as increased production of the pro-inflammatory adipokines, TNFα and IL-6 which have been linked with insulin resistance, hyperlipidemia and hypertension (Jump and Clarke 1999; Wajchenberg 2000). Recent studies in mice have also shown that obesity alters the balance between the T helper type 1 (Th1) and the humoral Th2 response, resulting in increased cytotoxic T cells and macrophages within adipose tissue, particularly in visceral fat depots (Lumeng, Maillard et al. 2009).

1.2 Dietary lipid

The development of metabolic syndrome is closely related to the kind of food being consumed. Dietary fat is an important macronutrient for growth and development and it functions as an important source of energy as well as playing an essential role in

maintaining the structure of cellular membranes, as fatty acids function as building blocks of the phospholipid bilayer. This is important as distortion of the membrane structure can affect its permeability and protein content. Over the last

decade, a number of studies have shown that dietary fats as well as endogenous lipids modulate cell differentiation and metabolism by affecting gene expression (Duplus, Glorian et al. 2000; Dussault and Forman 2000; Madsen, Petersen et al. 2005). Fatty acids derived from dietary fats have an important role in cell signaling and can also serve as precursors for steroid and postaglandin synthesis.

Triglyceride (TG) is the main form of lipid in the body and consists of a molecule of glycerol esterified with 3 fatty acids. TG is derived either from the diet or is synthesized endogenously; both fat and liver can synthesize and store TG. TG plays an important role in metabolism as an energy source and is transported within the body. High levels of plasma TG have been linked to increased risk of developing atherosclerosis, heart disease and stroke (Labreuche, Touboul et al. 2009). Plasma TG which is derived from dietary lipid is transported from the small intestine to the liver in the form of chylomicrons. In the liver it is packaged as very-low-density lipoprotein (VLDL) for export from the liver. In peripheral tissues, TG is released from VLDL and broken down by lipoprotein lipase (LPL) in a process called lipolysis; into glycerol and free fatty acids, which are then stored or utilized in tissues (Reshef, Olswang et al. 2003). Other than TG, dietary fats include cholesterol which is essential for maintaining cellular membrane structure and permeability. Like TG, cholesterol is also synthesized *de novo* if dietary intake is not sufficient to meet demands. Cholesterol is found in animal fat which is also high in saturated fat. Once ingested it is packaged by the liver as low density lipoprotein (LDL) and released into the bloodstream for transport to tissues, including adipose tissue for storage, and skeletal muscle for consumption. LDL is generally considered to be “bad” cholesterol as numerous studies have shown that elevated plasma LDL levels are associated with increased risk of cardiovascular disease (Beaven and Tontonoz 2006). In contrast, high density lipoprotein (HDL), the so-called “good” cholesterol, is associated with reduced risk of cardiovascular disease (Grundy 1998). HDL is responsible for transporting excess cholesterol from peripheral tissues to the liver in a process termed reverse cholesterol transport (Beaven and Tontonoz 2006). In liver, excess cholesterol is catabolized to bile acids (Beaven and Tontonoz 2006).

1.2.1 Dietary fats, saturation, chain length and bioactivity

In general, there are two types of dietary fats; saturated and unsaturated fat. The rate of fat absorption is influenced by the structure of the fatty acid (Fig. 1.1) (Ramirez, Amate et al. 2001). Saturated fats (having no double bonds in their structure) are usually found within animal products and processed food (e.g. fast food) which is high in hydrogenated oil. Saturated fat such as stearate, which is composed of 18 carbons with no double bonds (C18:0), are most strongly associated with the development of insulin resistance. Diets high in saturated fatty acids have been shown to impair glucose metabolism by decreasing glucose uptake in muscle and lipid packaging and export in the liver (Sampath and Ntambi 2005). In contrast, unsaturated fats (having one or more double bonds in their structure), found commonly in plants and oily fish-derived foods, are associated with improved insulin sensitivity and reduced risk of metabolic disease (Manco, Calvani et al. 2004). Dietary unsaturated fats can be sub-divided into mono-unsaturated (one double bond, e.g. oleic acid; C18:1) and poly-unsaturated fat (more than one double bond). Poly-unsaturated fats such as linoleic (n-6) and linolenic (n-3) acid that are found in vegetable oils are essential constituents of the human diet.

As well as influencing cellular metabolism by contributing fatty acids and metabolites, some lipids can directly influence gene expression through binding themselves or through metabolite binding to lipid-sensitive nuclear receptors including the peroxisome proliferator-activated receptors (PPARs) (see below) and the oxysterol receptors, the liver X receptors (LXRs) (see below). PPARs and LXRs as well as the receptor for glucocorticoids (GR), are expressed in cells and tissues with key roles in energy homeostasis and inflammation (Bookout, Jeong et al. 2006; Fievet and Staels 2009), suggesting that they are important regulators of both metabolism and inflammation.

1.2.2 Carbohydrates

Apart from dietary fat, dietary carbohydrates are also macronutrient that are critical in the development of obesity. Various studies have shown that a low carbohydrate diet is associated with improved insulin sensitivity, lower fasting plasma TG levels and weight loss (Bradley, Spence et al. 2009; Leite, DeOgburn et al. 2009).

In general, carbohydrates are subclassified into mono-, di- and poly-saccharide according to the number of sugar units present, with each having a different impact on

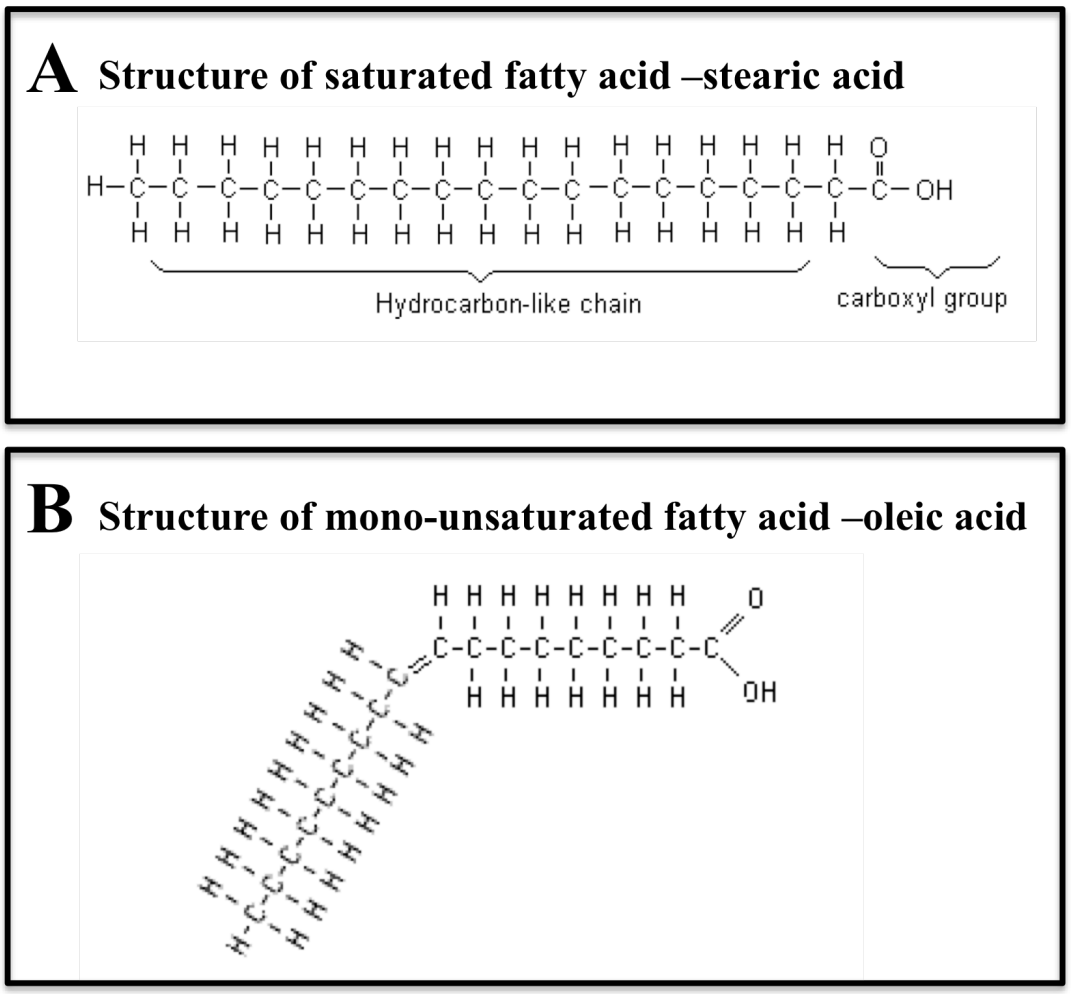

Figure 1.1 The molecular structure of saturated and mono-unsaturated fatty acids

Fatty acids essentially consist of carboxylic acid with long hydrocarbon chains. There are two types of fatty acids; saturated and unsaturated. (A) Saturated fatty acids have all bonding positions between carbons filled by hydrogens with no double bonds. An example of saturated fat, stearic acid, is shown above. (B) Unsaturated fatty acids have one or more double bonds between the carbons; oleic acid, an example of a mono-unsaturated fatty acid is shown.

Adapted from http://science.csustan.edu/stone/2090/stearic.htm and http://www.tripleomegaplus.com/drho/Chapter2a.html

metabolism. Under conditions of normal energy balance, dietary carbohydrates are absorbed in the intestine and delivered to the liver. Carbohydrates are also utilized by tissues as an energy source and can be temporarily stored by liver and muscle in the form of glycogen. However, when dietary carbohydrates become excess to energy requirements, the rate of *de novo* lipogenesis within the liver is increased, channeling excess glucose to fatty acids, which are subsequently combined with glycerol to form TG and are secreted as VLDL from the liver.

1.2.3 Regulation of lipid and glucose homeostasis

As mentioned above, dietary fats can affect gene expression and cellular differentiation associated with metabolism through different nuclear receptors; including the PPARs and LXRs as well as other transcription factors such as sterol-regulatory element-binding protein (SREBP). The PPARs regulate fatty acid oxidation, adipogenesis and inflammation (Jump and Clarke 1999; Madsen, Petersen et al. 2005). Three isoforms have been identified; PPARα, PPARγ and PPARδ (Barish 2006). PPARα is expressed predominantly in liver, kidney, heart and brown adipose tissue and is involved in regulation of lipid and carbohydrate metabolism (Barish 2006). PPARγ is widely expressed but is highest in adipose tissue, skeletal muscle and macrophages (Barish 2006). It is required for adipogenesis as well as regulation of lipid storage in adipocytes (Beaven and Tontonoz 2006; Sharma and Staels 2007). PPARδ is also widely expressed including in skeletal muscle, heart, small intestine and vascular smooth muscle cells (Beaven and Tontonoz 2006). Reflecting their importance in lipid metabolism, PPARs are the target for various hypolipidemic drugs including fibrates, which target PPARα and thiazolidinediones (TZD) which target PPARγ (Hansson and Libby 2006). Another nuclear receptor, LXRs were also found to play an important role in lipid, cholesterol and carbohydrate metabolism (Steffensen and Gustafsson 2004). Two isoforms exist, LXRα and LXRβ, both of which are activated by oxysterols (Barish 2006). LXRα is found principally in liver, adipose tissue, small intestine, adrenal gland and macrophages whereas LXRβ is expressed ubiqutinously (Barish 2006). Activation of LXRs promotes reverse cholesterol transport and eleminates excess cholesterol from the body (Fievet and Staels 2009). Mice that lack LXRα have been

shown to have impaired expression of proteins involved in fatty acid metabolism including the transcription factors SREBPs (see below) and stearoyl CoA desaturase (SCD-1), a key enzyme responsible for converting saturated fatty acids to mono-unsaturated fatty acids (Juvet, Andresen et al. 2003).

In addition to the nuclear receptors, an important role in lipogenesis and cholesterol homeostasis has been ascribed to SREBPs, which binds to sterol response elements in genes involved in cholesterol and lipid synthesis (Jump and Clarke 1999). Three isoforms have been identified; SREBP-1a and 1c are derived from the same gene by different promoter usage (Osborne 2000) and are important in promoting lipid synthesis by regulating genes required for lipid synthesis, while SREBP-2 regulates genes responsible for cholesterol homeostasis (Osborne 2000). NFκB, on the other hand is an important pro-inflammatory transcription factor that regulates expression of numerous cytokine genes (e.g. TNF-α and IL-6) and has been linked to inhibition of the insulin signaling pathway (Jump and Clarke 1999). NFκB is activated by many stimuli including activation of toll-like receptors (TLRs). TLR4 is expressed in adipose tissue and macrophages, as well as being activated by specific pathogen associated molecules such as lipopolysaccharide (LPS), it is activated by nutritional fatty acids contributing to the insulin resistance that occurs in response to obesity (Lee, Sohn et al. 2001; Shi, Kokoeva et al. 2006).

1.3 Glucocorticoid Hormones

Glucocorticoids (GC) are endogenous steroid hormones generated in the adrenal gland. Cortisol is the major active GC found in human and most mammals. Rodents produce a different active form, corticosterone. GCs have profound effects on physiological systems; maintaining homeostasis, energy balance and regulating the stress response (Datson, Morsink et al. 2008; McEwen 2008). GCs are also important for the development and maturation of tissues. They modulate intermediary metabolism, the immune system and influence behaviour (McEwen, Conrad et al. 1997; Chourbaji, Vogt et al. 2008; Kolber, Wieczorek et al. 2008). It is essential that GC secretion and action be tightly regulated in order to prevent detrimental effects. GC insufficiency (Addison's disease) is characterized by weight loss, muscle weakness, hypotension, hypoglycemia, skin hyperpigmentation and depression. On the other hand, Cushing's

Syndrome occurs after chronic exposure to high levels of circulating GCs, either due to excessive (unregulated) endogenous production of GC or administration of GC as medical treatment. The pathological characteristics of Cushing's disease include redistribution of body fat (visceral obesity), dyslipidaemia, hypertension, muscle wasting, thin skin, insulin resistance/type 2 diabetes, salt and water retention, osteoporosis, anxiety and depression.

1.3.1 Glucocorticoid Synthesis and Secretion

In humans, GCs are released from the adrenal gland in a diurnal pattern with peak levels in the morning and nadir levels in the evening and with an inverse pattern in rodents. GCs are synthesised in, and released from, the zona fasiculata / reticularis of the adrenal cortex, under the control of the hypothalamic-pituitary-adrenal (HPA) axis (Fig. 1.2). Excitation of the hypothalamus (e.g. by stress) causes the release of corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamic praventicular nucleus (PVN) into the portal vein. CRH and AVP are then transported to the anterior pituitary gland where CRH binds to corticotrophin releasing hormone 1 receptors and AVP to vasopressin receptor 1, which both stimulates the release of adrenocorticotrophic hormone (ACTH) into the circulation as well as increasing the synthesis of proopiomelanocortin (POMC), the precursor polypeptide. ACTH then binds to melanocortin receptor type 2 in the adrenal cortex to stimulate the synthesis and release of GC (Cole, Blendy et al. 1995; Buckingham 2006).

Plasma GC levels are tightly regulated by a negative feedback mechanism which mainly acts at the hypothalamus to inhibit CRH and AVP secretion and at the pituitary to suppress ACTH release and repress POMC transcription. The feedback mechanism is sensitive to increased levels of circulating GC and regulated by both glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (Reul and de Kloet 1985). GR predominantly regulates stress-induced cortisol secretion and the circadian peak, whereas MR regulates the basal circadian secretion of GC (Reul and de Kloet 1985).

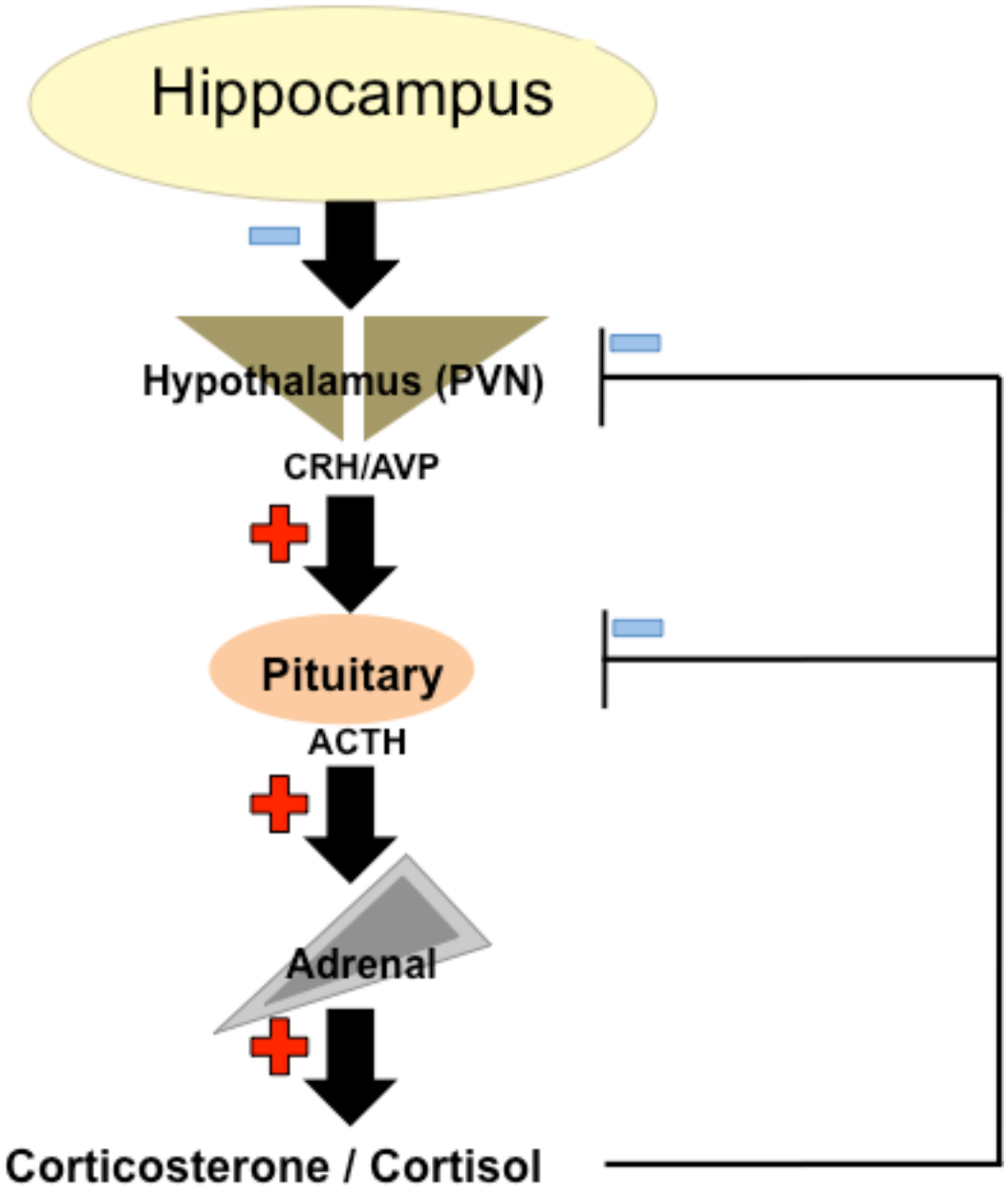

Figure 1.2 Regulation of glucocorticoid secretion in the hypothalamus-pituitary-adrenal (HPA) axis

Schematic diagram showing the HPA axis and the principal loci involved in glucocorticoid feedback control. Arrows with the "+" indicate positive actions leading to the production and secretion of glucocorticoid from the adrenal cortex. The secretion of glucocorticoid is auto-regulated by negative feedback (indicated by "-") which occurs at two levels; via suppressing the release of corticotrophin releasing hormone (CRH) from the hypothalamus and inhibiting adrenocorticotropic hormone (ACTH) release and synthesis in the anterior pituitary.

1.3.2 Mechanism of glucocorticoid action

GCs act on target cells through binding to intracellular receptors. As well as GR (also called the "type II" glucocorticoid receptor), GCs can also bind to MR (or the "type I" glucocorticoid receptor) (Bamberger, Schulte et al. 1996). GR has a lower affinity than MR and is glucocorticoid selective, whereas MR binds glucocorticoids (cortisol and corticosterone) as well as the mineralocorticoid, aldosterone. However, MR has a restricted distribution, being expressed mainly in classical mineralocorticoid target tissues such as the distal renal tubule and colon, as well as specific regions of brain and heart. In contrast, GR are widely distributed in the body. Like PPARs and LXR, both GR and MR belong to the superfamily of ligand-activated nuclear receptors (De Kloet, Vreugdenhil et al. 1998; Buckingham 2006). GR, like all the other nuclear receptors has 3 major domains (Fig. 1.3). The N-terminal domain contains "activating function" (AF)-1, required for activation of some target genes (Giguere, Hollenberg et al. 1986; Hollenberg, Giguere et al. 1987). A central highly conserved DNA binding domain contains two highly conserved zinc fingers, essential for DNA binding (Picard and Yamamoto 1987; Tsai, Carlstedt-Duke et al. 1988). The third domain of GR is the C-terminal ligand-binding domain which binds to hormonal ligands and contains a dimerization interface. It also contains an activation domain, AF-2, as well as sequences important for heat shock protein binding and silencing of the receptor (Hollenberg, Giguere et al. 1987; Dalman, Scherrer et al. 1991; Pratt 1993).

In the absence of ligand, GR is found in the cytoplasm as part of a multi-protein complex together with various heat shock proteins (e.g. hsp90, hsp70, hsp56, hsp40) and immunophilins (e.g. Cyp40, FKBP52) (Hollenberg and Evans 1988; Webster, Green et al. 1988; Hutchison, Scherrer et al. 1993). When unliganded, the GR/HSP complex exchanges constantly between the nucleus and cytoplasm (Hache, Tse et al. 1999). The binding of glucocorticoid to GR results in a conformational change in the receptor; GR dissociates from the HSP multi-protein complex and the receptor protein becomes hyper-phosphorylated (Dalman, Sanchez et al. 1988; Hoeck and Groner 1990). The nuclear localization signals within the ligand-binding domain of the receptor are unmasked, and the GC/GR complex translocates into the nucleus where it binds to glucocorticoid response elements (GREs) to stimulate or repress the

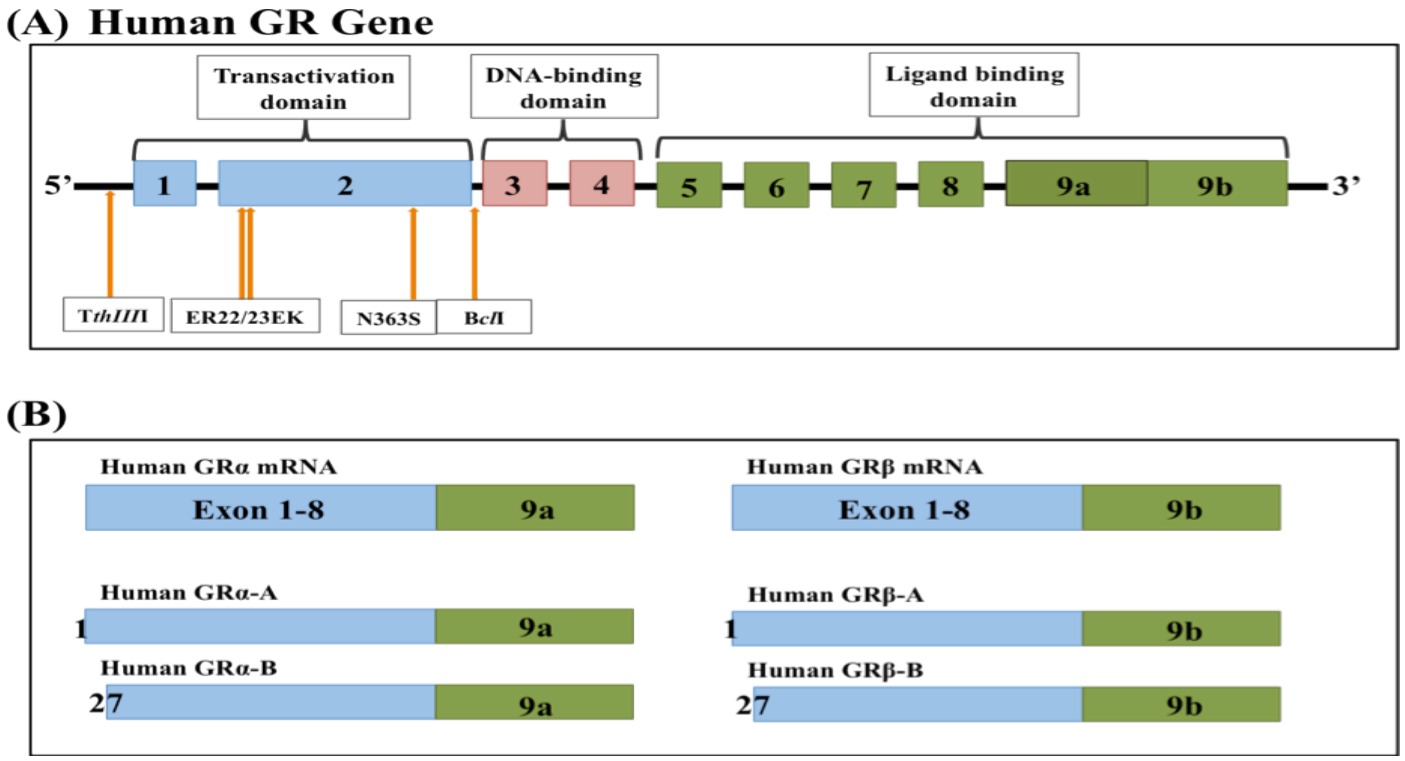

Figure 1.3: Human Glucocorticoid receptor gene structure

(A) Schematic diagram showing the structure of the human GR gene with the transactivation domain in blue, DNA binding domain in pink, ligand binding domain in green and the sites of key GR polymorphisms referred in the text shown by orange arrows. (B) Alternative splicing results in the formation of three isoforms; human GRα, GRβ and GRγ; GRγ has an extra amino acid in DNA binding domain (not shown for clarity), both GRα and GRβ contain the same exons 1-8 but different versions of exon 9, resulting in different C termini in the encoded proteins (see text for details). Both isoforms have two subtypes; GR-A and GR-B, as a result of alternative translation initiation (see text for detail). Adapted from (Schaaf and Cidlowski 2003)

transcription of target genes (Bamberger, Schulte et al. 1996; Schaaf and Cidlowski 2002; Lu and Cidlowski 2004).

There are multiple GR isoforms, all derived from a single gene and generated by alternative mRNA splicing and alternative translation initiation (Fig. 1.3B) (Lu and Cidlowski 2004; Lu and Cidlowski 2006). Each of the isoforms acts differently and may have a different role in regulating GC action within cells. GRα is the hormone-responsive active form and consists of 777 amino acids in humans (Weinberger, Hollenberg et al. 1985). GRβ is generated by alternative splicing of exon 9 resulting in a truncated C-terminal domain which does not bind ligand (Schaaf and Cidlowski 2002). Both proteins contain the same N-terminal and DNA binding domain, but differ in the C terminus, with GRα containing a 50 amino acid sequence and GRβ containing a unique 15 amino acids sequence (Schaaf and Cidlowski 2002). GRβ has been reported to act as a dominant negative repressor of GRα activity by forming GRα/GRβ heterodimers and is postulated to be increased in GC-resistant disease (Schaaf and Cidlowski 2002). However, although GRβ mRNA is found in humans it does not exist in rodents and its relevance to human disease is controversial (Oakley, Webster et al. 1997; Otto, Reichardt et al. 1997). GRγ is another GR splice variant which has an additional arginine located between exon 3 and 4 (Rivers, Levy et al. 1999). This variant has been reported to repress GC activation in a carcinoma cell line (Haarman, Kaspers et al. 2004).

In addition, each GR splice isoform generates additional subtypes of GR protein via alternative translation initiation (Fig. 1.3B) (Lu and Cidlowski 2004). This results in the generation of GR-A (initiating at the first ATG; Met-1) and GR-B, which initates at the second ATG (Met-27), located 71 bp 3' of the first start codon (Yudt and Cidlowski 2001). Recently, more isoforms; GR-C1, C2, C3, D1, D2 and D3 have been identified with varying lengths at the N-terminus (Lu and Cidlowski 2006).

The primary determinants of GC action are GR density and ligand availability, the latter determined by the activity of the HPA axis (as described above) and the cellular activity of 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes (described below) (Seckl, Morton et al. 2004).

1.3.3 Physiological effects of glucocorticoids

GCs affect many physiological processes. They are well known for their anti-inflammatory and immunosuppressive actions and are the most commonly used drugs to treat a range of inflammatory and autoimmune disorders including asthma and rheumatoid arthritis (McEwen, Conrad et al. 1997). However, their use as anti-inflammatory agents is limited by their effects on metabolic processes (reviewed below).

1.3.3.1 Immune response

GCs influence both innate and adaptive immune responses (McEwen, Conrad et al. 1997). GCs promote neutrophilia but also cause lymphopenia and induce apoptosis in lymphocytes, eosinophils and basophils (McEwen, Conrad et al. 1997). Acutely, they exert both anti-inflammatory and pro-inflammatory effects on macrophages and they also alter differentiation and activation state of leukocytes, including macrophages (McEwen and Magarinos 1997). GCs modulate genes involved in the initial immune response by suppressing Th1-cellular immunity and promoting Th2-humoral immunity (Mittelstadt, Galon et al. 2002). GCs also reduce the expression of various pro-inflammatory cytokines and chemoattractants (e.g. IL-1, TNFα, IL-6, IL-8, IL-3 and IL-4) and increase the expression of anti-inflammatory cytokines (e.g. IL-10 and transforming growth factor-β (TGF-β)) (Franchimont 2004).

1.3.3.2 Metabolism

The metabolic actions of GCs are closely related to hepatic and peripheral insulin resistance, dyslipidemia, hyperglycemia and obesity. During fasting and exercise, when glucose and insulin levels are low, GCs increase blood glucose levels by inhibiting glucose uptake and utilization in peripheral tissues, by antagonizing anabolic insulin action and by promoting hepatic glucose production (Dallman, la Fleur et al. 2004). Such actions illustrate that GC play an important role in glucose homeostasis. GCs also stimulate insulin secretion *in vivo* through central nervous system (CNS) actions that remain incompletely understood (Dallman, Pecoraro et al. 2005)

However, they also act directly on pancreatic β cells to inhibit insulin secretion, as shown in mice over-expressing GR within pancreatic β cells (Delaunay, Khan et al. 1997). In addition, they impact upon insulin signaling by stimulating accumulation of the sphingolipid ceramide in liver and adipose tissue which has been shown to inhibit insulin signaling by inhibiting Akt/protein kinase B (PKB) phosphorylation and activation (Summers and Nelson 2005), as well as reducing glucose transport via translocation of membrane bound glucose transporter GLUT4 to an internal location (Horner, Munck et al. 1987; Delaunay, Khan et al. 1997). GCs also increase hepatic glucose production by stimulating hepatic gluconeogenesis through induction of transcription of phosphoenolpyruvate carboxykinase (PEPCK), tyrosine aminotransferase (TAT) and glucose-6-phosphatase (G-6-Pase) (Hanson and Reshef 1997). Mice which lack GR within hepatocytes become hypoglycemic during fasting and show lower expression of genes involved in gluconeogenesis (Opherk, Tronche et al. 2004). In addition, GCs are also associated with hepatic fat accumulation as demonstrated in adrenalectomised rats treated with dexamethasone which show increased triglyceride (TG) synthesis, reduced fatty acid oxidation and accumulation of lipid within liver (Cole, Wilcox et al. 1982).

In adipose tissue, GCs affect lipid metabolism and reduce insulin sensitivity. Patients with Cushing's syndrome show redistribution of body fat from peripheral to central depots (Walker and Andrew 2006). In addition, GCs exert different actions on different adipose depots. For example, in central/visceral fat depots, GCs promote pre-adipocyte differentiation and increase lipogenic activity and cellular hypertrophy (Ailhaud, Amri et al. 1991; Samra, Clark et al. 1998). In peripheral fat depots (such as subcutaneous fat), GCs increase lipoprotein lipase (LPL) mRNA expression and stimulate lipolysis by inducing hormone sensitive lipase, resulting in the breakdown of TG and the release of fatty acids and glycerol from adipose tissue (Slavin, Ong et al. 1994; Mattsson and Olsson 2007). In brown adipose tissue, GCs decrease thermogenesis by down-regulating uncoupling protein (UCP-1) expression (Sell, Deshaies et al. 2004) . Furthermore, GCs also been shown to influence the insulin signaling cascade within adipose tissue. Recent studies have shown GC treatment down-regulates insulin receptor substrates (IRS)-1 and-2 levels, blunting the insulin-signaling pathway (Caperuto, Anhe et al. 2006; Gathercole, Bujalska et al. 2007). In

addition, markers of insulin signaling, such as phosphoinostitide 3 (PI3) kinase activity and Akt phosphorylation, were also suppressed by GC in primary rat adipocytes (Buren, Liu et al. 2002; Buren, Lai et al. 2008). Studies have also shown that GCs influence insulin signaling in a tissue-specific manner as they down-regulate insulin signaling proteins and glucose uptake capacity in omental but not in subcutaneous human adipocytes (Lundgren, Buren et al. 2004). In skeletal muscle, GCs modulate glucose metabolism and protein degradation. These biological effects are usually apparent either during fasting or stress. Under these conditions, increased levels of GCs inhibit glucose uptake, suppress muscle protein synthesis and induce protein degradation, mobilizing amino acids from muscle protein to provide substrate for energy generation via hepatic gluconeogenesis (Wing et al 1993). These catabolic effects of GCs have been associated with muscle atrophy, as observed in patients with Cushing's syndrome or those on steroid therapy (Schakman, Gilson et al. 2008).

1.3.4 Developmental and other effects of glucocorticoids

GCs also have a fundamental role in the development and maturation of organs. They are essential for foetal lung maturation (Garbrecht, Klein et al. 2006) and mice that are homozygous for a targeted disruption of the GR gene (GR^{hypo}) die soon after birth due to severe lung atelectasis (Cole, Blendy et al. 1995). GC, are essential for the expression of the adrenaline-synthesizing enzyme, phenylethanolamine-N-methyl transferase (PNMT) in the adrenal medulla (Michelsohn and Anderson 1992). In addition, GCs are reported to affect the proliferation of adrenergic cells and suppress neuronal markers in sympathoadrenal progenitors, shifting them towards a chromaffin cell phenotype (Finotto, Krieglstein et al. 1999). However, both GR^{hypo} and GR^{null} mice were shown to have normal numbers of adrenal chromaffin cells, despite the loss of GR-mediated signaling, but had no expression of PMNT (Finotto, Krieglstein et al. 1999). Other than development, GCs also have an important role in stress response and in behaviour. GR is widely expressed within the brain (including in the hippocampus, amygdala, hypothalamus, thalamus, cerebellum and cortex) with highest expression found in

amygdala and the hippocampus, both areas important for memory, learning, mood and behaviour (McEwen 2005).

Under normal conditions, GC levels are tightly regulated by the HPA axis. Physical or emotional stress activates the HPA axis, increasing GC secretion. In the short term, this is a protective mechanism as GCs suppress inflammation and increase the availability of energy. But in the long term, high GC levels result in structural remodeling in the brain (Johnson, Farb et al. 2005). Cushing's patients with elevated GC levels exhibit memory deficit, anxiety and depression (Sonino, Fava et al. 1993). Conversely, chronic GC deficiency is associated with aggressive behaviour (Kim and Haller 2007).

1.3.5 Glucocorticoid receptor transgenic mice

To elucidate the various and tissue-specific physiological roles of GR, a number of transgenic mouse models have been generated. The first GR transgenic mouse model was created by expressing antisense GR RNA under the control of a human neurofilament promoter (Pepin, Pothier et al. 1992). This resulted in ~50-70% reduction in GR levels within the brain and ~40% reduction elsewhere, including within the liver (Pepin, Pothier et al. 1992). These mice exhibited HPA axis hyperactivity and developed obesity, with increased fat mass and plasma TG levels, despite reduced food intake (Pepin, Pothier et al. 1992; Richard, Chapdelaine et al. 1993). As this model exhibited different degrees of "knockdown" of GR between tissues and only displayed a partial reduction in GR levels within tissues (Pepin, Pothier et al. 1992), the actual impact of GR function could not be fully investigated. Later, mice homozygous for a hypomorphic allele of GR (GR^{hypo}) were created by insertion of a neomycin phosphotransferase cassette into exon 2 of the GR gene (Cole, Blendy et al. 1995). This resulted in 90% of the homozygous mice dying at birth with respiratory failure (Cole, Blendy et al. 1995). However, 10% of the homozygous mice survived and displayed GC insensitivity, a hyperactive HPA axis and adrenal hypertrophy (Cole, Blendy et al. 1995; Cole, Myles et al. 2001). The variability in phenotype was later shown to be due to incomplete inactivation of the GR gene (Cole, Myles et al. 2001). To ensure complete inactivation of GR, mice were generated by deleting exon 3 which encodes part of

the DNA binding domain (GR ^{null}). All of these mice died at birth due to respiratory failure as a result of lung atelectasis (Finotto, Krieglstein et al. 1999). Since then, various transgenic mouse models (summarized in Table 1.1) including conditional (tissue-specific) deletion of GR have been created to overcome the neonatal lethality of the complete inactivation of GR.

1.3.6 Polymorphisms of glucocorticoid receptor

A number of polymorphisms have been identified within the GR gene locus, which are associated with altered GR sensitivity, altered hypertension, body fat distribution, body composition and metabolic profile (van Rossum and Lamberts 2004; Gross and Cidlowski 2008).

The BclI polymorphism is a restriction fragment length polymorphism located in intron 2, in which a G to C polymorphism occurrs at nucleotide 646 resulting in fragments of 2.3 and 3.9 kb (van Rossum, Russcher et al. 2005). The C allele is considered the wide-type allele as it occurs more frequently (65%) (van Rossum and Lamberts 2004).This polymorphism is associated with increased GC sensitivity (Fleury, Beaulieu et al. 2003; van Rossum, Koper et al. 2003). When compared to homozygous GG carriers, GC carriers have increased visceral fat and are hypertensive (Watt, Harrap et al. 1992; Panarelli, Holloway et al. 1998; Rosmond, Chagnon et al. 2000). Another polymorphism that has been associated with increased GC sensitivity is the N363S polymorphism (Koper, Stolk et al. 1997; Huizenga, Koper et al. 1998). This single nucleotide polymorphism (SNP) is located in codon 363 within exon 2 altering ATT to AGT (Huizenga, Koper et al. 1998), resulting in a replacement of asparagine with serine. This polymorphism is located in the N terminus of the receptor and has been linked with higher body mass index (BMI), tendency to lower bone mineral density and increased insulin sensitivity with dexamethasone (Huizenga, Koper et al. 1998; Lin, Wang et al. 2003; Roussel, Reis et al. 2003). However, the impact of this polymorphism is controversial as some studies have found no association with increased BMI or increased GC sensitivity (Echwald, Sorensen et al. 2001; Rosmond, Bouchard et al. 2001)

The ER22/23EK GR polymorphism affects codons 22 and 23 within exon 2 of GR (van Rossum, Feelders et al. 2004). This polymorphism consists of two

Table 1.1 GR transgenic mice and their phenotype

Mouse	GR expression	Phenotype	Ref
GR^{hypo}	Target insertion mutation in exon 2	• Most die at birth • Abnormal adrenal development • Normal thymus and normal thymocyte development • Glucocorticoid resistant • Altered proportions of lung epithelial cells • Reduced cateolamine levels • Hyperactive HPA axis (↑ACTH, CRH mRNA, POMC mRNA) • Elevated plasma corticosterone	(Cole, Blendy et al. 1995; Purton, Boyd et al. 2000; Cole, Myles et al. 2001; Cole, Solomon et al. 2004)
GR^{null}	Target deletion of exon 3	• All die at birth	(Finotto, Krieglstein et al. 1999)
GR-*i* mice	Antisense GR mice	• Obese • Increased body fat content • Increased in plasma TG levels • Unaltered plasma glucose and insulin levels • Reduction in food intake and energy expenditure • Cognitive impairment • Partial block in T cell development • Altered thymic development	(Pepin, Pothier et al. 1992; Richard, Chapdelaine et al. 1993; Montkowski, Barden et al. 1995; Morale, Batticane et al. 1995;

Mouse	GR expression	Phenotype	Ref
GR-*i* mice (Cont.)		• Decreased locomotion in dark phase • Sustained increase in TNFα after endotoxin • Enhanced ACTH response to stress • Resistant to experimental autoimmune encephalomyelitis	Linthorst, Karanth et al. 1999; Sacedon, Vicente et al. 1999; Marchetti, Morale et al. 2002)
GR-TKO	Reduced GR level in immature thymocytes using antisense rat GR driven by the lck promoter	• 50% GR mRNA reduction in thymus • Reduction in thymus size • Reduction of $CD4^+CD8^+$ cells • Normal plasma corticosterone levels	(King, Vacchio et al. 1995; Tolosa, King et al. 1998)
RIP1-GR	Overexpression of GR in pancreatic β cells using the insulin promoter	• Reduced glucose tolerance but normal fasting blood glucose • Reduced insulin response to glucose load	(Delaunay, Khan et al. 1997)
GR^{NesCre}	Lack of GR within the central nervous system (using Cre/loxP system, nestin-Cre)	• Elevated CRH levels in the PVN • Elevated plasma corticosterone levels • Reduced anxiety behavior and altered stress response • Increased % of body fat before weaning • Reduction in body weight • 75% reduction in fat mass at adulthood	(Tronche, Kellendonk et al. 1999; Kellendonk, Eiden et al. 2002)

Mouse	GR expression	Phenotype	Ref
GR ^{NesCre} (Cont.)		• Altered fat distribution • Reduced metabolic efficiency and food intake • Increased plasma insulin and leptin levels in adult mice	
FBGRKO	Forebrain-specific disruption of GR using Cre/loxP system; (forebrain-specific CamKIIα promoter-cre)	• 60% reduction in forebrain GR levels at 2 months of age • Complete deletion of GR at 4-6 months • Hyperactive HPA axis with increased plasma ACTH and corticosterone levels • Normal CRH levels • Elevated AVP mRNA levels in the PVN • Exhibit depression-like behaviour • Reduction in hippocampal neurogenesis	(Boyle, Brewer et al. 2005; Boyle, Kolber et al. 2006; Kronenberg, Kirste et al. 2009)
GR^{dim}	Targeted point mutation in the GR which prevents homodimerisation action of GR	• Mice viable at birth • Increase in POMC and ACTH levels • Normal adrenal development • Elevated plasma corticosterone levels • Reduced expression of some GC-activated genes • Some anti-inflammatory actions of GR intact • Abnormal wound repair	(Reichardt, Kaestner et al. 1998; Tuckermann, Reichardt et al. 1999; Oitzl, Reichardt et al. 2001; Gorospe, Naidu et al. 2002; Tuckermann, Kleiman et al. 2007)

Mouse	GR expression	Phenotype	Ref
$GRN^{+/-}$ (Cont.)		• Resistant to immunosuppressive effects of dexamethasone in experimental autoimmune encephalomyelitis (EAE)	et al. 2008)
YGR mice	Overexpression of GR with yeast artificial chromosome (2 extra copies)	• ~2 fold elevation in GR mRNA within the hippocampus • Reduction in plasma corticosterone levels • Reduction in plasma corticosterone under stress • Resistant to stress • Enhanced survival following endotoxin • Increased thymocyte sensivity to pro-apoptotic effects of GC	(Reichardt, Tuckermann et al. 2000; Ridder, Chourbaji et al. 2005)
Lck-AS-GR	Underexpression of GR within thymus and T cells (Antisense rat cDNA under the control of the proximal lck promoter	• Reduced thymocyte number • Increased apoptotic activity in thymus	(Pazirandeh, Xue et al. 2002)
Lck-s-GR	Overexpression of GR within thymus and T cells Sense rat cDNA driven by the proximal lck promoter	• Increased number of thymocytes • Increased T cell number	(Pazirandeh, Xue et al. 2002)

Mouse	GR expression	Phenotype	Ref
hCD2-GR	Conditional overexpression of rat GR in thymocytes using a tetracycline-controlled CD2 promoter	• Reduction in thymocyte number • Increase in thymocyte apoptosis	(Pazirandeh, Jondal et al. 2005)
TGRKO	Using Cre/LoxP system to generate T cell-specific deletion of GR (Lck promoter-Cre)	• Increased mortality following immune activation • Increased susceptibility and worse disease in EAE	(Brewer, Kanagawa et al. 2002; Brewer, Khor et al. 2003; Wust, van den Brandt et al. 2008)
DT mice	Overexpression of human GR in heart using tetracycline-inducible system in conjunction with cardiac-specific αMHC promoter	• Abnormal electrocardiogram • Exhibit atrio-ventricular block in electrical coupling	(Sainte-Marie, Nguyen Dinh Cat et al. 2007)

Mouse	GR expression	Phenotype	Ref
GR_{M610L}	Knock-in Human GR Poi§nt mutation that increase sensitivity for GCs	• Reduction in plasma corticosterone and ACTH • Reduction in adrenal size • Increased sensitivity to GCs	(Zhang, Geller et al 2009)

linked, single nucleotide changes with no change in amino acid at codon 22 (GAG to GAA and a change from arginine (R) to lysine (K) in codon 23 (Koper, Stolk et al. 1997; van Rossum, Koper et al. 2002).. *In vitro* studies have suggested that the ER22/23EK polymorphism alters the mRNA structure, favouring translation initiation at AUG-1 (Met-1) rather than AUG-2 (Met-27) (see section 1.3.2), suggesting that more of the less transcriptionally active GR-A isoform is made from individuals with this polymorphism (Russcher, van Rossum et al. 2005). This polymorphism is associated with reduced GCs sensitivity, along with a better metabolic profile with improved insulin sensitivity and lower total cholesterol levels (van Rossum, Koper et al. 2002; Wust, Van Rossum et al. 2004; Koeijvoets, van der Net et al. 2008). Within human elderly male population, the ER22/23EK polymorphism was associated with a lower tendency to develop dementia, increased insulin sensitivity, lower levels of C-reactive protein (associated with cardiovascular disease) and lower mortality (van Rossum, Feelders et al. 2004; van Rossum and Lamberts 2004). In a younger population, male ER22/23EK carriers were taller with more muscle mass and in female carriers, the waist:hip ratio was found to be smaller than in non-carriers (van Rossum and Lamberts 2004). Although most studies have indicated that the ER22/23EK polymorphism is associated with a better body composition and metabolic profile, a recent study has also linked it with a more aggressive multiple sclerosis disease course (van Winsen, Manenschijn et al. 2009).

A fourth polymorphism was found within the GR gene promoter region; the T*thIII*I polymorphism (van Rossum and Lamberts 2004). It has a single nucleotide change from T to C at 3807bp upstream from the GR mRNA start site (van Rossum and Lamberts 2004).This polymorphism on its own shows no association with insulin sensitivity, plasma glucose levels and lipid concentration. However, when it coexists with the ER22/23EK polymorphism, it is associated with lower insulin and cholesterol levels (van Rossum, Roks et al. 2004).

1.4 Systemic glucocorticoids, Cushing's disease and the metabolic syndrome

Cushing's disease is accompanied by excess plasma GC. However, the more common metabolic syndrome of visceral obesity, hypertension, insulin resistance /

type II diabetes and dyslipidemia is not (reviewed in Seckl, Morton et al. 2004). The striking phenotypic similarity between the common metabolic syndrome and the much rarer Cushing's disease raised speculation that dysregulation of GC action also occurs in metabolic syndrome (reviewed in Seckl, Morton et al. 2004).

1.4.1 Peripheral glucocorticoid metabolism and 11β-HSD1

As mentioned above, despite the phenotypic similarities between Cushings's disease and metabolic syndrome, obesity is not characterized by plasma cortisol excess (Seckl, Morton et al. 2004). Indeed, although there is some evidence for HPA axis activation in obesity, plasma levels of GC may even be low due to increased GC clearance by the A-ring reductase pathways (Livingstone, Jones et al. 2000, reviewed in Seckl, Morton et al. 2004). However, it has become clear in recent years that idiopathic obesity is associated with an increased level of GC reamplification within the adipose tissue, by the intracellular enzyme 11β-dehydroxysteroid dehydrogenase (11β-HSD1) (reviewed in Seckl, Morton et al. 2004 and see below). Thus, despite normal circulating GC levels, the similarities between cushingoid and idiopathic obesity are hypothesized to result from increased GC action within adipose tissue (Seckl, Morton et al. 2004). Note that liver GC regeneration by 11β-HSD1 is a major contributor to plasma GC levels (Paterson, Holmes et al. 2007) and is generally lower in obesity (Livingstone, Jones et al. 2000).

1.4.2 Pre-receptor glucocorticoid metabolism

11β-HSD catalyses the interconversion of active GC (mainly cortisol in humans and corticosterone in rodents) and inert 11-keto forms; cortisone in human and 11-dehydrocorticosterone (11-DHC) in rodents (reviewed in Seckl 2004) (Fig. 1.4). Two isozymes exist, 11β-HSD type 1 and type 2. Each of these enzymes has a distinct physiological role and tissue distribution (reviewed in Seckl, Morton et al. 2004; Tomlinson, Walker et al. 2004).

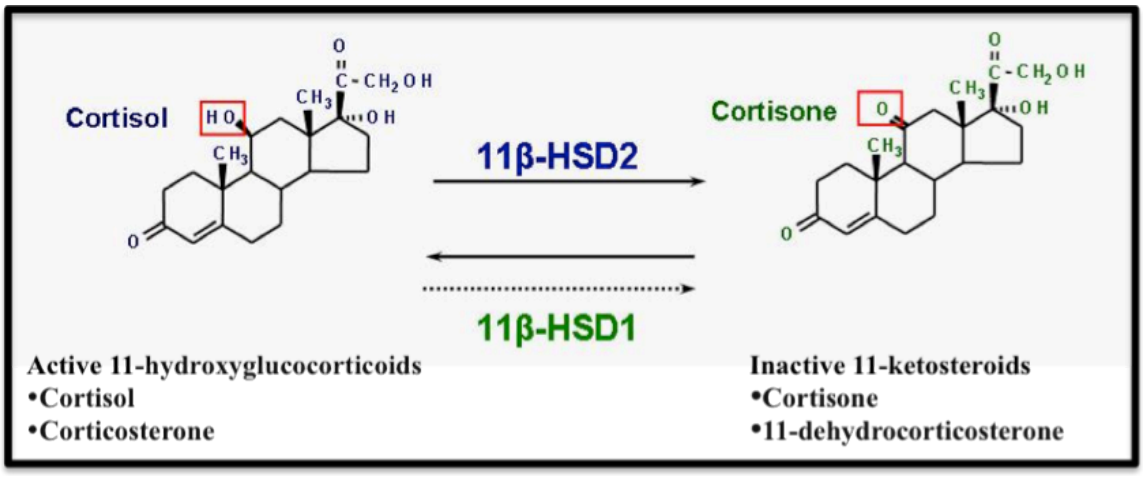

Figure 1.4 Interconversion of active and inactive glucocorticoids by 11β-hydroxysteroid dehydrogenase (11β-HSD)

In vivo, the conversion of active to inactive GC is catalyzed by 11β-HSD2; a high affinity (for cortisol), nicotinamide adenine dinucleotide (NAP+) - dependent enzyme. It is highly expressed in the kidney, sweet glands, salivary glands, colon and the placenta. The regeneration of active GCs is catalyzed by 11β-HSD1, which is an NADPH- dependent enzyme and it is widely expressed in many tissues (e.g. liver, adipose tissue, lung and brain) (Ricketts, Shoesmith et al. 1998; Seckl, Morton et al. 2004).

Adapted from
http://www.pharma.unibas.ch/institutes/systemtoxicology/research/endogenous_toxicity/Tox_Fig4.jpg

1.4.2.1 11β-dehydroxysteroid dehydrogenase type 2 (11β-HSD2)

11β-HSD2 is a high affinity, nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenase enzyme which inactivates GCs (Brown, Chapman et al. 1996). During development, 11β-HSD2 is widely expressed in the developing fetus and in placenta (Diaz, Brown et al. 1998), protecting the fetus from maternal GCs which otherwise reduce birth weight and possibly cause lifelong hypertension, hyperglycemia and anxiety behaviour (Lindsay, Lindsay et al. 1996; Benediktsson, Calder et al. 1997; Nyirenda, Lindsay et al. 1998; Kotelevtsev, Brown et al. 1999; Holmes; Abrahamsen et al. 2006; Cottrell and Seckl 2009). During adulthood, 11β-HSD2 is most abundant in mineralcorticoid-target tissues such as the kidney, sweat glands, salivary glands, colon and placenta (Hammer and Stewart 2006). In mineralocorticoid target tissues, 11β-HSD2 prevents GC occupation of MR which otherwise would bind GCs with high affinity (Stewart, Corrie et al. 1988; Funder, Pearce et al. 1990). This confers aldosterone specificity upon MR where 11β-HSD2 is co-expressed with MR (Stewart, Corrie et al. 1988; Funder, Pearce et al. 1990). MR only acts as a glucocorticoid receptor in tissues where it is not co-expressed with 11β-HSD2 – mainly the hippocampal formation of brain and the heart (Reul, Gesing et al. 2000; Kellendonk, Gass et al. 2002)

1.4.2.2 11β-dehydroxysteroid dehydrogenase type 1 (11β-HSD1)

11β-HSD1 is a 34 kDa glycoprotein found within the lumen of the endoplasmic reticulum (ER) and is anchored to the membrane by its N-terminus (Agarwal, Rogerson et al. 1995). This enzyme has a lower affinity for cortisol and corticosterone than 11β-HSD2 and is an NADPH-dependent reductase within intact cells, generating active GC. It is highly expressed in liver (Agarwal, Monder et al. 1989), brain (Moisan, Seckl et al. 1990), lung (Hundertmark, Ragosch et al. 1993), adipose tissue (Napolitano, Voice et al. 1998), and skeletal muscle and vascular smooth muscle (Walker, Yau et al. 1991; Monder 1993). 11β-HSD1 has also been found to be expressed in immune cells. It is induced upon macrophage

differentiation from monocytes (Thieringer, Le Grand et al. 2001) and is induced in macrophage cell lines following activation by LPS (Thieringer, Le Grand et al. 2001). In a number of cell types, 11β-HSD1 expression is increased by pro-inflammatory cytokines such as TNFα and IL-1 (reviewed in Chapman, Coutinho et al. 2009). Recently it has been proposed that 11β-HSD1 modulates inflammatory processes in rheumatoid arthritis and atherosclerosis (Hermanowski-Vosatka, Balkovec et al. 2005; Schmidt, Weidler et al. 2005; Hardy, Filer et al. 2006; Hardy, Rabbitt et al. 2008). Administration of a selective 11β-HSD1 inhibitor to Apolipoprotein E deficient (*Apoe*$^{-/-}$) mice slowed the formation of atherosclerotic lesions (Hermanowski-Vosatka, Balkovec et al. 2005) although more recent studies have not replicated this finding (Lloyd, Helmering et al. 2009). Data from human studies as well as various rodent models have highlighted the importance of this enzyme in the pathogenesis of metabolic syndrome and obesity. Many studies have shown that obese humans have increased 11β- HSD1 in their adipose tissue, with a strong association between BMI and adipose 11β-HSD1 expression (reviewed in Wake, Rask et al. 2003, Seckl et al 2004, Stewart 2005). Some rodent models of obesity (e.g. Zucker rats, leptin deficient *ob/ob* mice) also have increased levels of 11β-HSD1 in adipose tissue (Livingstone, Jones et al. 2000). However, an inverse correlation between BMI and 11β-HSD1 activity in primary omental human pre-adipocytes has been shown (Bujalska, Gathercole et al. 2008). Mice which are 11β-HSD1-deficient (11β-HSD1$^{-/-}$) mice are viable, fertile and have a normal lifespan (Kotelevtsev, Holmes et al. 1997). Importantly, they are protected from the adverse metabolic effects of high fat diet and have improved glucose tolerance and insulin sensitivity (Kotelevtsev, Holmes et al. 1997; Morton, Holmes et al. 2001; Morton, Paterson et al. 2004). Mice with liver-specific overexpression of 11β-HSD1 (using the *Apoe* enhancer-promoter) were found to be mildly insulin-resistant, dyslipidemic and hypertensive, without obesity (Paterson, Morton et al. 2004). However, overexpression of 11β-HSD1 specifically in adipose tissue of transgenic mice (using the aP2 enhancer-promoter) caused visceral obesity, hyperinsulinemia, hypertension

and insulin resistance and has provided a mouse model of the metabolic syndrome (Masuzaki, Paterson et al. 2001; Masuzaki, Yamamoto et al. 2003). Interestingly, in both rats and mice, high fat diet (both short term and up to 18 weeks), reduced 11β-HSD1 expression specifically within adipose tissue (Morton, Ramage et al. 2004; Drake, Livingstone et al. 2005). It has been proposed that this down-regulation of 11β-HSD1 within adipose tissue is an important mechanism to combat obesity (Morton, Ramage et al. 2004).

1.5 Aim of the thesis

Obesity, particularly abdominal (visceral) obesity, is associated with increased risk of hypertension, insulin resistance and diabetes mellitus, hyperlipidaemia, and cardiovascular disease (the metabolic syndrome). Both human and animal studies have shown that regulation of GC production and or receptor density may be an important factor in determining visceral adiposity.

1.5.1 Hypothesis

GC action (GR density and 11β-HSD1-determined ligand availability) within adipose tissue is an important determinant of adipose tissue distribution and mass.

1.5.2 Aims of the thesis:

To test this hypothesis, 2 approaches were undertaken:

1. To investigate the effects of dietary fat upon adipose parameters of GC action, the effect of different types of dietary fat upon expression of 11β-HSD1 and GR in adipose tissues and liver of mice were examined.
2. To test whether GR density in adipose tissue is an important determinant of fat mass and distribution, a transgenic model designed to have increased GR levels specifically within adipose tissue was created and the effect of chronic high fat diet upon adipose GC action was investigated in these mice.

2 Materials and Methods

2.1 Materials

Chemicals and molecular biology reagents

All chemicals were purchased from Sigma-Aldrich Company Ltd or BDH Merck Ltd unless stated otherwise (supplier addresses can be found in Appendix A). Other materials were obtained as stated below:

1kb DNA ladder	Invitrogen Life Technologies
20 x SSC	Invitrogen Life Technologies
Anti-rabbit SPA reagent	PerkinElmer LAS (UK) Ltd
Bacto yeast extract	Becton, Dickinson and company
Bacto tryptone	Becton, Dickinson and company
Blotting grade blocker non fat dry milk	Bio-Rad Laboratories Ltd
Caesium chloride	Invitrogen Life Technologies
Calf intestinal phosphatase	Invitrogen Life Technilogies
Deoxynucleotide triphosphates (dNTPs)	Promega Corporation
DNeasy tissue kit	Qiagen Ltd
Ethanol	Hayman Ltd
ECL™ western blotting detection reagent	Amersham Pharmacia Biotech UK Ltd
Glucose hexokinase reagent	Thermo fisher scientific Inc
Glucose	Thermo fisher scientific Inc
High DNA mass™ ladder	Invitrogen Life Technologies
Hybond N+ nylon membrane	Amersham Pharmacia Biotech UK Ltd
DNA Polymerase I (Klenow) Fragment	Promega Corporation
Low melting point agarose	Invitrogen Life Technologies
LightCycler® 480 probes master	Roche Diagnostic Ltd
NICK columns	Amersham Pharmacia Biotech UK Ltd
Nitrocellulose/filiter paper sandwich	Invitrogen Life Technologies
NuPAGE® antioxidant	Invitrogen Life Technologies
NuPAGE® LDS sample buffer (4x)	Invitrogen Life Technologies

NuPAGE® MOPS SDS running buffer	Invitrogen Life Technologies
NuPAGE® Novex 4-12% bis-Tris Gel	Invitrogen Life Technologies
NuPAGE® sample reducing agent (10x)	Invitrogen Life Technologies
NuPAGE® transfer buffer	Invitrogen Life Technologies
Phenol	Fisher Scientific UK Ltd
Protein assay reagent A	Bio-Rad Laboratories Ltd
Protein assay reagent B	Bio-Rad Laboratories Ltd
Protein standard II	Bio-Rad Laboratories Ltd
QIAEXII gel extraction kit	Qiagen Ltd
Reverse transcription system	Promega Corporation
Rediprime II random primer labeling kit	Amersham Pharmacia Biotech UK Ltd
Riboprobe in vitro transcription system	Promega Corporation
RNAse Zap	Applied Biosystems
RNAsin® ribonuclease inhibitor	Promega Corporation
RNA wash concentrate	Anachem Ltd
RNAID matrix	Anachem Ltd
RPA III^{TM} kit	Applied Biosystems
Seakem LE agarose	Cambrex Corporation
SeeBlue® Plus2 pre-stained standard	Invitrogen Life Technologies
TaqMan® Gene expression assay	Applied Biosystems
*Taq*bead™ hot start polymerase	Promega Corporation
Triglyceride	Thermo fisher scientific Inc
Triglyceride liquid stable reagent	Thermo fisher scientific Inc
TRIzol®	Invitrogen Life Technologies
Tween 20	Bio-Rad Laboratories Ltd
NICK columns	Amersham Pharmacia Biotech UK Ltd
[α^{32}P]-dCTP (3000 Ci/mmol, 10mCi/ml)	PerkinElmer LAS (UK) Ltd
[^{3}H]-corticosterone	PerkinElmer LAS (UK) Ltd

2.1.1 Solution and buffers

1kb ladder	30µl 1kb ladder, 60µl glycerol, 120µl H_2O, 5µl loading dye
2 x HBS (500ml)	8g NaCl, 0.2g Na_2HPO_4, 6.5g HEPES, pH7.0
5M potassium acetate (500ml)	127.21g potassium acetate, 57.5ml glacial acetic acid
10 x MOPS (1 litre)	83.7g MOPS, 8.2g Sodium acetate, 3.72g EDTA, pH 7.0
10 x TAE (1 litre)	242g Tris base, 57.1ml glacial acetic acid, 0.5M EDTA, pH8.0
10 x TBE (1 litre)	108g Tris base, 55g boric acid, 40ml 0.5M EDTA
10 x TBS (1 litre)	24.4g Tris base, 80g Nal, 38ml HCl
20 x SSC (500ml)	87.7g NaCl, 44.1g citric acid, pH 7.0
Alkaline SDS solution	0.2M NaOH, 1% SDS
Borate buffer	0.133 M boric acid, 67.5mM NaOH +HCl, pH 7.4 containing 0.5% bovine serum albumin (BSA), 1% methanol and 0.1% ethylene glycol
Depc treated H_2O	5 drops diethyl pyrocarbonate (depc) added to 500ml H_2O; left to stand 1h then autoclaved
GTE (500ml)	50mM glucose, 10mM EDTA, 25mM Tris, pH8.0
TE buffer (500ml)	10mM Tris, 1mM EDTA, pH8.0
Transfection assay buffer (50ml)	0.359g Tricine, 0.514g dithiothreitol (DTT), 20µl 0.5M EDTA, 2.43g $MgCO_3$, 267µl 1M $MgSO_4$, 10mg coenzyme A, pH7.5
Transfection lysis buffer (50ml)	25mM Tris phosphate (pH8.0), 2mM DTT, 1% Triton X 100, 10% glycerol
LB (1 litre) Luria Broth (LB)	10g tryptone, 5g yeast extract, 5g NaCl, autoclaved immediately
LB/Amp agar plates (1 litre)	10g tryptone, 5g yeast extract, 5g NaCl, 10g agar, solution was autoclaved, and 100mg/ml ampicillin added once agar had cooled to ~45ºC, then plates

	poured immediately
Loading dye	2g Ficoll 400, 100mg SDS, 2.5mg bromophenol blue, 25mg xylene cyanol, 10ml 0.1M EDTA (pH8.0)
Phosphate Buffer (500ml)	13.1g Sodium phosphate monohydrate, 42.6g anhydrous Na_2HPO_4, 0.93g EDTA
Phosphate Buffered Saline (1 litre)	8.00g NaCl, 0.2g KCl, 1.44g Sodium phosphate monohydrate, 0.24g Potassium dihydrogen phosphate, pH 7.4
Protein extraction buffer (1 litre)	50mM HEPES, 8g NaCl, 0.2g $MgCl_2$, 0.15g $CaCl_2$, 0.42g NaF, 0.37g EDTA, 2.29g Sodium pyrophosphate
Special depc. water	10µl 100mM DTT, 1µl RNasin, 89ul of depc treated H_2O

2.1.2 PCR Primers used for genotyping and detection of transgene expression

aP2-GR (expected PCR product size 376bp)

Forward	5'-GCTGCTGCACATTGCTTGTGG-3'
Reverse	5'-GGAGCCTTTCGAGAAATCAAGG -3'

β-Globin (expected PCR product size 494bp)

Forward	5'-CCAATCTGCTCACACAGGATAGAGAGGGCAGG-3'
Reverse	5'-CCTTGAGGCTGTCCAAGTGATTCAGGCCATCG-3'

PstI digest (expected PCR product size 100bp)

Forward	5'-CAGGAGTCTCACAAGACACTTC-3'
Reverse	5'-TCACCTCCAGCAGTGACACC-3'

2.1.3 Antibodies and neutralizing peptide used for Western blotting

Anti-GR rabbit polycolonal antibody (PAI-512)	Cambridge BioScience Ltd
Anti-GR rabbit polycolonal antibody (M-20)	Santa Cruz Biotechnology, Inc.
PA1-512 Neutralizing peptide	Cambridge BioScience Ltd
Anti-β-tubulin monoclonal antibody	Sigma-Aldrich Company Ltd
Goat anti-mouse IgG-HRP (Sc-2005)	Santa Cruz Biotechnology, Inc.
Goat anti-rabbit IgG-HRP (Sc-2004)	Santa Cruz Biotechnology, Inc.

2.2 Methods

2.2.1 Animals

All experiments were approved by The University of Edinburgh ethical committee and were carried out according to the UK Animals (Scientific Procedures) Act 1986. All experimental mice were housed in standard conditions on a 12h-light and 12h-dark cycle. The dietary experiments (Chapter 3) used adult (6-8 weeks) C57BL/6J male mice (n=12/group) given one of the following diets (all from Research Diets, Inc. New Brunswick, NJ); control low fat with sucrose (Research Diets D12329), control low fat with cornstarch (D12328), or high fat diets; oleic acid-enriched (D04070902), safflower oil-enriched (D04070903), stearate-enriched (D04070901), 58% high fat (D12331) and 45% high fat (D12451), for 1 week acclimatization followed by 4 weeks with body weight and food intake measurement. Towards the end of the experiment, mice were separated into two groups and killed by cervical dislocation at 0800h (n=6) or 2000h (n=6). Blood was taken for measurement of plasma: corticosterone, insulin and glucose level. Liver, kidney, adrenal and various fat depots, gonadal (Gon), subcutaneous (Sc), mesenteric (Mes), retroperitoneal (RP) and brown adipose tissue (BAT) were collected, snap frozen on dry ice and weighed. RNA was extracted from fat depots as described in section 2.2.7; mRNA expression of both GR and 11β-HSD1 was measured by northern blotting (section 2.28). In addition, 11β-HSD1 activity was also measured by Lynne Ramage.

To test the effect of diet on transgenic mice (Chapter 4), 5 transgenic F_1 males were mated to C57BL/6J females to generate F_2 offspring. Offspring were weaned on to either 58% high fat diet (Research Diets D12331) or 11% low fat diet (D12328). Ear notches were taken at weaning for genotyping (section 2.11). Both male and female mice ($n \geq 12$/group) were separated into different cages according to their genotype. Body weight and food intake were measured throughout the 6 month study period. At week 15 of the study, 6 mice from each group were selected for blood pressure measurements which were carried out weekly for 10 weeks. Glucose tolerance tests were carried out at week 20 of the study. Mice (n=6/group) were fasted for 6h then blood was taken for plasma glucose assay (session 2.2.4). Initially,

10µl blood was taken from a tail nick, into EDTA-coated microtubes. Mice were then injected intraperitoneally (i.p.) with 2mg glucose/g body weight. Further blood samples (10µl/sample) were taken at 15min, 30min, 60min and 120min after the injection. Blood was cntrifuged at 2000rpm for 10 min at 4°C, plasma extracted and kept at -80°C for later analysis. Insulin tolerance tests were carried out at week 24 of the study. Mice (n=6/group) were fasted for 6h then blood was taken for plasma insulin assay. Initially, 10µl blood was taken via a tail nip then a dose of 2mU/gram of body weight was injected i.p.: Further 10µl blood samples were taken at 15min, 30min, and 60min. Plasma was extracted from the blood as described above. At the end of the study, mice were culled by cervical dislocation between 0730h and 0900h. Blood was taken for measurement of plasma corticosterone levels (section 2.2.3) in plasma extracted as described above. Liver, kidney, adrenal, tail and various fat depots; gonadal (Gon), subcutaneous (Sc), mesenteric (Mes), retroperitoneal (RP) and brown adipose tissue (BAT) were collected, snap frozen on dry ice and weighed.

2.2.2 Measurement of plasma insulin levels

Plasma insulin levels were measured using ultra-sensitive rat insulin ELISA kits (Crystalchem, Downers Grove, IL, USA), according to the manufacturer's instructions. A series of insulin standards ranging from 0-5000µg/µl was used to produce a standard curve. A 96 well plate was coated with mouse anti-insulin monoclonal antibody, which bound to insulin within the samples or standard. Guinea pig anti-insulin antibody was then applied to the wells to "sandwich" insulin. Unbound material was removed by washing and horseradish peroxidase (HRP)-conjugated anti-guinea pig antibody was added then the wells were washed again to remove the excess antibody. O-phenylenediamine substrate solution was then added to the wells (which changes color if HRP-conjugated complexes are present). The colour change was measured at 492nm using a plate-reader spectrophotometer (Molecular Devices, OPTImax) and the insulin concentration within the samples determined from the standard curve.

2.2.3 Measurement of plasma corticosterone (B) levels

Radioimmunoassay was used to determine the total plasma B concentration as described (Harris, Kotelevtsev et al. 2001). Plasma was diluted 1:10 in borate buffer and heated at 75ºC for 30min to denature corticosterone binding globulin in the samples. A series of B standards ranging from 0-320nM was used to produce a standard curve. In a 96 well plate, 20µl of standards or samples were incubated in duplicate with 25µl of B antibody mixture (1 in 10,000 dilution antibody raised in rabbit, PerkinElmer) and 25 µl of [^{3}H]-B in borate buffer (specific activity 80Ci/mmol; final concentration 1.5nM) to give a total volume of 70µl. The plate was then covered with parafilm and incubated at room temperature for 1h. 50µl of anti-rabbit scintillation proximity assay (SPA) beads was added to each of the samples and the plate re-sealed with sealant film and incubated for 10h. During the incubation the antibody binds to both unlabelled B and [^{3}H]-B. Antibody bound by both [^{3}H]-B and SPA beads emit a signal detected by the LKB Wallac Microbeta counter. The B concentration in the samples was then determined from the standard curve using multicalc software assay zap version 3.1. Intra and inter assay variation were 9.4 and 9.2% respectively. Compared with corticosterone (100%), cross reactivities for progesterone, deoxycorticosteron and cortisol are 7.7, 6.5 and 5.3% respectively.

2.2.4 Measurement of plasma glucose levels

A series of glucose standards ranging from 0-400mg/dL was used to produce a standard curve. In a 96 well plate, 2µl of standards or sample plasma were mixed with 250µl of glucose hexokinase reagent and incubated at 37°C for 3min. The reagent causes the glucose within the sample to be phosphorylated and NADH is generated. The absorbance of NADH within the plate was then measured at 340nm using a plate-reader spectrophotometer (Molecular Devices, OPTImax). The glucose concentration within the samples was determined from the standard curve.

2.2.5 Measurement of plasma Free Fatty acid (NEFA) levels

An enzymatic colour assay was used to determine free fatty acid (NEFA) levels in plasma. A standard curve was produced from a series of non-esterified fatty

acid (NEFA) standards (Thermo), ranging from 0-2.0mEq/L. In a 96 well plate, 4µl of standard or samples were mixed with 80µl of reagent A from Wako NEFA C test (Thermo) and incubated at 37°C for 10 min. Then 160µl of reagent B was added and the plate was incubated for a further 10min at 37°C, followed by 5min incubation at room temperature. The plate was then read at 550nm in the spectrophotometer (Molecular Devices, OPTImax). Plasma NEFA concentration was then determined from the standard curve.

2.2.6 Measurement of liver triglyceride levels

Frozen liver samples were homogenized in 10 vol (w/v) of isopropanol, shaken at room temperature for 45min, and then centrifuged at 3000rpm for 10min. The supernatant was removed and kept at 4°C. An enzymatic colour assay was used to determine triglyceride levels within samples. A standard curve was created from a series of triglyceride standards (Thermo), ranging from 0-2.5mmol/l. In a 96 well plate, 15µl of standard or samples were incubated in duplicate with 200µl of infinity triglyceride liquid stable reagent (Thermo) at 37°C for 5min. The samples were then read at 500nm in the plate reader spectrophotometer (Molecular Devices, OPTImax). The triglyceride concentration was then determined from the standard curve.

2.2.7 RNA extraction

Prior to RNA extraction, in order to protect RNA samples from RNase contamination, all the apparatus used was autoclaved and all solutions prepared with depc H_2O. For RNA extraction, TRIzol reagent (Invitrogen, Paisley, UK) was used. Frozen tissue samples were weighed and ~50mg homogenized in 800µl of TRIzol. The homogenate was either used immediately or frozen at -80ºC for later extraction. 200µl of chloroform was added to the homogenate then centrifuged at 12,000g for 1min to remove any debris. The supernatant was then vortexed for 15s and centrifuged at 12,000g for 15min at 4ºC. The upper (aqueous) layer was removed, mixed with 30µl of RNAID matrix (Anachem, Luton, Bed, UK) and agitated for 5min before centrifuging in a microfuge at maximum speed for 1min. The supernatant was removed and 500µl of RNA wash (Anachem) was added to the

pellet to dislodge it. This was then followed by centrifugation at 14,000g for 1 min. The washing step was repeated 3 times to ensure the pellet was clean. The pellet was resuspended in 20µl of "special depc. water" and incubated at 55ºC for 6min with a quick vortex before a further 6min of incubation. Finally, samples were centrifuged at 14,000g for 2.5min at 4ºC and 20µl of the supernatant aliquoted into 3 separate tubes, snap frozen on dry ice and stored at -80ºC for future use. RNA was quantified using a GeneQuant spectrophotometer.

2.2.7.1 Assessment of RNA concentration

The integrity of the extracted RNA was verified by RNA gel electrophoresis. For each sample, 3µg RNA were mixed with 2.5µl 10x MOPS buffer, 2.5µl formaldehyde and 10µl deionised formamide. The mixture was incubated at 65°C for 15min to denature the RNA then rapidly placed on ice. 2µl depc treated loading buffer was added to each sample. The samples were then loaded on a denaturing 1% agarose 1xMOPs/18% formaldehyde gel. The agarose was first melted in H_2O in a microwave, cooled slightly then MOPS and formaldehyde were added and the volume adjusted to the required amount with H_2O. The gel was allowed to set, then placed into an electrophoresis than filled with 1x MOPs buffer. Samples were then loaded onto the gel and run at 80V for ~3-4h bromophenol blue dye reached $\sim^2/_3$ of the length of the gel. The integrity of the RNA was then visualized under UV light at 260nm.

2.2.8 Northern Analysis of RNA

Prior to northern blotting, all equipment was treated with RNaseZap in order to prevent RNase contamination. RNA was electrophorised on denaturing 1% agarose gels as described above (section 2.2.7). An overnight capillary transfer was then set up as shown in Fig 2.1. to transfer denatured RNA from the gel to a nylon membrane for hybridisation, using 20xSSC as the transfer buffer. Following transfer the membrane was rinsed in 2xSSC and left to dry on Whatman No.3 filter paper for 2h. The RNA was then fixed onto the membrane by UV-crosslinking (Spectronics Corporation, power at 1200 x 100µw/cm^2).

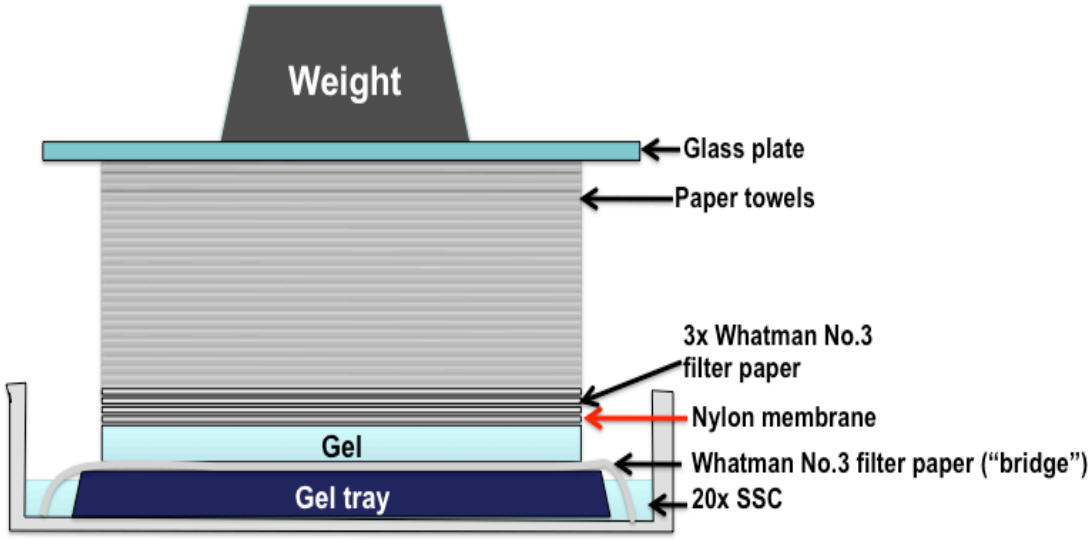

Figure 2.1 Schematic diagram of the northern blot transfer setup

Schematic diagram showing the transfer step of Northern blot. After gel electrophoresis, RNA was transferred by capillary transfer to a nylon membrane (Hybond N). A "bridge" (long piece of Whatman No.3 filter paper) was soaked in 20x SSC and was placed on top of the RNAse free gel tray. The ends of the filter paper were immersed in 20x SSC, which was used as the transfer buffer. The gel containing the denatured RNA was inverted and placed on top of the "bridge". The nylon membrane was then placed onto the gel being careful to remove any bubbles trapped between the nylon membrane and the gel, followed by 3 pieces of Whatman No.3 filter paper. To ensure the capillary transfer would occur, layers of dry paper towels were placed on the top. In addition, a glass plate was placed on top with extra weight above it to maintain a flat and even transfer. The whole setup was then left overnight at room temperature to ensure the majority of the RNA would transfer from the gel to the nylon membrane.

2.2.8.1 Hybridization

Membranes were prehybridised at 65ºC with 18ml of phosphate buffer and 9ml of 20% SDS in a hybridization bottle. 1ml of sonicated salmon testes DNA (10mg/ml) was denatured at 100ºC for 10min and added to the hybridization bottle. Prehybridisation was carried out at 65ºC for 3h. A radiolabelled cDNA probe was made for the gene of interest using a rediprimeII random-prime labeling kit. 25ng DNA template was diluted to 45µl with TE buffer. The probe was then denatured at 100ºC for 15min and immediately cooled on ice for a further 10min. Denatured DNA was then added to the reaction tube together with 5µl of [$^{32}\alpha$P]-dCTP. The reaction was incubated at room temperature for 2h then labeled cDNA was purified through a Nick column. Briefly, columns were washed with 3ml TE buffer before use, then the labeling reaction was applied to the column and allowed to run in. Two aliquots of 400µl TE were then applied to the column. The first eluate was discarded and the second eluate, containing the purified probe, was collected. 1µl of the purified probe was added to 1ml of scintillant and counted. Generally, probes obtained 100,000 cpm/µl. Before adding the entire probe to the hybridisation bottle, it was denatured at 100ºC for 10min. The hybridization mixture was then left overnight at 55ºC.

The hybridization mixture was then disposed of and the membrane was washed to remove any non-specifically bound probe. Three 50ml washes were preformed; an initial wash at room temperature for 15min with 2xSSC, 0.1%SDS then followed by 2 washes, each for 15min at 65ºC, with 1xSSC, 0.1%SDS then 0.5xSSC, 0.1%SDS. The washed membrane was then wrapped in Saranwrap and exposed to a phosphorimager screen (Fuji imaging plate) for 10min then read using a Fuji BAS phosphorimager. mRNA levels were quantitated using Aida software (Advance image data analyzer Version 3.44.035 Raytect, Shaffield).

2.2.9 Ribonuclease Protection Assay (RPA)

RPA is a method to detect and quantify RNA using a radioactive probe. Single stranded RNA is digested by RNase, whereas double stranded RNA (probe hybridized to target mRNA) is protect from RNase digestion.

2.2.9.1 Probe preparation

^{32}P-labelled cRNA probes were synthesized *in vitro* in 20µl reactions containing 4µl 5x transcription buffer, 0.4mM DTT, 2µM each ATP, CTP, UTP, 2µl 0.1mM GTP, 20U RNasin, 1µg DNA Template, 1µl Sp6 polymerase, 3µl [$^{32}\alpha$P]-GTP. Reactions were incubated at 40°C for 120min. 1U RQ1 RNase free DNase (Promega) was added to stop the reaction by incubating at 37°C for 20min. Labeled probe was purified using Nick Columns. Usually 100,000 cpm was obtained from each purified probe.

2.2.9.2 Hybridisation and RNase Digestion

Each RNA sample (8µg, unless stated otherwise) was mixed with 0.1vol 5M ammonium acetate, 2.5vol ethanol and 100,000cpm of each labeled probe. Samples were placed at -20°C for 1h for RNA precipitation to occur then centrifuged at 12,000g for 15min to recover the pellet. The supernatant was removed and 10µl of hybridisation buffer III (provided in the RPAIII kit) was added to resuspend the pellet. Samples were then denatured at 95°C for 3min and hybridized at 42°C for 16h.

RNase digestions were carried out either with RNase T_1 alone (cleaves at G residues; reduced specificity to detect mouse and rat GR mRNA) or with RNase A/T_1 mix (cleaves at G, T and C residues to give higher specificity for exact sequence match to probe). RNase (either T_1 or A/T_1) was diluted with RNase Digestion Buffer III (provided in the RPAIII kit). 150µl of the diluted RNase was then added to each sample and incubated at 37°C for 30min. RNase digestion was stoppped by adding 200µl inactivation III buffer (provided in the RPAIII kit), 75µl ethanol and 5µg of yeast tRNA. The samples were placed at -20°C for 1h to precipitate RNA then centrifuged at 13,000rpm for 15min. The supernatant was removed and the pellet air dried for ~1 min before being resuspended in 7.5µl of gel loading buffer III (provided in the RPAIII kit). Samples were heated at 95°C for 3min then placed on ice before loading onto a denaturing polyacrylamide gel.

2.2.9.3 Denaturing polyacrylamide gel eletrophoresis

Prior to making the gel, glass plates, gel spacers and comb were cleaned with detergent then wiped with 100% ethanol. The vertical gel eletrophoresis tank was set up and the percentage of the polyacrylamide gel used depended on the size of the fragment of interest. For RPA assay, normally a 5% polyacrylamide gel was used. For a 25ml gel, 12.5% acrylamide : bisacrylamide (29: l ratio) was mixed with 10.5g urea and 2.5ml 10X TBE. Sufficient H_2O was added to dissolve the urea and the solution filtered and topped up to 25ml with H_2O. Before pouring the gel, 150µl of 10% ammonium persulfate and 40µl Tetramethylethylenediamine (TEMED) were added to the gel solution to catalyze polymerization. The solution was immediately poured between the glass plates and a comb inserted. Once the gel had set, it was placed in the vertical electrophoresis tank with 1X TBE as running buffer. Before loading the samples, the gel was pre-run at 25mA for 30min. Samples were loaded, then electrophoresis continued at 250V for 2h. Following electrophoresis (when the dye reached the bottom of the gel), the gel was disassembled, transferred from the glass plate onto 3MM filter paper and dried under vacuum for 2h. The dried gel (covered in Saranwrap) was exposed to a phosphorimager screen (Fuji imaging plate) for 15min, which was then scanned in a Fuji BAS phosphorimager. mRNA levels were quantitated using Aida software (Advance image data analyzer Version 3.44.035). A representative autoradiograph of a test RPA is shown in Fig 2.2.

2.2.10 Realtime PCR

cDNA was synthesized from RNA as described in section 2.2.11.1 and specific cDNA levels were measured using the Roche LightCycler 480 (Roche Diagnostic Ltd, Burgess Hill, West Sussex, UK). Initially, a test plate was carried out to assess the cDNA dilution needed and select the appropriate internal control. cDNA was pooled from each sample and an initial standard curve was produced by serial dilution of the pooled cDNA; neat, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:168 and 1:336. Each sample was meaured in triplicate in a total reaction volume of 10µl; 5µl LightCycler 480 Probes Master mix, 4.5µl cDNA and 0.5µl Taqman® gene

expression assay (Applied biosystems). Dilution of the cDNA selected for measurement was chosen to give amplification at around 20-27 cycles. Appropriate internal controls (ß-actin, TBP or 18S) were selected for each individual experiment that showed no difference between the groups within that experiment.

2.2.11 Competitive RT-PCR to determine relative abundance rat and mouse GR mRNAs

2.2.11.1 cDNA synthesis

For reverse transcription reactions, 2µg of RNA sample was diluted to 10µl with nuclease-free H_2O (Promega) and incubated at 70°C for 10min to disrupt secondary structure. 10µl master mix (4µl $MgCl_2$, 2µl 10x reverse transcription buffer (Promega), 2µl 10mM dNTP mix, 0.5µl recombinant RNasin® ribonuclease inhibitor (Progmega), 15U AMV reverse transcriptase, 0.5ug oligo(dT) primer) was added, with nuclease free water to a total of 20µl. Reactions were then incubated at 42°C for 20min, 95°C for 5min and 4°C for 5min and stored at 4°C for later use.

2.2.11.2 PCR reactions and PstI digestion of rat GR cDNA

1µl of RT reaction was used in the PCR reaction. PCR reactions were carried out as described in section 2.11.1 with PstI digest primers (section 2.1.3). The PstI primers were an identical match to both rat and mouse GR cDNA and amplify a 100bp PCR product from both. To distinguish rat GR PCR product from mouse, 17µl of the PCR reaction was restricted in a 20µl volume containing 2µl 10x restriction buffer H (Promega), 10U PstI (Promega) and digested at 37°C for 5h. The PstI digested PCR product gave a 49bp fragment. The undigested PCR product and the PstI-digested PCR product were assessed by electrophoresis on a 2.5% agarose gel (Section 2.2.12.1).

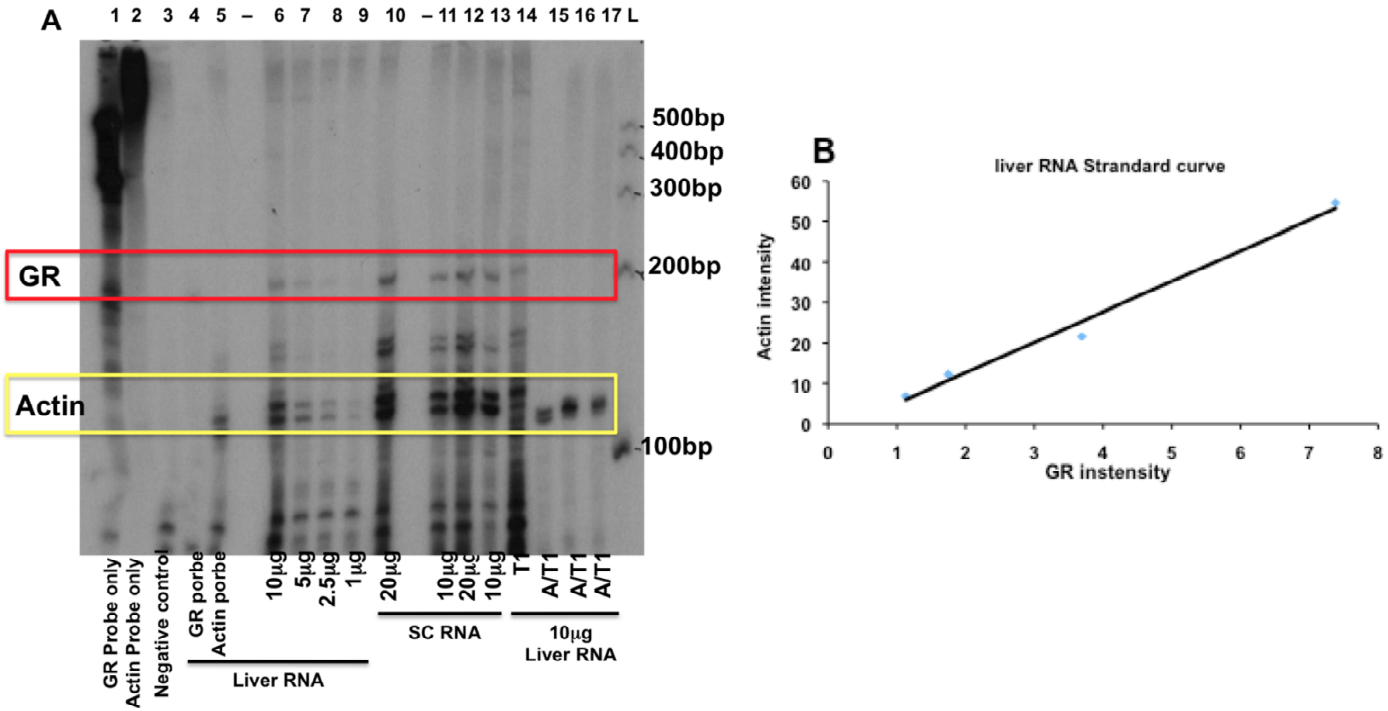

Figure 2.2 Optimization of RNase protection assay (RPA)

Representative autoradiograph showing a test RPA used to assess the amount of sample RNA and RNase needed. (A) 6h exposure showing GR and actin (internal control) protected fragments. Lanes 1 and 2 contained reactions of probe (GR or actin; respectively) with no RNase. Lane 3-14 all contain reactions digested with 1/25 dilution of RNase T_1. Lane 3 contained yeast tRNA, GR and actin probe (negative control). Lanes 4 and 5 contained 10µg of mouse liver RNA with probe (GR or actin; respectively) with RNase T_1. Lanes 6-14 contain various concentrations of liver or Sc RNA extracted from a non-tg mouse (Lanes10;11 and 12;13 contain RNA from adipose tissue of 2 difference mice). In sach case, the RNA was made to a total of 10ug with yeast tRNA. Lanes 15-17 contained 10µg of liver RNA with various dilutions of RNaseA/T_1 (1/50, 1/100, 1/200; respectively) L= ladder. (B) Graph showing a linear relationship between the levels of GR and actin products obtained from phosphoimager quantification of the gel shown above.

2.2.12 Recombinant DNA techniques

2.2.12.1 Restriction enzyme digests and gel electrophoresis

For optimal enzyme digestion, different enzyme buffers (supplied with the enzyme) were used for different enzymes. A typical 15µl enzyme digest contained 1.5µl 10x enzyme buffer, 1µl (10U) enzyme, 5-10µg DNA of interest. The DNA/enzyme mixture was then incubated at 37ºC for 1h.

The percentage of agarose gel made depended on the size of DNA fragment of interest. Agarose was dissolved in 0.5 x TBE or 1 x TAE and dissolved by boiling in a microwave. For every 100ml of agarose gel, 2µl of ethidum bromide (10mg/ml) was added. Molten agarose was poured into a gel tray containing an appropriately sized comb, to set. The gel was then placed into an electrophoresis tank filled with 0.5 x TBE or 1 x TAE. DNA samples were mixed with 1µl loading dye and loaded onto the gel, 1 kb ladder was loaded in parallel. The gel was then run at 70-100V for ~1h untilthe fragment of interest was separated by electrophoresis. For excision of DNA the band was visualized under UV light at 360nm. For analytical digest, DNA was visualized at 260nm. DNA fragments for cloning or microinjection were separated by electrophoresis in 0.8-1.5% (depending on the size of fragment to be extracted) low melting point agarose gels in 1x TAE. The fragment of interest was visualized at 360nm, excised from the gel and extracted using a Qiaex II agarose gel extraction kit (Qiagen).

2.2.12.2 Filling in 3' or 5' overhang DNA ends

For filling in 3' recessed ends following restriction enzyme digest, reactions were adjusted to 1x HindIII restriction buffer and 0.25mM dNTPs added together with 2-3 U Klenow enzyme. Reactions were incubated at room temperature for 15min and terminated by heating at 75ºC for 15min. For the 3' overhangs, Klenow was added first without dNTPs and incubated at room temperature for 15min (to remove the 3' overhang). dNTPs were added as above and the reaction continued as above.

2.2.12.3 Removal of 5" phosphate from DNA

For digestion with CIP, restriction enzyme reactions were diluted >2 fold with TE (to reduce NaCl content below 25mM), then 1U of CIP was added and the reaction incubated at 37ºC for 5min. The enzyme was inactivated by incubation at 65ºC for 15min.

2.2.12.4 Ligation of DNA

Ligations typically contained 0.5-2µg DNA, comprising vector and insert in a ~1:3 molar ratio, and were carried out in 10µl with 1 x T4 ligase buffer (supplied with the enzyme) and 1U of T4 DNA ligase for sticky end ligation or 3-5U for blunt end. Ligations were incubated at 12-15ºC for up to 48h.

2.2.12.5 Transformation

Prior to the transformation, competent cells were made. A single HB101 colony was selected from a plate and placed into 2ml of LB then incubated in a shaking incubator at 37ºC for 16h. 0.5ml of this starter culture was then diluted into 50ml LB then incubated in a shaking incubator at 37ºC for ~1.5h to grow to mid log phase. The culture was centrifuged at 6K rpm (rotor JA20, Beckman J2-MC centrifuge) for 5min at 5ºC. The cell pellet was then resuspended in 20ml 0.1M $CaCl_2$ and the suspension was placed on ice for 10min. The cells were then centrifuged at 6K rpm (rotor JA20, BeckmanJ2-MC) for 5min at 5ºC. The cell pellet was then resuspended in 2ml 0.1M $CaCl_2$ and left on ice for ≥12h. For transformation, 200µl of competent cells were mixed with ~5µl of ligation mixture, left on ice for 20-30min, then heated at 42ºC for 50s before transferring to ice for 2-3min. The cells were plated out on an LB/AMP plate and incubated at 37ºC for ~16h.

2.2.12.6 Plasmid prep (miniprep)

A single transformed bacterial colony was selected from the plate and placed into 2ml of LB containing 100µg/ml ampicillin then incubated in a shaking incubator at 37ºC for 8-16h to grow to late log phase. The culture was centrifuges at 13k rpm

(Heraeus, biofuge) for 1min at room temperature. The cell pellet was resuspended in 100µl GTE, 200µl of alkaline/SDS solution was then added to lyse the bacterial cells and the suspension was placed on ice for 10min. 150µl of postassium acetate solution was added to the suspension mix, mixed well and placed on ice for a further 15min. The suspension was spun at 13k rpm (Heraeus, biofuge) for 5min. The supernatant was transferred to a fresh tube and 450µl phenol/chloroform (1:1) added, vortexed, 400µl of the aqueous (upper) layer was transferred to a fresh tube and 1ml ethanol added to precipitate the DNA. After 1min, the precipitated DNA was collected by centrifugation at 13l rpm (Heraeus, biofuge) for 2min. The pellet was air-dried at room temperature, then resuspended in 50µl TE buffer, 1µl RNase A (20 µg/ml).

2.2.12.7 Plasmid prep (maxiprep)

A single transformed bacterial colony was selected from the plate and placed into 2ml of LB containing 100µg/ml ampicillin then incubated in a shaking incubator at 37ºC for 6h to grow to late log phase. This starter culture was then added to 500ml LB containing 100µg/ml ampicillin and grown overnight with shaking at 37ºC. The culture was centrifuged at 6k rpm (rotor JA14, Beckman J2-MC centrifuge) for 5min at 4ºC. The cell pellet was then resuspended in 12ml of GTE. To lyse the bacterial cells, 25ml of alkaline/SDS solution was added and the suspension placed on ice for 10min. 16ml of potassium acetate solution was added to the suspension, shaken well to mix and placed on ice for a further 10min. The cell lysate was centrifuged at 6k rpm (rotor JA14, Beckman J2-MC) for 10min and the supernatant filtered through gauze to remove floating precipitate. Plasmid DNA was precipitated from the supernatant by the addition of 32ml isopropanol followed by incubation at room temperature for 30min. The precipitated DNA was collected by centrifugation at 10k rpm (rotor JA14, Beckman J2-MC) for 3min. The pellet was air-dried at room temperature for 2h, then resuspended in 2.2ml of TE. 3g CsCl and 100µl Ethidium Bromide (10mg/ml) were then added and the suspension transferred to a heat-seal ultracentrifuge tube. It was then centrifuged at 70k rpm (rotor TLK 100.3, Beckman Optima™ TLX ultracentrifuge) for 18h. The ethidium bromide stained band containing plasmid DNA was extracted through the side of the tube using a syringe

and needle, transferred to a fresh tube and topped up with TE/CsCl solution made by adding exactly 1g of CsCl to each 1ml TE. The DNA prep was centrifuged again at 70k rpm (rotor TLK 100.3, Beckman Optima™ TLX ultracentrifuge) for 16h or at 100k rpm for 4h. The plasmid band was removed as before, ethidium bromide extracted from the DNA with repeated isopropanol extractions (until no more pink color was detectable) and plasmid DNA dialysed against several changes of TE over 24h.

2.2.13 Transfection of mammalian cells

B103 neuroblastoma cells (Steinbach, Schubert et al. 1974) were kindly provided by V. Kelly. Cells from two 75cm^2 flasks were harvested by centrifugation at 1k rpm (Heraeus labofuge 400R) for 5min at room temperature. The pellet was resuspended in 15ml fresh medium (DMEM containing 10% foetal calf serum (FCS), 100U/ml penicillin and 100U/ml streptomycin). The cell number was determined using a hameocytometer and the volume of cells adjusted to give a final concentration of 3 x 10^5 cells/ml. 1ml of the resuspended cells was placed in each 60mm dish with 3ml of medium and incubated overnight at 37ºC, 5% CO_2. The medium was replaced 1h prior to transfection. For each transfection, a DNA mix was made consisting of 37µl 2M $CaCl_2$ and 10µg DNA adjusted to 300µl with H_2O. 20min prior to transfection, the DNA mixture was slowly added with agitation to 300µl of 2xHBS; a fine precipitate was formed. This transfection mixture was incubated at room temp for 15min, then added dropwise to a dish of cells. The cells were incubated with the transfection mixture overnight. The next day, the medium in each dish was replaced with fresh medium and again incubated overnight. The following day, transfected cells were harvested; medium was removed carefully from each dish and cells were washed in 3ml of PBS. 300µl of lysis buffer was then added to each dish and left for 15min. Lysed cells were scraped from each dish and centrifuged at 13k rpm for 1min at room temperature in a microfuge. Both luciferase and β-galactosidase assays were carried out on aliquots of lysates.

2.2.13.1 Luciferase assay

Assays were carried out in duplicate. Each tube contained 5µl 100mM ATP pH7.0, 100µl luciferase assay buffer and 40µl lysate. Light emission was read in a luminometer (Berthold) following injection of 105µl 1mM luciferin.

2.2.13.2 β-galactosidase assay

Assays were carried out in duplicate using a commercial kit (Galacto-light kit, Tropi, Bedford MA). Each tube contained 67µl $^{1}/_{100}$ dilution galacto reaction buffer (supplied with the kit) and 10µl of lysate. Reactions were incubated at room temperature for 20 min and light emission measured using a luminometer (as above) which injected 105µl acceleration solution (supplied with the kit).

2.2.14 Creation of the aP2-GR construct

pGL3-basic (Promega) was used as the vector for the construct. The initial cloning steps were carried out by Val Kelly (creating all the pVL plasmids). First, pGL3-basic was restricted with SalI and ligated to a pair of complementary oligonucleotides of the following sequence: 5'-TCGAGCGGCCGCGTTTAAACG-3' and 3'-CGCCGGCGCAAATTTGCAGCT-5' to create pVL295. This recreated the SalI site at one end only and also added NotI and PmeI restriction sites. Next, the SmaI-XbaI fragment of pVL295 was removed and ligated to rat GR cDNA to form pVL296; rat GR cDNA was encoded on a SalI–SspI (GR exon 2) fragment from pVL152 and a SalI-XbaI fragment from EGFP-rGR (Prima, Depoix et al. 2000) encoding the rest of rat GR cDNA. Finally, the aP2 enhancer/promoter was inserted in 2 stages; pVL296 was restricted with KpnI/SstI and ligated to a ~600bp KpnI-SacI fragment from paP2 (Ross, Graves et al. 1993) (a gift from B. Spiegelman) to form pJan1 (Fig.2.3). Next, pJan1 was restricted with Sst1, treated with calf intestinal phosphatase (CIP) to prevent religation of pJan1, and ligated to a 5000bp SstI aP2 fragment from paP2 to form the final aP2-GR construct pJan2 (Fig.2.4). The construct was verified using XbaI and EcoRV which generated the predicted fragments of 1876, 3200, 2750, 3660 and 918 and 8300b.p., respectively (Fig.2.5).

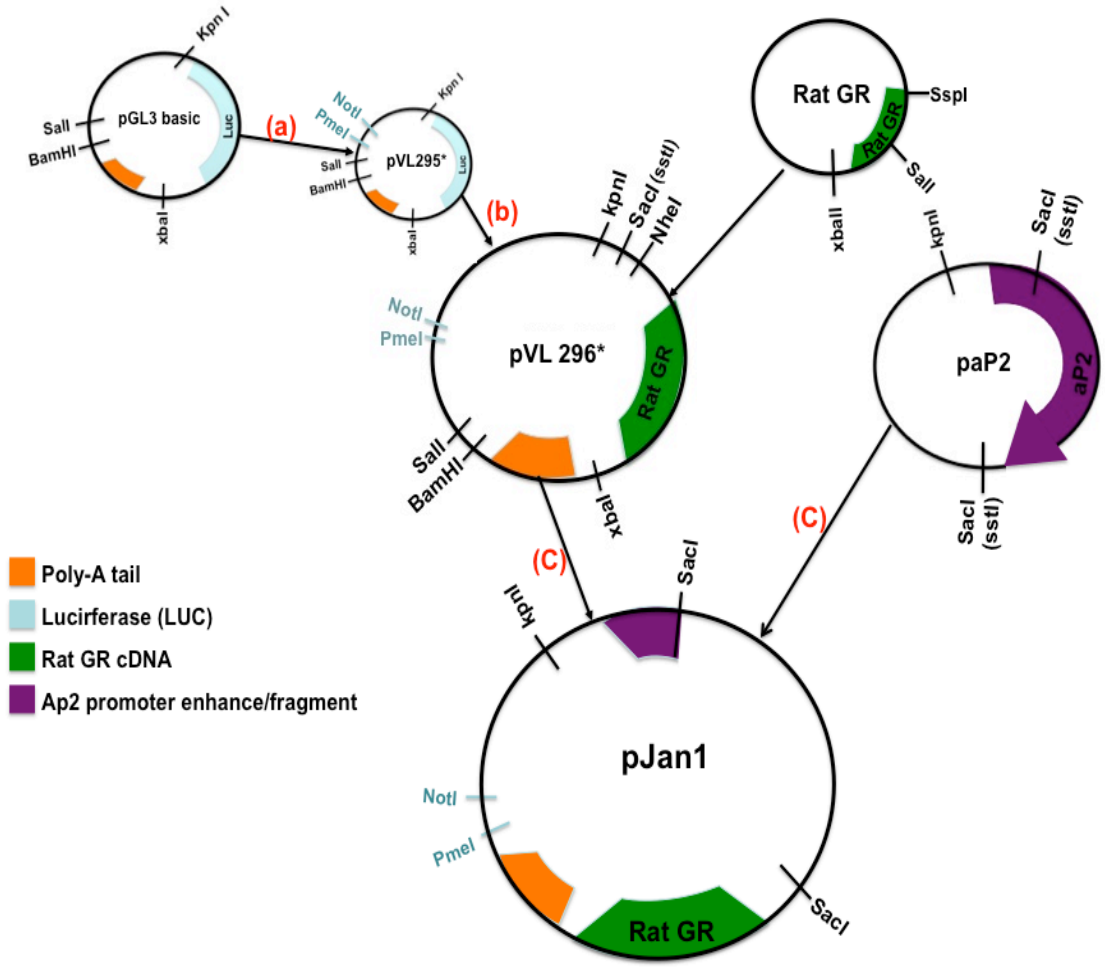

Figure 2.3 Schematic diagram of the structure and the formation of pJan1

Plasmid pJan1 was formed by sequentially (a) modifying the enzyme sites within pGL3 basic to add NotI and PmeI sites on a linker, creating PVL295*; (b) replacing the luciferase cDNA (LUC) with a KpnI-XbaI fragment encoding "sense" rat GR cDNA (PVL296*). Then (c) a KpnI-SacI fragment encoding a section of the aP2 promoter enhancer/fragment (400bp) was added to the modified plasmid to create pJan1. *PVL 295 and 296 were created by Val Kelly.

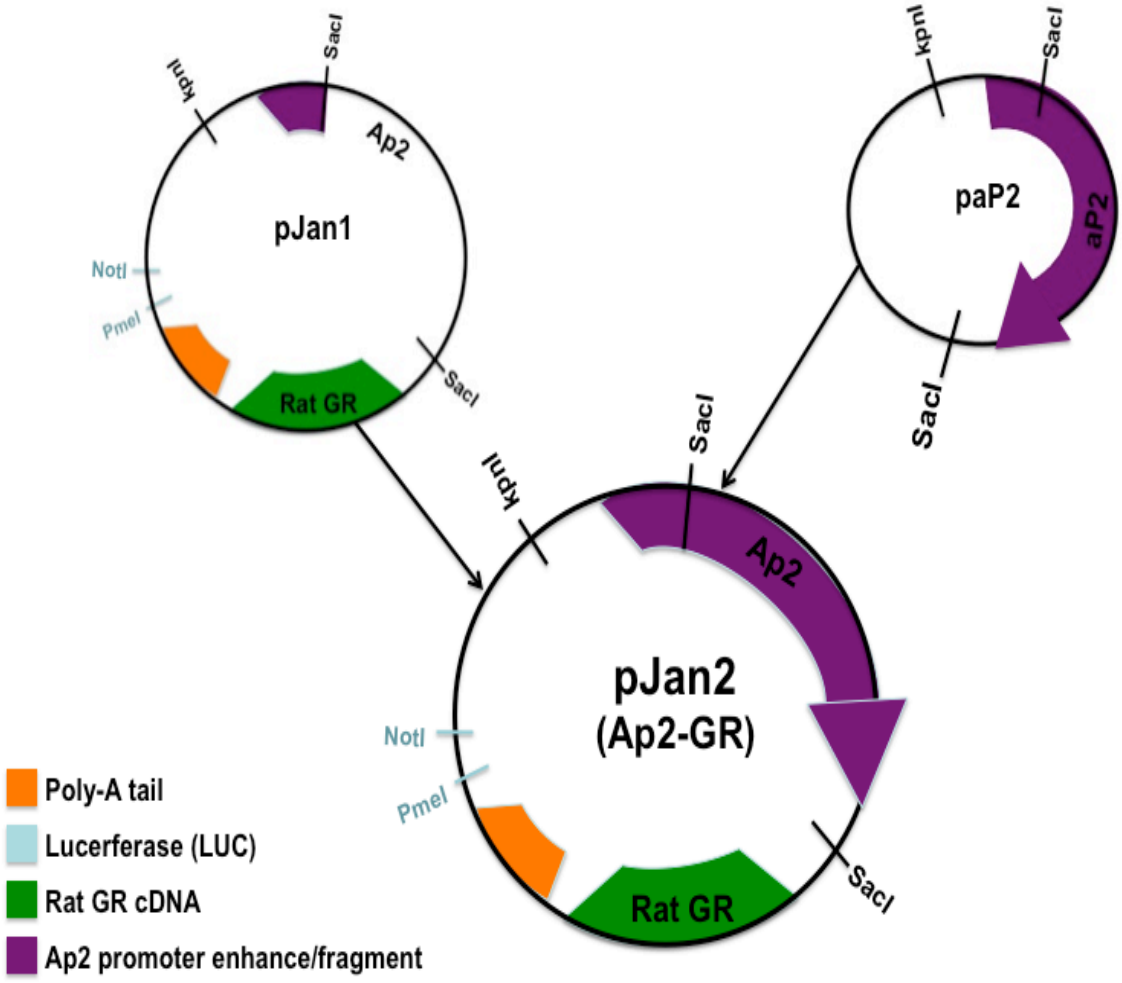

Figure 2.4 Schematic diagram of the structure and the formation of the Ap2-GR transgene.

Plasmid Ap2-GR was formed by SacI cleavage of pJan1 and insertion of a 5000bp SacI fragment from paP2 encoding the aP2 promoter/enhancer, to create pJan2. The transgene containing the fragment was then extracted by digestion with KpnI and PmeI.

2.2.15 Creation of the SV40-GR construct

To establish that the reconstructed GR cDNA was functional, a second construct was created using a similar approach. The aP2 enhancer/promoter in pJan2 was replaced with the SV40 viral promoter/ enhancer from pJ3 (Morgenstern and Land 1990). pVL296 was linearised with NheI to generate a fragment of 5560bp which was blunt ended by fillinf in the ends with Klenow (section 2.2.11.2) then ligated to a 400b.p. PvuI-SmaI fragment encoding the SV40 promoter enhancer from pJ3. The resulting construct, pJan3 (Fig.2.6A) was verified using SalI/Xba double digest, which created fragments of 3300, 2200 and 480bp (Fig.2.6B). The construct was transfected into B103 cells, which have negligible endogenous GR (Freeman, Munn et al. 2004) to assay GR activity.

2.2.16 Creation of aP2-GR founder mice

A KpnI/PmeI double digest was used to isolate the 11kb aP2 promoter/enhancer-GR fragment to be injected Chapter4. The 11kb fragment was excised from a gel and isolated using a Qiaex gel extraction kit (Qiagen) according to the manufacturer's instructions. 10ng/µl of isolated fragment was microinjected into the pronuclei of C57BL/6J x CBA/C3H F_1 embryos and placed into foster mothers to produce "Founder" mice, by the re-derivation service, University of Edinburgh. Transgenic founder mice were identified by PCR carried out on genomic DNA extracted from either ear or tail clips using PCR primers that span the junction between the aP2 fragment and the GR cDNA (unique to the transgene) which produce a 376bp PCR product. Each of the founders was backcrossed to C57BL/6J mice to produce the F_1 generation.

Genotyping

2.2.16.1 Genotyping of aP2-GR transgenic mice by PCR

Genomic DNA was extracted from tail biopsies or from ear clips taken at weaning using a DNeasy® tissue kit (Qiagen) according to the manufactur's

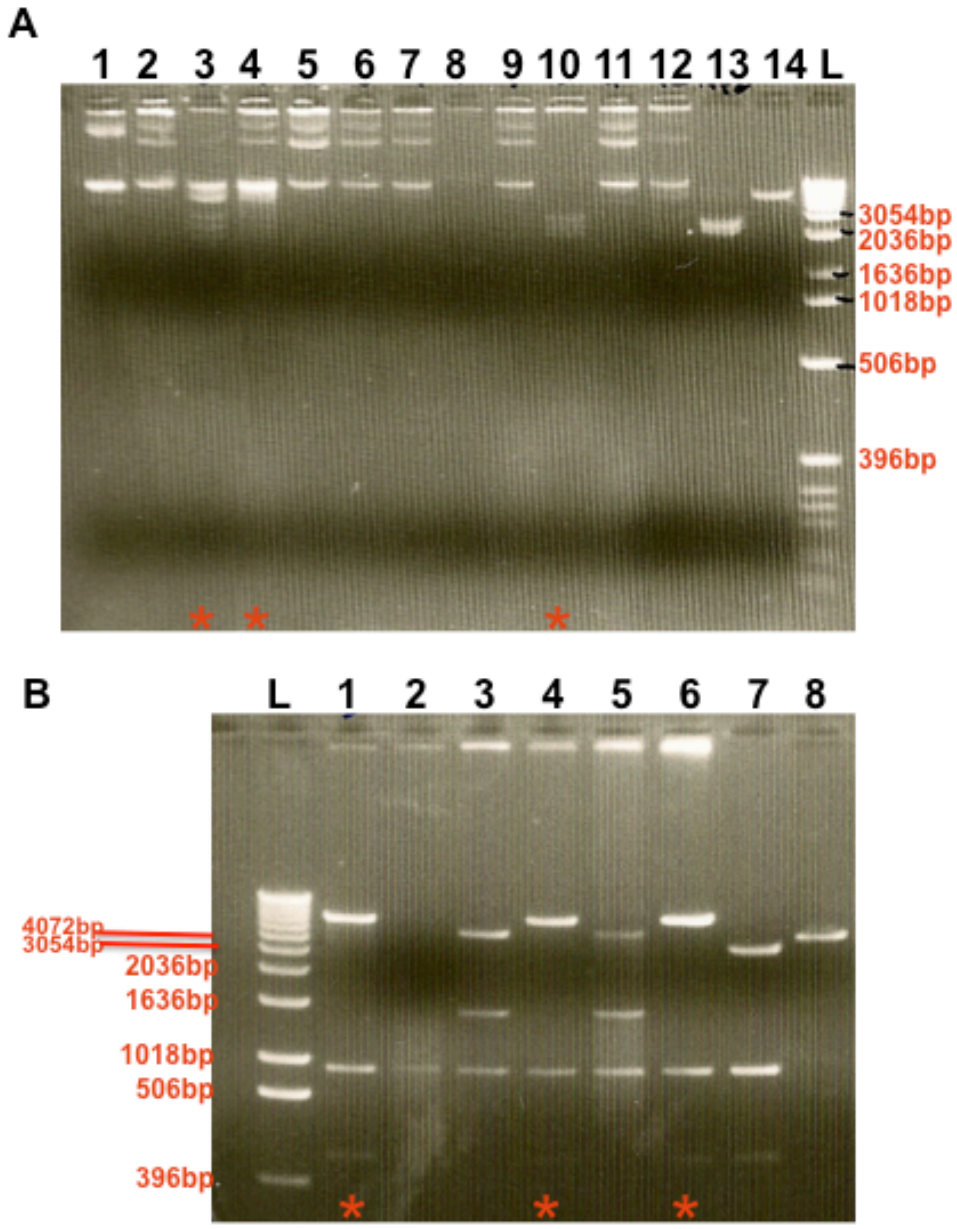

Figure 2.5 Restriction digest to identify plasmid Ap2-GR

Plasmid Ap2-GR (pJan2) was identified by restriction digest with (A) Xbal I (correct plasmid predicted to give fragments of 1876, 3200, 2750, 3660bp) and (B) EcoRV (correct plasmid predicted to give fragments of 918 and 8300bp). Each lane contains DNA from a different miniprep and lane marked "L" was loaded with 1kb ladder. *Indicates lanes in which the plasmid contains the correct predicted sized of fragments after digestion.

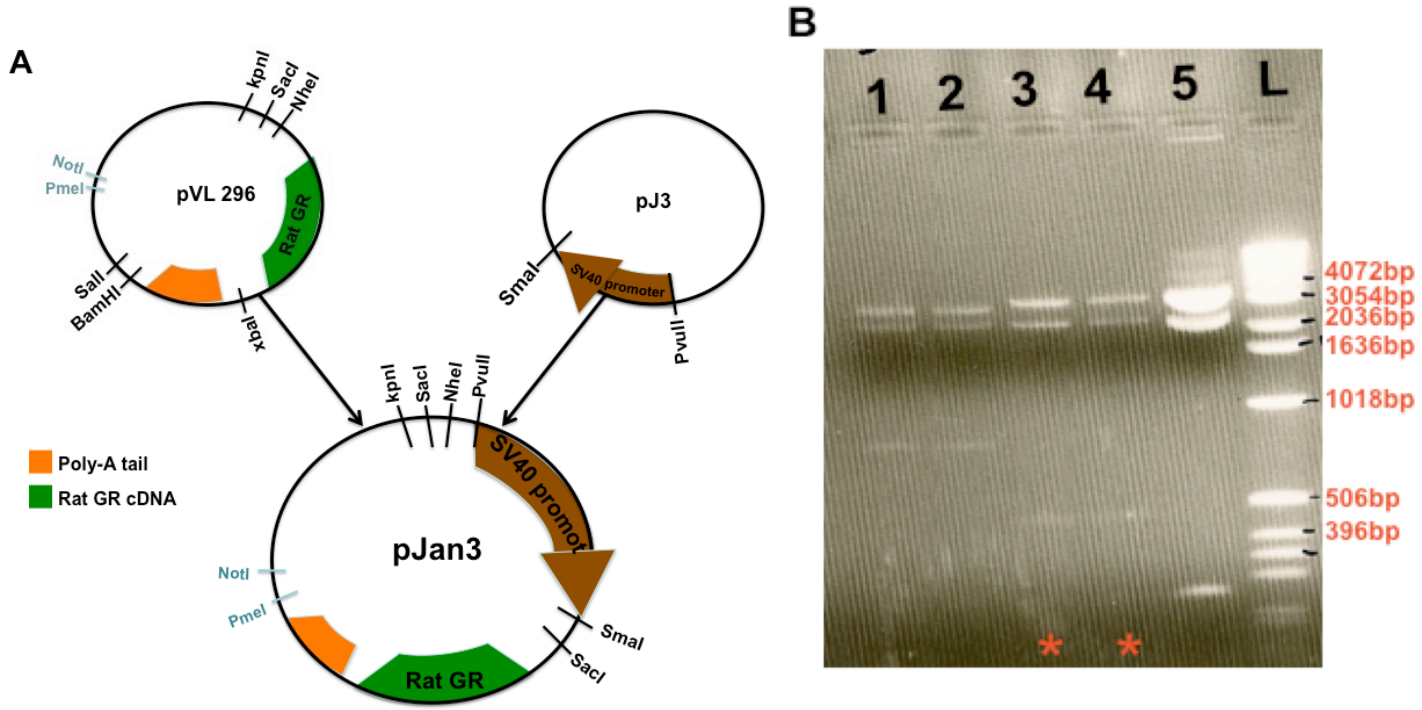

Figure 2.6 Schematic diagram of the structure and the verification of the SV40-GR construct

(A) Plasmid SV40-GR (pJan3) was formed by digesting pVL296 with NheI, filling in the sticky ends with Klenow fragment of DNA polymerase I to form blunt-end linear DNA, 5" phosphates with CIP, then ligating to a SV40 promoter fragment extracted from pJ3 by digesting with PvuI and SmaI. (B) DNA gel verifying the plasmid which the plasmid which contained the correct predicted fragments of 480, 2200 and 3300bp using SalI/XbaI double digest. * Indicated lanes in which the plasmid gives the correct predicted sizes of fragments after digestion.

instructions. DNA preparation from ear clips was identical to that from tail clips except that quantities were scaled down 10x.

In order to minimize non-specific priming, Taqbead hotstart polymerase (Promega) was used. PCR reactions were carried out in 0.5ml PCR reaction tubes and contained 1mM dNTPs, 1.5mM $MgCl_2$ (supplied with the polymerase), 1x polymerase reaction buffer (supplied with the polymerase), 0.1µM each primer (section 2.1.3), 1µl of genomic DNA and 1 Taqbead. The final volume of the PCR reaction was made to 50µl with ultra-pure H_2O. Initial denaturation was 96ºC for 8 min, then a total of 35 cycles of 94ºC 30s; 60ºC 90s; 72ºC 2 min were carried out, with a final incubation for 10 min at 72ºC. The aP2-GR primers amplify a 370 bp PCR product. Globin primers (section 2.1.2) , used as a positive control, amplify a 480bp PCR product. PCR products were assessed by electrophoresis of 20µl of the reaction on a 1.2% agarose gel (section 2.10.1).

2.2.17 Protein methods

2.2.17.1 Western blot

2.2.17.1.1 Protein extraction

For protein extraction, tissue samples were weighed and 50mg of tissue was homogenized in 600µl of protein extraction buffer. The homogenate was then centrifuged at 4k rpm (rotor F-45-11, Eppendorf microentrifuge 5415R) for 10min at 4°C to remove any debris. The supernatant was then removed and 50µl aliquots frozen at -20°C for future use and protein quantification.

2.2.17.1.2 Protein Quantification

Protein concentration was determined using the Bio-Rad DC protein assay (Bio-Rad). This is a colorimetric assay for protein determination. Sample concentrations were determined from a standard curve created from a series of dilutions of bovine serum albumin (BSA), ranging from 0-1.2mg/ml. To ensure all samples lay within the standard curve, a test assay was carried out using the most diluted sample within the set. This sample was compared with the highest protein standard (1.2mg/ml) using the Bio-Rad protein assay. Dilution was required if the sample shared a similar

or greater colour to the standard. All samples were diluted according to the test assay and measured in duplicate in a 96 well plate. 5µl of diluted sample or standard was added to each well followed by 50µl of reagent A then 200µl of reagent B. The plate was then incubated at room temperature for 15min before reading in a plate-reader spectrometer at 750nm (Molecular device, OPTImax). The protein concentration was then determined from the standard curve.

2.2.17.1.3 Western analysis of protein

25µg of protein sample was adjusted to 6.5µl with H_2O and added to 6µl of a pre-prepared mix of 5µl NuPAGE® LDS sample buffer (Invitrogen) and 1µl of NuPAGE® reducing agent (Invitrogen). Samples and 10µl of Plus2® pre-stained protein markers (Invitrogen) were incubated at 70ºC for 10min to denature the protein. Samples were loaded onto a 4-12% Bris-Tris Novex® pre-cast Gel (Invitrogen) which locked to the buffer core in a vertical electrophoresis tank. The inner chamber was filled with 200ml of 1x Novex® MOPs running buffer (Invitrogen) and 500µl of NuPAGE® antioxidant (Invitrogen) and the outer chamber filled with 600ml of 1x Novex® MOPs running buffer. Samples were electrophoresed at 200V until the bromophenol blue band had migrated to the bottom of the gel. The gel was then removed form the plastic cassette and proteins transferred from the gel to a nitrocellulose membrane using a wet transfer system. The transfer was set up as shown in Fig 2.7. Transfer was effected at 30V for 90min. Following transfer, the membrane was rinsed in H_2O and left to dry on Whatman No.3 filter paper.

2.2.17.1.4 Antibody binding to western blot

To reduce non-specific antibody binding, the membrane was rehydrated with 1xTBS for 5 min blocker (Bio-rad), 0.1% Tween) for 1h at RT or overnight at 4°C with agitation. The blocking solution was then removed and the membrane was incubated with primary antibody diluted in blocking buffer (GR 1:400 or tubulin 1:5000) for 2h at room temperature with agitation. Next, the membrane was rinsed in blocking solution three times, each for 10min then incubated with agitation for 2h at

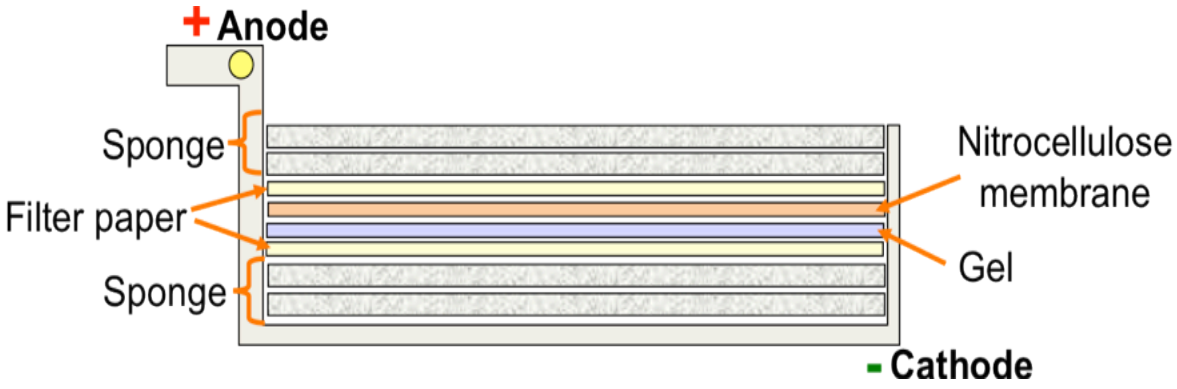

Figure 2.7 Schematic diagram of western blot transfer setup

Schematic diagram showing the wet transfer step of western blot used to transfer protein from SDS-PAGE gels to nitrocellulose membranes.

room temperature with secondary antibody diluted in blocker buffer. For the GR primary antibodies, the secondary antibody was goat anti-rabbit IgG-HRP (Santa-Cruz) , used at a dilution of 1:5000; for the tubulin primary antibody, the secondary antibody was goat anti-mouse IgG-HRP (Santa Cruz), used at a dilution of 1:10,000. Finally, the membrane was rinsed in 1x TBS for 15min, followed by three washes in 1x TBS, each for 5min. The secondary antibody was visualized by incubating the membrane in 1ml of ECL detection mix (equal volumes of reagents 1 and 2) for 1min before exposing against autoradiographic film for a period of time between 5 and 15min and then developing. Autoradiographs were scanned using ascanner and quantitated using Aida software (Advance image data analyzer Version 3.44.035).

2.2.17.1.5 Peptide binding assay

Mouse GR has a predicted molecular mass of ~86kDa, but migrates on SDS-PAGE at ~95kDa (Hutchison, Dalman et al. 1993). To determine which specific band within the western blot represented GR, the primary antibody was preabsorbed with the peptide used as immunogen (PA1-512, Cambride Bioscience). Two tubes of primary antibody were prepared, a "control" tube containing 1μg GR antibody with 2 ml of blocking solution and a "neutralized" tube containing 1μg GR antibody with 4μg neutralizing peptide in 2ml of blocking solution. Both tubes were incubated with agitation at RT for 1h. The primary antibody was then applied to the two separate blots as described in 2.12.1.4. By comparing the "control" blot with the "neutralized" blot the GR immunoreactive band was identified. (Fig.2.8)

2.2.18 Statistical analysis

For statistical analysis of data obtained in this theses, 2 statistical software were used, Sigma Stat 3.1 and GraphPad Prism 5.

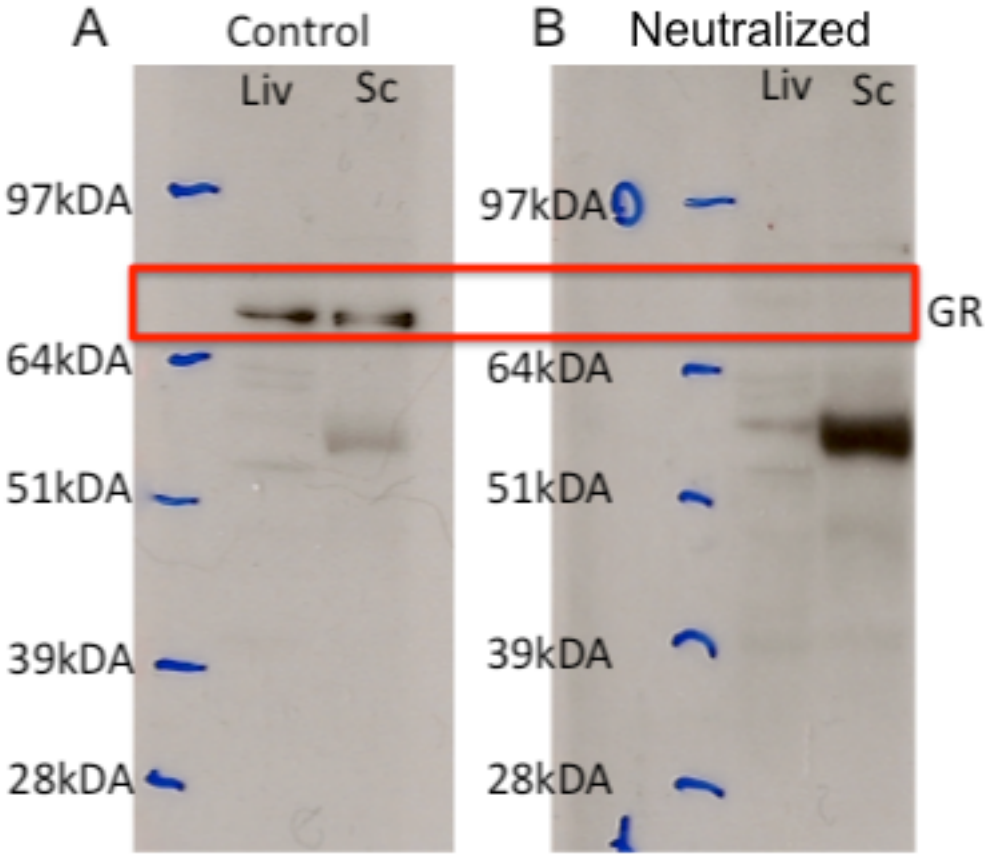

Figure 2.8 Validation of the GR antibody

To identify the GR immunoreactive band, the primary GR (PAI-512) antibody was preabsorbed against the peptide used to generate the antibody. Two identical western blots loaded with 30µg protein from liver (Liv) or Sc adipose tissue of non-transgenic mice were probed (A) with GR antibody and (B) with GR antibody preincubated with neutralising peptide. The band identified as GR is highlighted in red.

3 The Effects of Dietary Fats on Peripheral Glucocorticoid Action

Dietary fats are known to contribute to the development of obesity, hypertension, dyslipidemia and insulin resistance, collectively termed the metabolic syndrome. It appears that different types of dietary fatty acid can differentially impact on insulin sensitivity. In healthy humans, it has been shown that without altering total fat content, simply switching from a diet high in saturated fat to unsaturated fat results improves insulin sensitivity (Vessby et al 2001, Ricardi et al., 2004). Furthermore, stearate (18:0) an abundant dietary long chain saturated fat inhibits insulin signaling in muscle cells, whereas myristate (C14:0) a short chain saturated fat, and oleate (18:1) a long chain unsaturated fat, do not exert such effects (Chavez and Summers 2002).

Interestingly, a high fat diet regulates adipose 11β-HSD1 level (Morton, Ramage et al. 2004). Both acutely and chronically, mice fed high fat diet show a marked reduction in 11β-HSD1 mRNA levels and enzyme activity in all adipose depots (Morton, Ramage et al. 2004). It was suggested that the down-regulation of adipose tissue 11β-HSD1 expression upon high fat diet might be protective against obesity (Morton, Ramage et al. 2004), but the exact mechanism that caused this down-regulation was unclear. The work described in this chapter was designed to test the hypothesis that 11β-HSD1 and glucocorticoid receptor (GR) expression levels within adipose tissue are regulated by the composition of dietary fats. To address this, mice were fed diets enriched in long-chain saturated, mono-unsaturated or poly-unsaturated fatty acids, and their effects on systemic corticosterone levels, glucose/insulin homeostasis and tissue 11β-HSD1 mRNA levels were investigated.

3.1 Result

3.1.1 Study 1: Pair Feeding of HF Diet; Experimental Design

Male C57BL/6J mice aged 6-8 weeks, n=12 per group, were housed in pairs under standard conditions with 12h light and 12h dark. Mice were given one of three different high fat diets; enriched in saturated (stearate, 18:0), mono-unsaturated (oleic, 18:1) or poly-unsaturated fat (safflower, 18:2). In order to allow comparison, all three diets contained the same kcal/g and % of fat (Table 3.1). In addition, a

control diet enriched in sucrose was selected which contained a lower % of fat and kcal/g (Table 3.1). To exclude an effect from different calorie consumption, all groups were fed *ad libitum* for a week with food intake monitored. The control sucrose group was found to consume the lowest calories. All diet groups were given calorie-matched diet daily, with continuous adjustment to the average intake for the control sucrose group, for 3 weeks. Weight gain and food intake were monitored and recorded every day. At the end of the experiment, mice were culled at 2 time points; morning (8am) and evening (8pm). This approach was used to allow an insight into the effect of diet on circadian rhythm (plasma hormones) and the HPA axis.

3.1.1.1 Effect of HF diet upon body weight

During the first week of the study, when all groups were allowed *ad libitum* access to food, the control group had the lowest energy (kcal) intake (Fig 3.1). Therefore, for the remaining 3 weeks, all the other groups were pair-fed to the control group. Throughout the study, the stearate group routinely consumed all the food within 16 h, while all the other groups had food remaining at the time of monitoring and food replenishment (data not shown).

At the end of the experiment, the mean body weight of stearate-fed mice was 15% less than control diet-fed mice (Fig 3.2). The mean body weight of the group fed safflower oil enriched diet was highest throughout the study, but was not significantly different to the control diet group (Fig 3.2). The body weight of the oleic acid-fed group was similar to the control diet group (Fig 3.2).

To assess any differences in adipose tissue distribution and weight, the mesenteric (Mes), subcutaneous (Sc), epididymal (Epi), retroperitoneal (RP) and brown adipose depots (BAT) were dissected and weighed. In addition, kidney, liver and adrenal gland were dissected and weighed to assess changes in other organs possibly affected by diet. There were no differences in adipose tissue or organ weights between the control, safflower and oleic acid fed-groups, whether the animals were killed in the morning or the evening (Fig 3.3). However, in the stearate group, adipose tissue weights were significantly lower than in the control group (Fig 3.3). No differences were seen in any of the organ weights measured in the morning.

Table 3.1 Composition of diets used in study 1 and study 2

	Research diet No.	kcal/g	%kcal fat	%kcal Carbohydrate
Control (Sucrose)	D12329	4.07	11%	73%
Stearate	D04070901	5.05	45%	39%
High Oleic	D04070902	5.05	45%	39%
Safflower	D04070903	5.05	45%	39%
Control (Cornstarch)	D12328	4.07	11%	35%
45% mixed fat	D12451	4.73	45%	35%
58% mixed fat	D12331	5.56	58%	25.5%

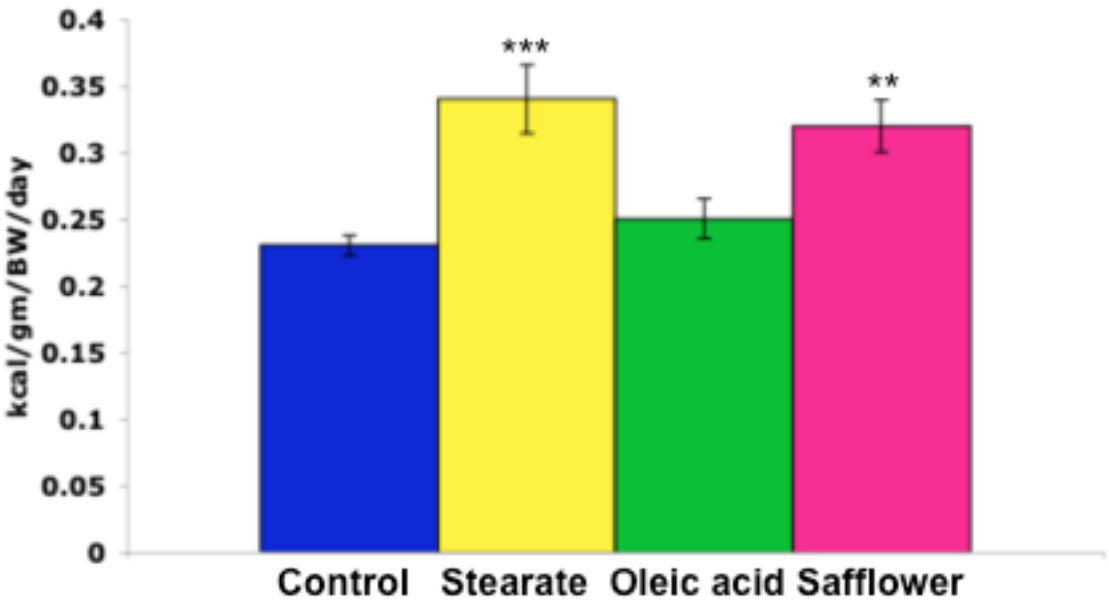

Figure 3.1 Caloric intake during the first week of the high fat diet

Total calorie intake per mouse in week 1. Control (■), Stearate (■), oleic acid (■) and safflower oil (■) Data are expressed as mean ± SE (n=6). **P<0.01, ***P < 0.001, compared to control diet (one way ANOVA followed by Dunnett post test).

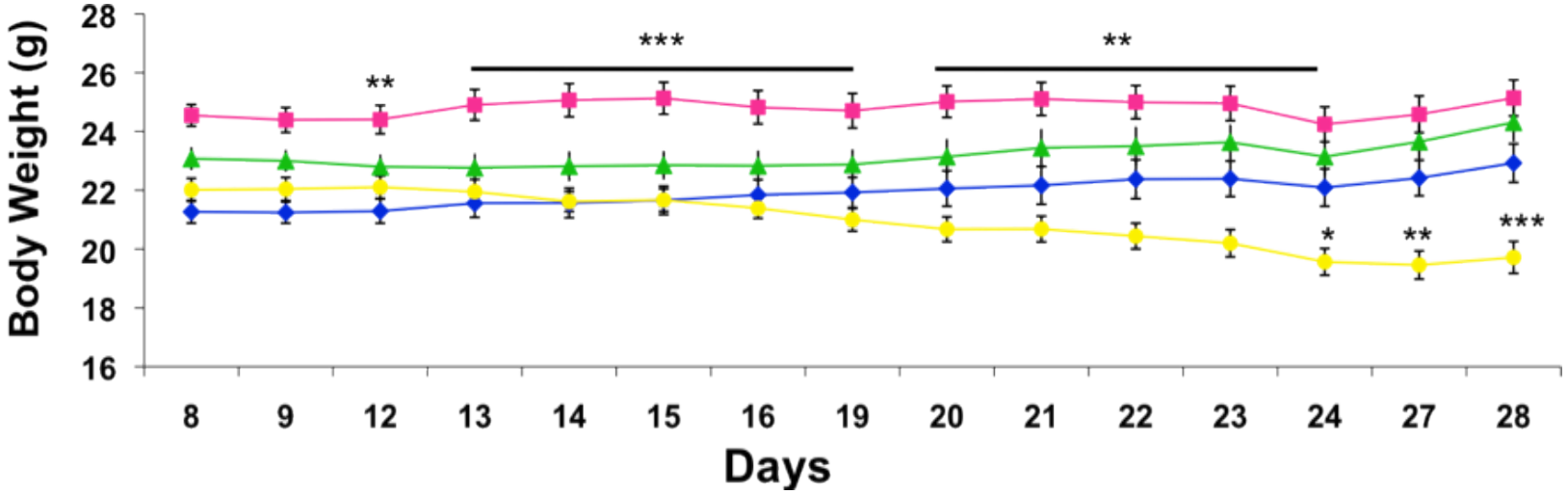

Figure 3.2 Longitudinal body weights

Body weight of mice fed control diet (●) or diets enriched in stearate (●), oleic acid (●) or safflower oil (●) during the entire 4 weeks of the study which included 1 week of free access to food to assess food intakes followed by 3 weeks of pair-feeding to the control group. *P<0.05, **P<0.01, ***P < 0.001, compared to control diet (two way RM ANOVA). Data are mean body weight of each group ± SEM**.**

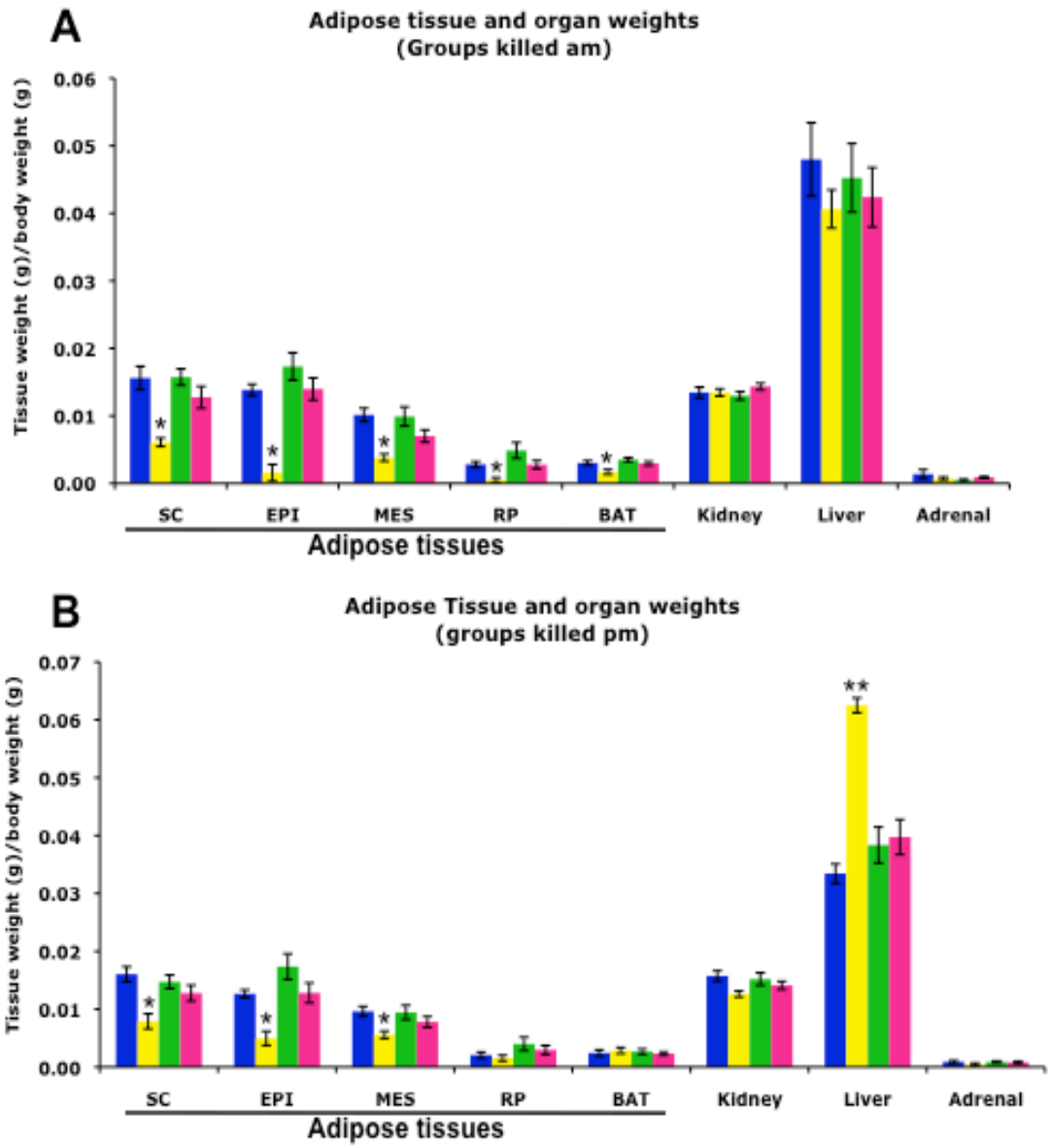

Figure 3.3 Effect of diets on adipose tissue and organ weights

Weights of adipose tissues from mice on control (■), stearate (■), oleic acid (■) and safflower oil (■) diets. Data from mice killed at 8am (A) or at 8pm (B). Data are mean ± SEM; * P<0.05, **p<0.01 ANOVA, compared to control diet.
Adipose tissues were: Subcutaneous (Sc), Epididymal (Epi), Mesenteric (Mes), Retroperitoneal (RP), Brown adipose tissue (BAT)

However, the liver weighed significantly more in the stearate group killed in the evening compared to the control diet group (Fig 3.3).

3.1.1.2 Effect of pair-feeding high fat (HF) diets upon plasma hormone levels

To determine the effect of the different HF diets upon plasma hormone levels, plasma corticosterone (Cort) and insulin levels were measured. In the morning, at the nadir level of Cort, the stearate group had 10-fold increased Cort levels compared to the control group (Fig 3.4A). Morning Cort levels in the oleic acid and safflower oil-fed groups were similar to the control group (Fig 3.4A). Although there was a trend for the oleic acid and safflower oil groups to have lower plasma Cort levels in the evening than the control group, this was not significant (Fig 3.4B).

Morning plasma insulin levels in the stearate group were significantly lower than in the control group (Fig 3.5A). Apart from that, there were no other significant differences between plasma insulin levels either in the morning or in the evening (Fig 3.5). Fasting plasma glucose levels were not different between groups, either in the morning or the evening (Fig 3.6).

3.1.1.3 Effect of HF diets upon 11β-HSD-1 mRNA and activity levels in adipose tissue

To assess whether any of the HF diets affected 11β-HSD1 expression, levels of 11β-HSD1 mRNA were measured by northern analysis of RNA isolated from Mes, Sc and Epi fat depots as well as liver. In addition, 11β-HSD1 enzyme activity was measured in homogenates of adipose tissues and liver (by Lynne Ramage). No group differences were seen between morning and evening 11β-HSD1 mRNA levels in Sc fat (Fig 3.7A). However in Epi fat, in the oleic acid fed group,11β-HSD1 mRNA levels were significantly higher in the evening compared to the morning

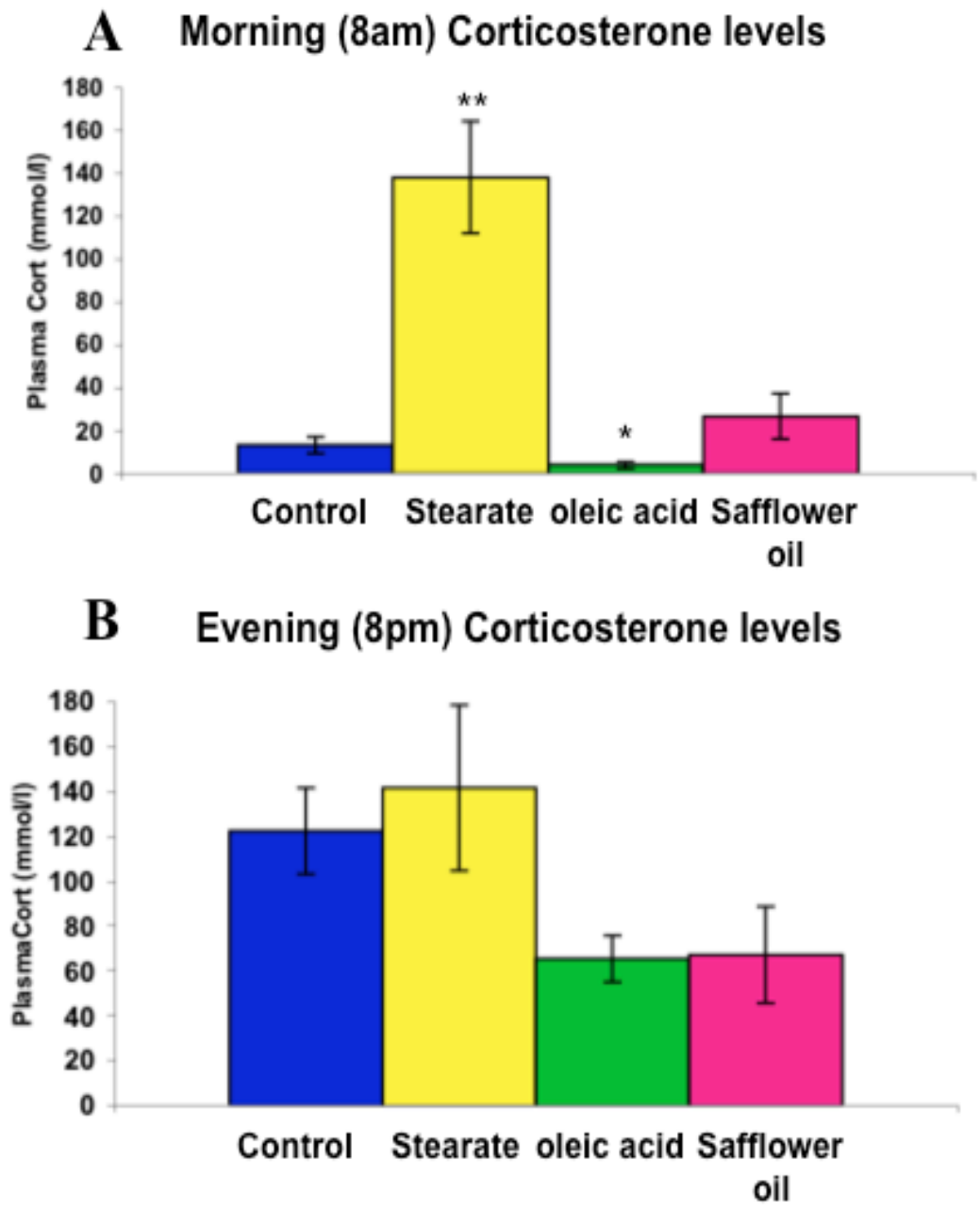

Figure 3.4 Consequence of high fat diet on plasma corticosterone level

Plasma Cort levels at 8am (A) and 8 pm (B) with control group (■), stearate group (■), high oleic acid group (■) and safflower oil group (■). Data are expressed as mean ± SE (n=6). *P < 0.05, ANOVA, compared to control diet.

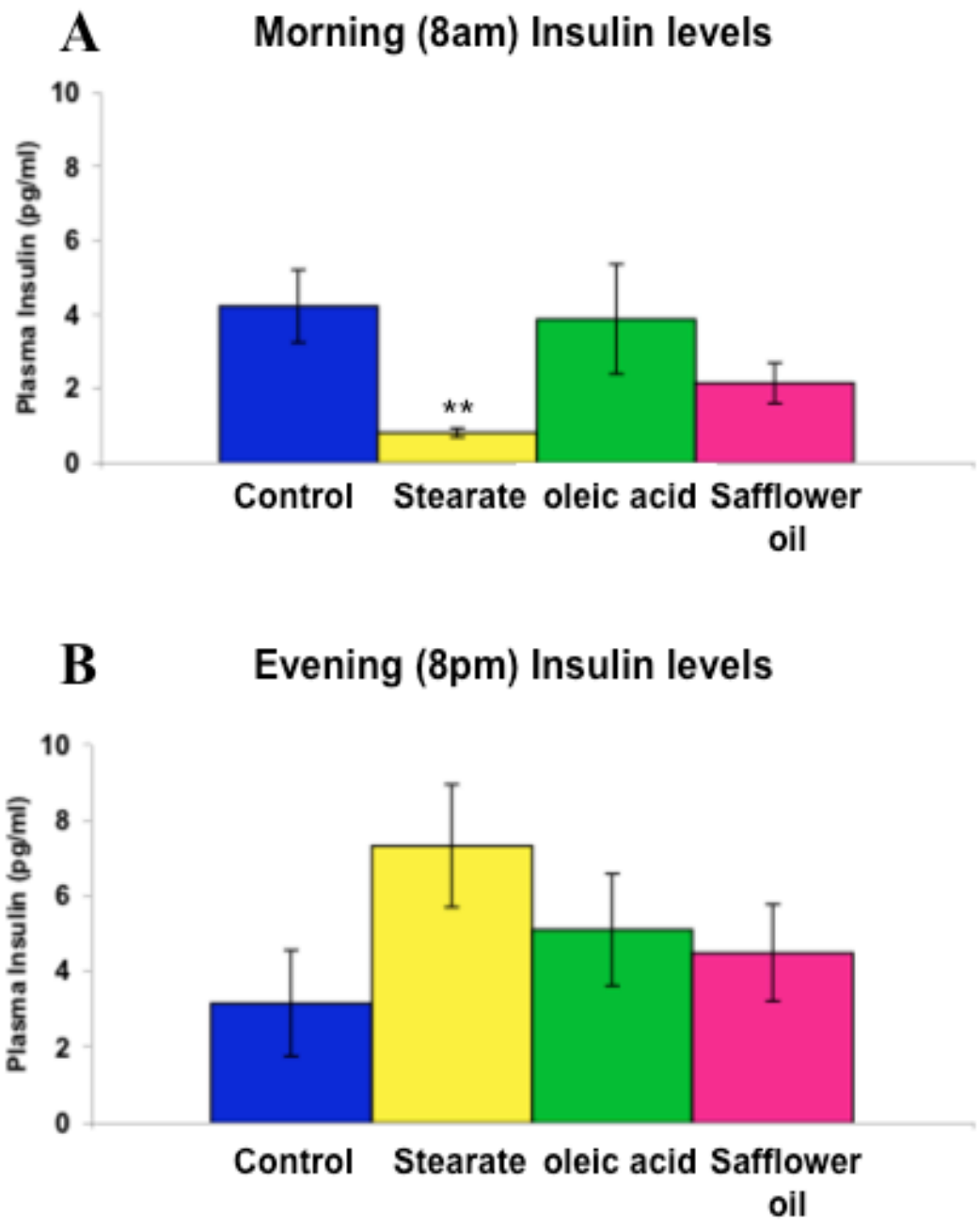

Figure 3.5 Consequence of high fat diet on plasma insulin levels

Plasma insulin levels were measured in control (■), stearate (■), high oleic acid (■) and safflower oil-fed groups (■) under basal conditions at 8am (A) and 8pm (B). Data are expressed as mean ± SE (n=6), **P < 0.005, ANOVA, compared to control diet.

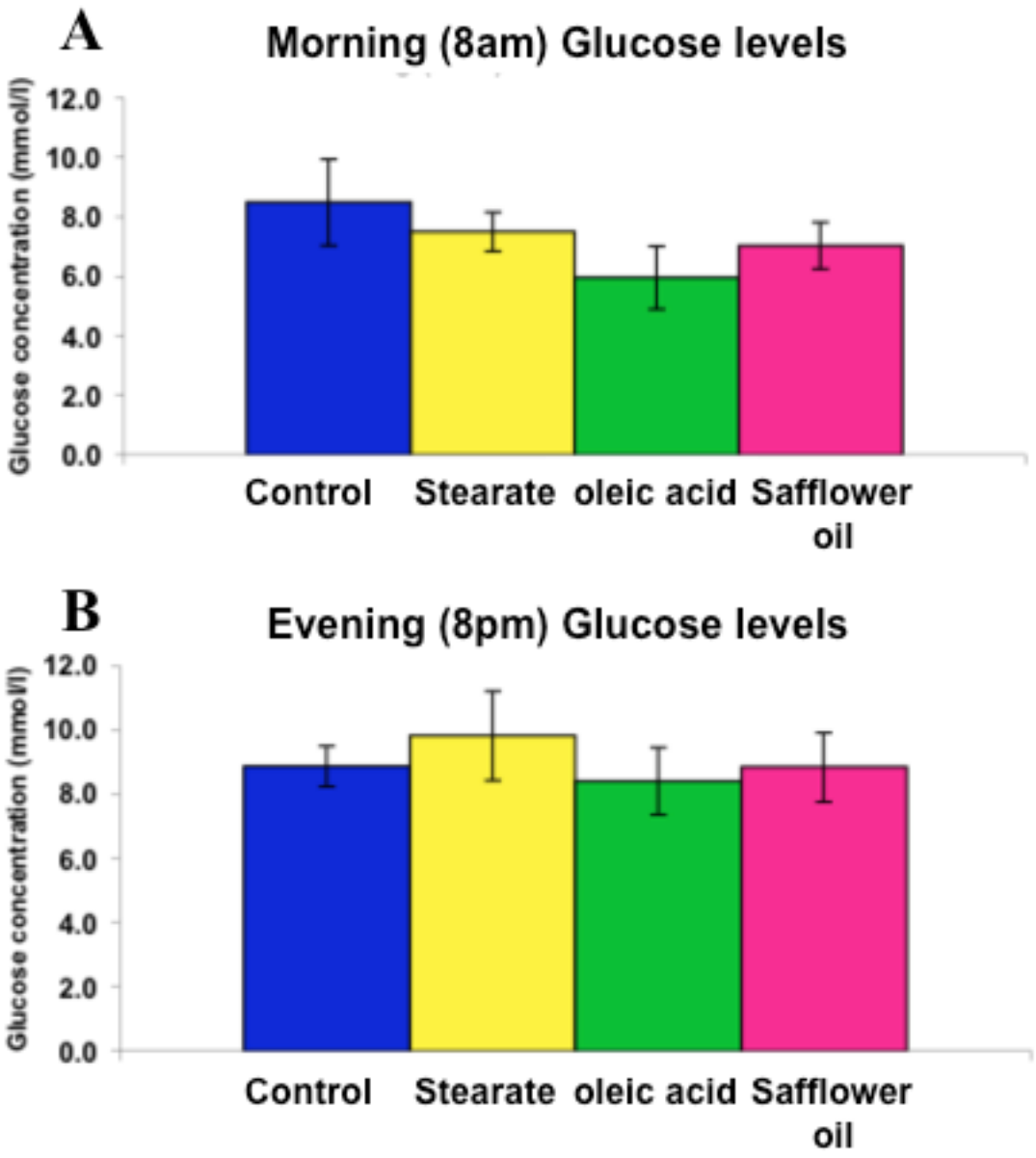

Figure 3.6 Consequence of high fat diet on fasting glucose levels

Fasting plasma glucose levels were measured in Control (■), stearate (■), oleic acid (■) and safflower oil (■) groups at 8am (A) and 8pm (B). Data are expressed as mean ± SE (n=6). There were no significant difference (1-way ANOVA) between groups.

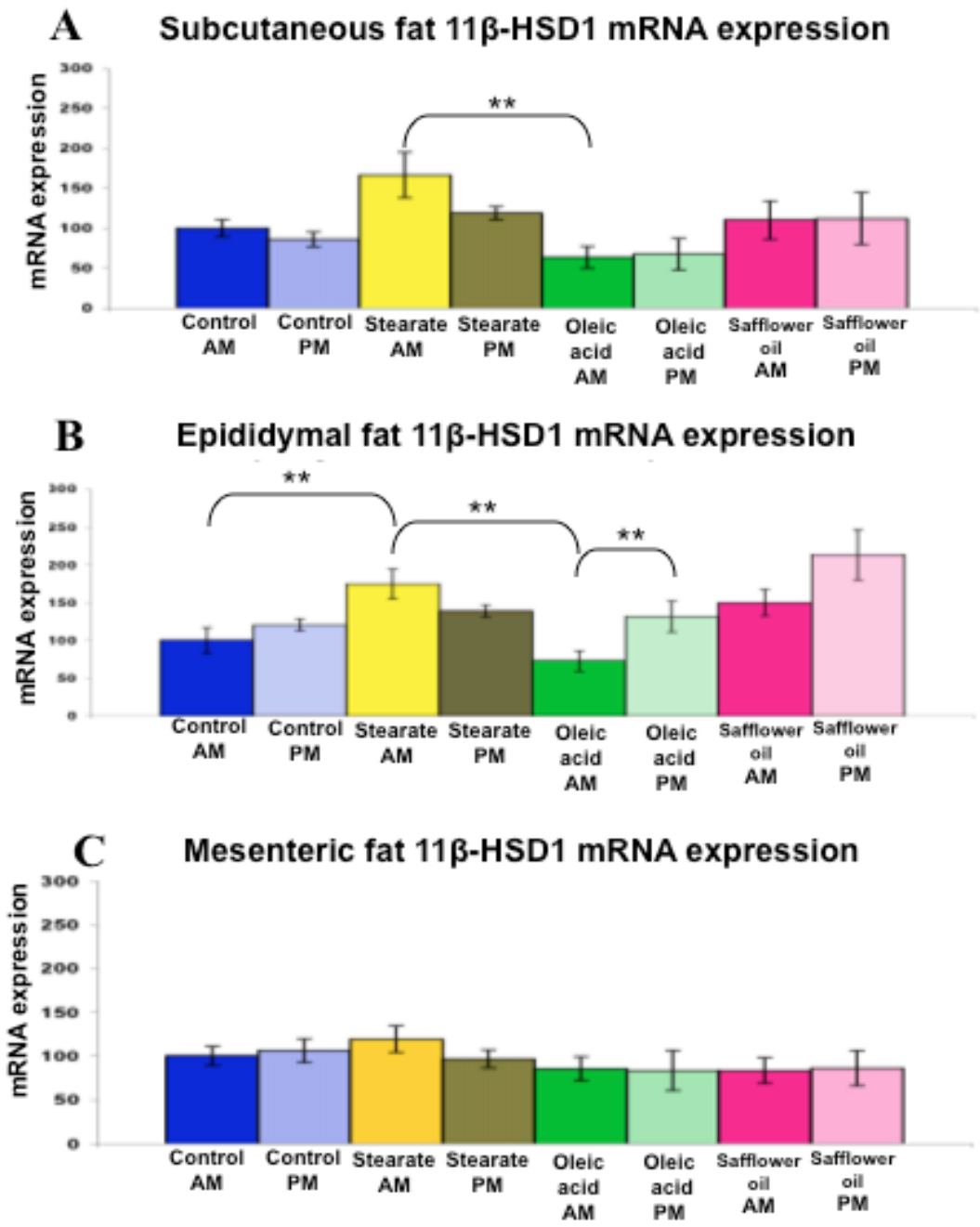

Figure 3.7 Effect of high fat diet on 11β-HSD1 mRNA expressions

11β-HSD1 mRNA levels were measured in control (■), stearate (■), oleic acid (■) and safflower oil-fed (■) groups killed at 8am (dark bars) or 8pm (light bars). 11β-HSD1 mRNA levels were measured relative to 18S RNA and are expressed as % of control killed at 8am, arbitrarily set to 100%. 11β-HSD1 activity assays were performed by L. Ramage. 11β-HSD1 activity is expressed as % conversion of substrate (Cort) to product (11dehydro cort). Data are expressed as mean ± SE (n=6); with mice on control diet arbitrarily set to 100%. * indicates a significant difference (ANOVA) between the indicated groups with **P< 0.01.

(Fig 3.7B). In addition, in Epi fat, morning 11β-HSD1 mRNA levels in the stearate group were increased compared to the control group (Fig 3.7B). In the evening, all 3high fat groups exhibited a similar level of 11β-HSD1 mRNA expression in Epi fat to the control group (Fig 3.7). There were no differences between groups or am versus pm in 11β-HSD1 mRNA levels in mes adipose tissue (Fig 3.7C)In the three fat depots, there were no differences between 11β-HSD1 enzyme activity measured in the morning and evening, within each diet group (Fig 3.8). However, an increase in 11β-HSD1 activity was observed in the stearate-fed group compared with the control diet-fed group, in all 3 depots (Fig 3.8).

In all three depots, 11β-HSD1 activity in the morning was significantly higher in the stearate-fed group than in the oleic acid-fed group (Fig 3.8). A similar pattern was also observed between the stearate- and safflower oil-fed groups, with higher 11β-HSD1 activity in the stearate group. However, the difference between stearate- and safflower oil-fed groups only achieved significance in the Sc and Epi fat depots (Fig 3.8).

3.1.1.4 Effect of HF diets upon GR and 11β-HSD1 mRNA levels in liver

To determine the effect of HF diet on glucocorticoid action within liver, GR and 11β-HSD1 mRNA levels were measured (Fig 3.9). Within each diet group, a significant difference between 11β-HSD1 mRNA levels in morning and evening was observed in both the stearate–fed and oleic acid-fed groups; in the stearate group, a 2-fold increase in 11β-HSD1 mRNA expression was found in the evening compared to the morning. However, the reverse pattern was observed in the oleic group, with a significant decrease in 11β-HSD1 mRNA in the evening compare to the morning (Fig 3.9A). In the evening, 11β-HSD1 mRNA levels in the stearate group were 3-fold higher than the control group (Fig 3.9A). Although a similar pattern of expression of GR mRNA was observed, none of the differences were significant (Fig 3.9B).

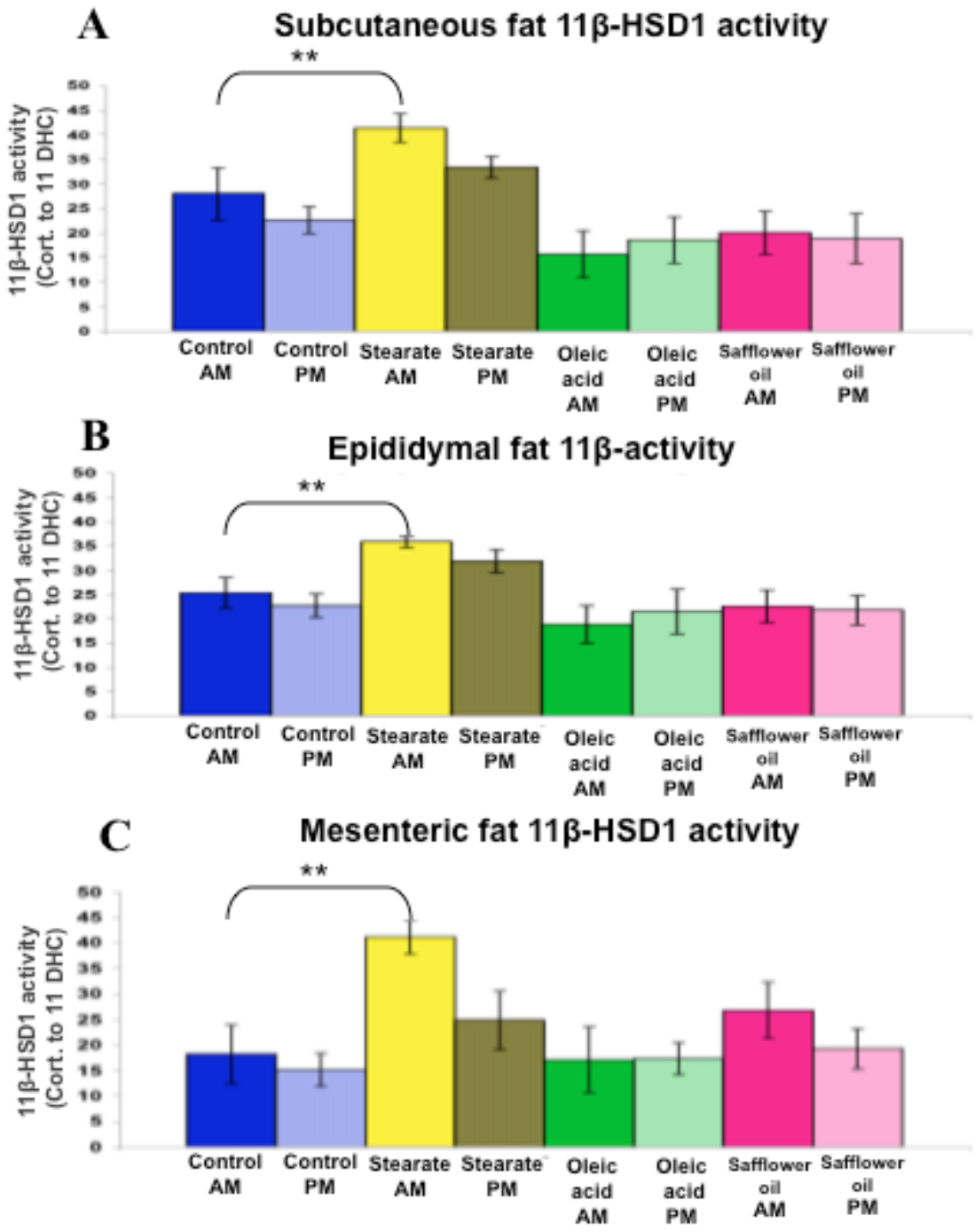

Figure 3.8 Effect of high fat diet on 11β-HSD1 activity in adipose tissues

11β-HSD1 enzyme activity were measured in control (Con)(■), stearate (ST)(■), high oleic acid (HO)(■) and safflower oil (SA)(■) groups killed at 8am (solid bars) or 8pm (spotted bars). 11β-HSD1 activity assays were performed by L. Ramage; 11β-HSD1 activity is expressed as % conversion of substrate (Cort) to product (11dehydro-cort).

Data are expressed as mean ± SE (n=6); with mice on control diet arbitrarily set to 100%. * indicates a significant difference (ANOVA) between the indicated groups with **P< 0.01.

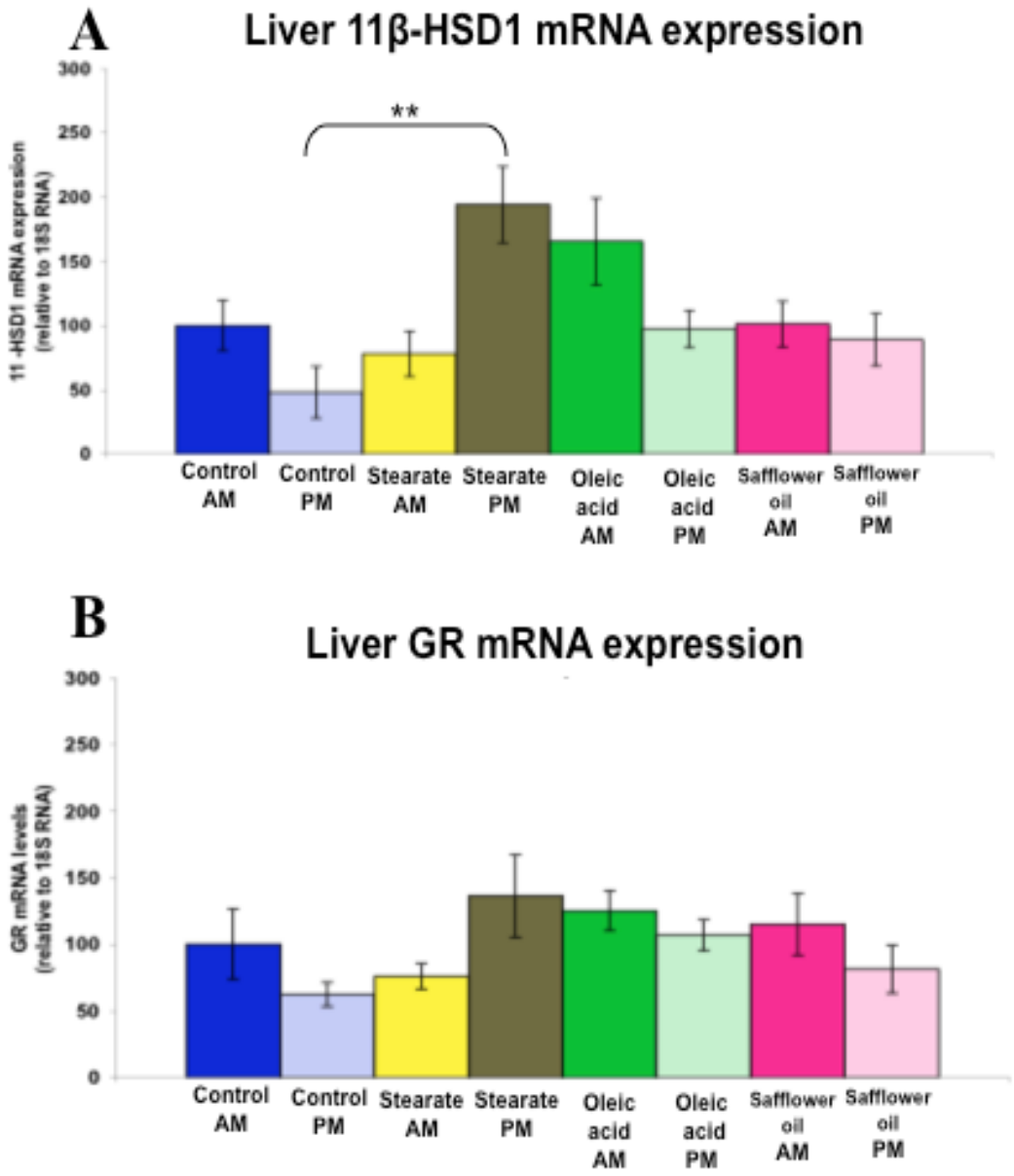

Figure 3.9 Effect of high fat diet on 11β-HSD1 and GR mRNA expression in liver

Both HSD1 mRNA expression (A) and GR mRNA expression was measured in mice fed in control diet (■) or diets enriched in stearate (■), High oleic acid (■) or safflower oil (■) in both 8am (dark bars) and 8pm (light bars). mRNA expression was measured relative to 18s RNA and is expressed as % of control killed at 8am, arbitraity set to 100%. Data are expressed as mean ± SE (n=6). ** P<0.01 (ANOVA).

3.2 Study 2: *Ad libitum* Feeding of HF diet: Aims and design

Study 1 showed that diets enriched in different fatty acids resulted in alterations in glucocorticoid action. However, the results were limited by the possible adverse effect of pair-feeding, particularly in the stearate group which lost weight during the study. Furthermore, none of the groups showed the down-regulation of adipose 11β-HSD1 activity previously observed in mice fed a 58% fat diet (mixed fat content) compared with an 11% fat (cornstarch-enriched) control diet (Morton, Ramage et al. 2004). Therefore a second study was carried out, in which diets were fed *ad libitum* to rule out the effect of calorie restriction. Additionally, in this study, to rule out a difference between control diets (cornstarch versus sucrose-enriched) both control diets were included. For comparison with the original study (Morton, Ramage et al. 2004), the same 58% fat diet (mixed fat) was selected. To exclude effects due to differences in % of kcal as fat, a 45% fat (mixed fat, identical composition to the 58%) diet was also selected. This 45% fat diet contains the same % of fat as the selectively enriched fatty acid diets used in study 1. Mice (n=6/group) were allowed *ad libitum* access to food throughout the study, which was carried out over 4 weeks. Body weight and food intake were recorded daily. Five days prior to the end of the experiment, blood was taken in the evening to measure plasma corticosterone levels. Three days prior to the end of the experiment, mice were fasted for 6h and blood was taken for measurement of fasting plasma glucose and insulin. This approach would allow an insight into insulin sensitivity. At the end of the experiment, mice were sacrificed in the morning, and blood was taken for measurement of plasma corticosterone levels. Tissue was collected as described in Study 1. RNA was extracted from tissues and both 11β-HSD1 and GR mRNA levels determined.

Results

3.2.1.1 Effect of *ad libitum* feeding HF diet upon body weight

After 4 weeks of *ad libitum* feeding, the body weight of the sucrose-enriched control group did not differ from the cornstarch-enriched control group. Mice fed oleic acid, safflower oil, 45% fat or 58% fat diets all gained significantly more body

weight than the control groups (Fig 3.10). Despite consumption of comparable amounts of calories, stearate-fed was the only group which lost weight during the study (Fig 3.10 and 3.11).

To assess any difference in adipose tissue distribution and weight, adipose tissues were dissected as described in Study 1. In addition, liver and kidney were also dissected as described in Study 1. There were no differences in adipose tissue or organ weights between the two control groups fed cornstarch or sucrose diets (Fig 3.12). In mice fed oleic acid or 58% fat diets, Sc, Epi and Mes fat depot weights were significantly greater than in the control cornstarch group (Fig 3.12). In contrast, in the stearate group, the Sc, Epi and Mes fat, weights were significantly lower than the cornstarch control group (Fig 3.12). Fat weight in the other groups (safflower oil or 45% fat) did not differ significantly from the cornstarch control group (Fig 3.12). No differences were seen in brown adipose tissue or organ weights between the groups (Fig 3.12).

3.2.1.2 Effect of *ad libitum* feeding HF diet upon plasma glucose hormone levels

In order to assess the effect of diet upon glucose homeostasis, fasting plasma insulin and glucose levels were measured (Fig 3.13). No differences in fasting plasma glucose/insulin levels were seen between the control sucrose and the control cornstarch group (Fig 3.13B). Compared to the cornstarch control group, the stearate-fed group had significantly lower fasting plasma insulin and glucose levels (Fig 3.13A), whereas, mice fed the oleic, safflower and 58% fat diets all showed a significant increase in both fasting insulin and glucose levels, suggesting a tendency towards insulin resistance (Fig 3.13A).

No difference in plasma corticosterone levels was seen between the control sucrose and the control cornstarch group either in the morning (nadir) or evening (peak) (Fig 3.14). Compared to the control cornstarch group, the oleic acid safflower oil, 45% fat and 58%-fat fed groups showed suppressed morning plasma corticosterone levels (Fig 3.14A). Peak plasma corticosterone (Cort) levels did not differ in any group compared to the control cornstarch group (Fig 3.14B). However,

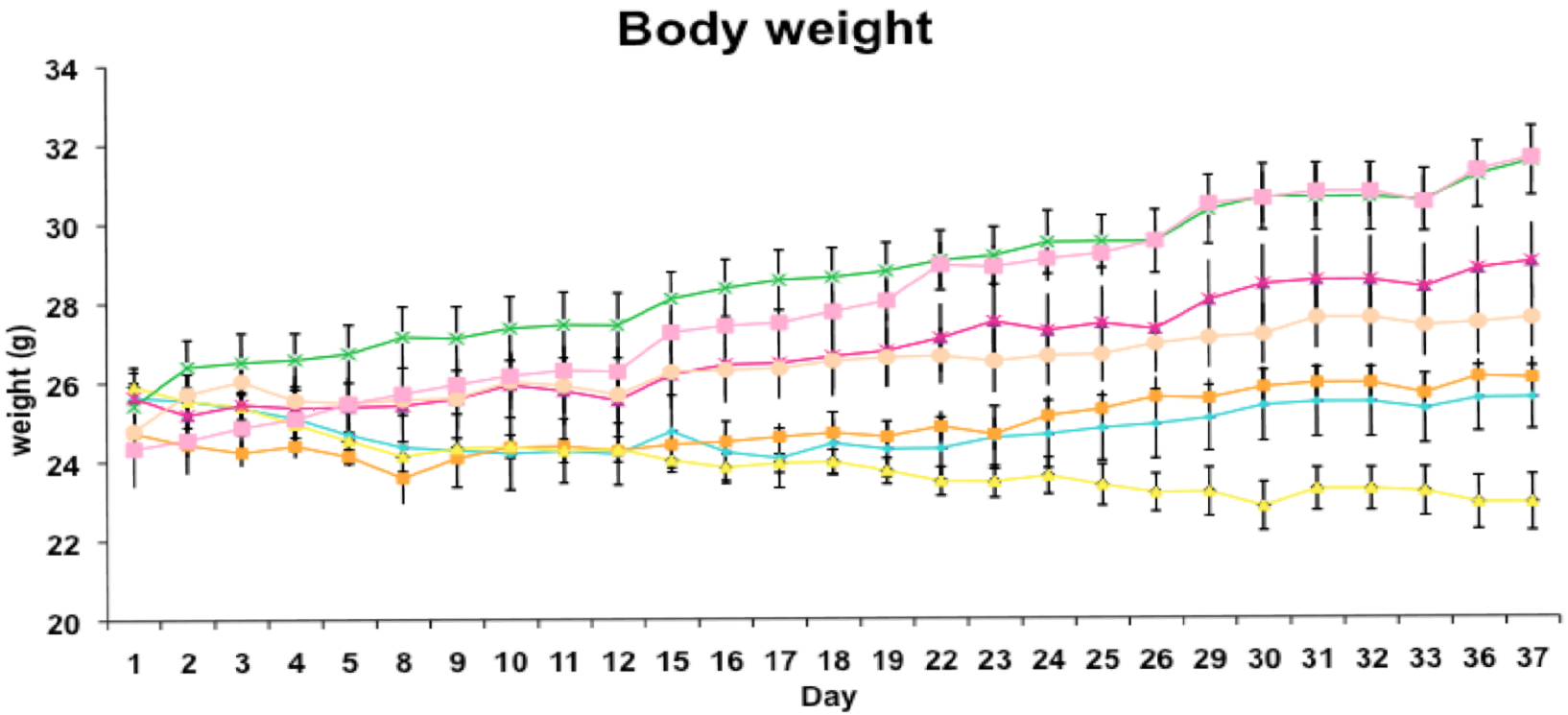

Figure 3.10 Longitudinal body weight

Body weight of mice fed control cornstarch diet (●) control sucrose diet (●) or diets enriched in stearate (●), oleic acid (●), safflower oil (●), 45% fat (●) and 58% fat (●) during the entire 4 weeks of the study
Data are mean body weight of each group ± SEM. From day 11 onward, mice fed oleic diet were significantly heavier than mice fed the control cornstarch diet (days 11,12; $p<0.05$ and days 15-37; $p<0.01$). In addition, mice fed stearate diet were significantly lighter than mice fed the control cornstarch diet on days 36 and 37; $p<0.05$.

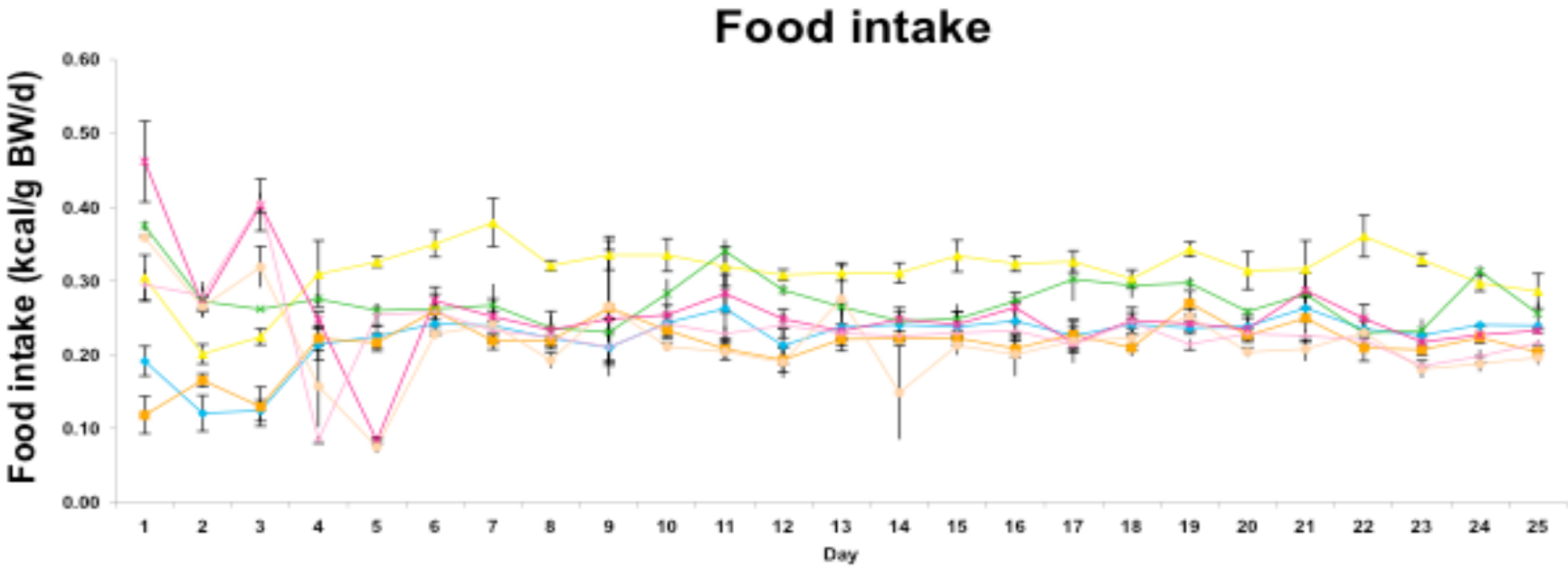

Figure 3.11 Food intake measured throughout the *ad libitium* diet study

Food intake (kcal/gmBW/Day) was measured on mice fed Control cornstarch diet (■), control sucrose diet (■), stearate diet (■), high oleic acid diet (■), safflower oil-enriched diet (■), 45% fat diet (■) and 58% fat diet (■) between day 1-37. Data are expressed as mean ± SE (n=6). There were no significant differences in food intake between groups

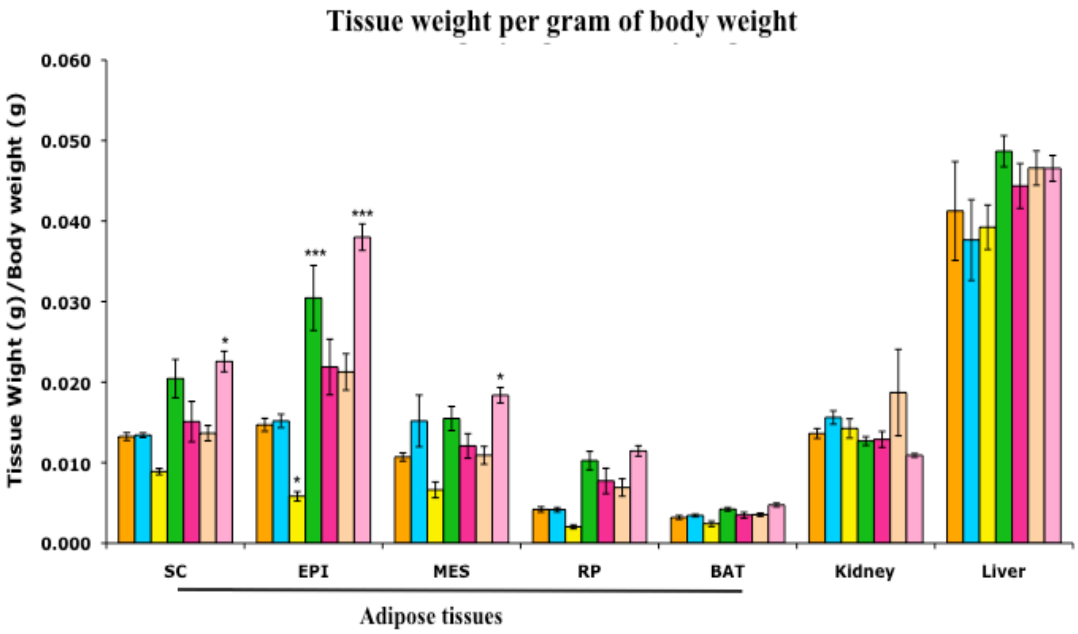

Figure 3.12 Effect of high fat feeding on organ and adipose tissue weights

Weights of adipose tissue and organs from mice fed control cornstarch (■), control sucrose (■), stearate (■), high oleic acid (■), safflower oil (■), 45% fat (■) or 58% fat (■) diets. Data are expressed as mean ± SEM (n=6). *P<0.05, ***P<0.001, compared to control cornstarch diet (ANOVA).

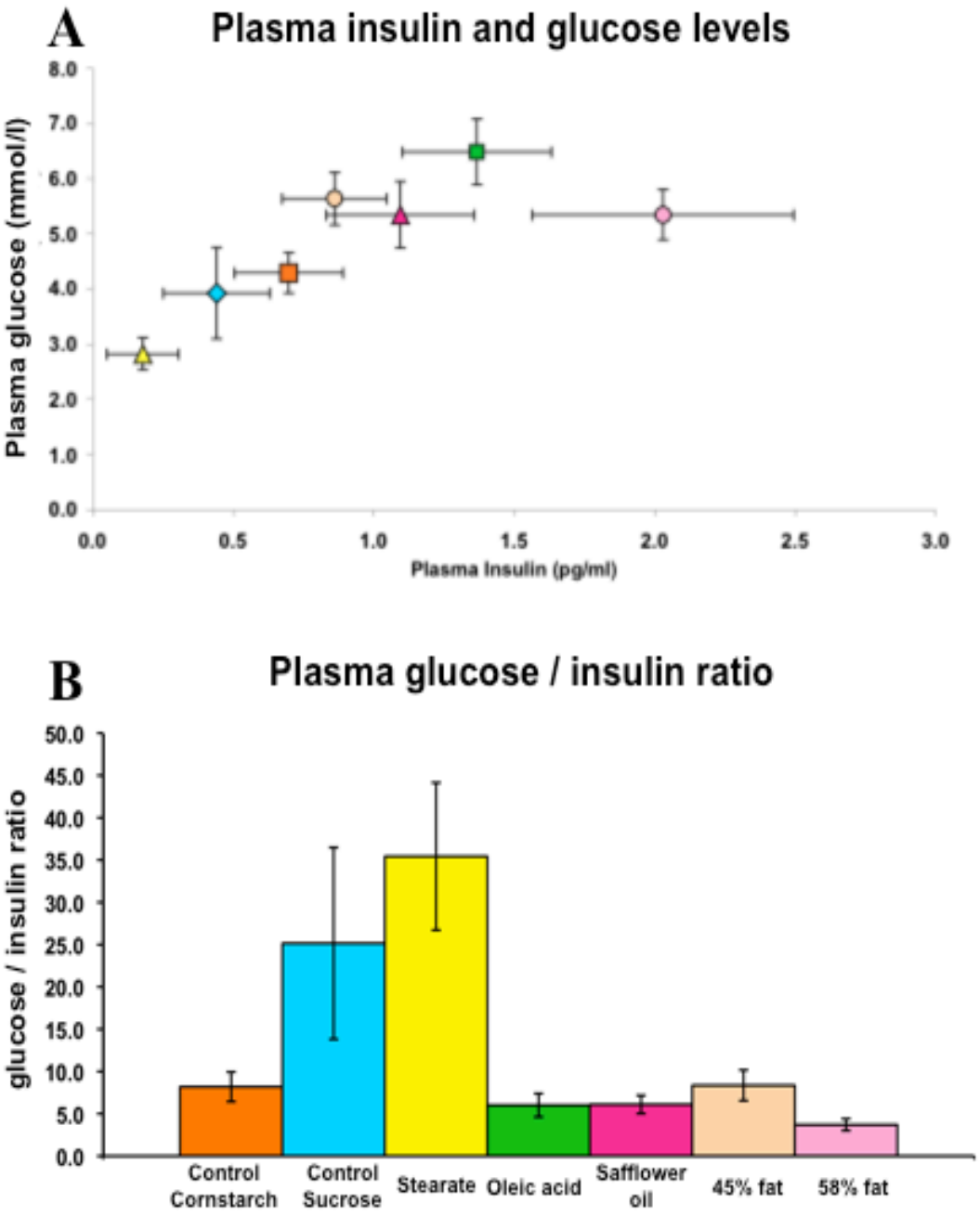

Figure 3.13 Effect of high fat feeding on plasma insulin and glucose level

The relationship between fasting plasma glucose and insulin levels (A) and glucose/insulin ratio (B) in mice fed control cornstarch (■), control sucrose (■), stearate (▲), high oleic acid (■), safflower oil (▲), 45% fat (●) and 58% fat (●) diets. Data are expressed as mean ± SE (n=6). *P < 0.05, compared to control diet (one way ANOVA followed by Dunnett post hoc test).

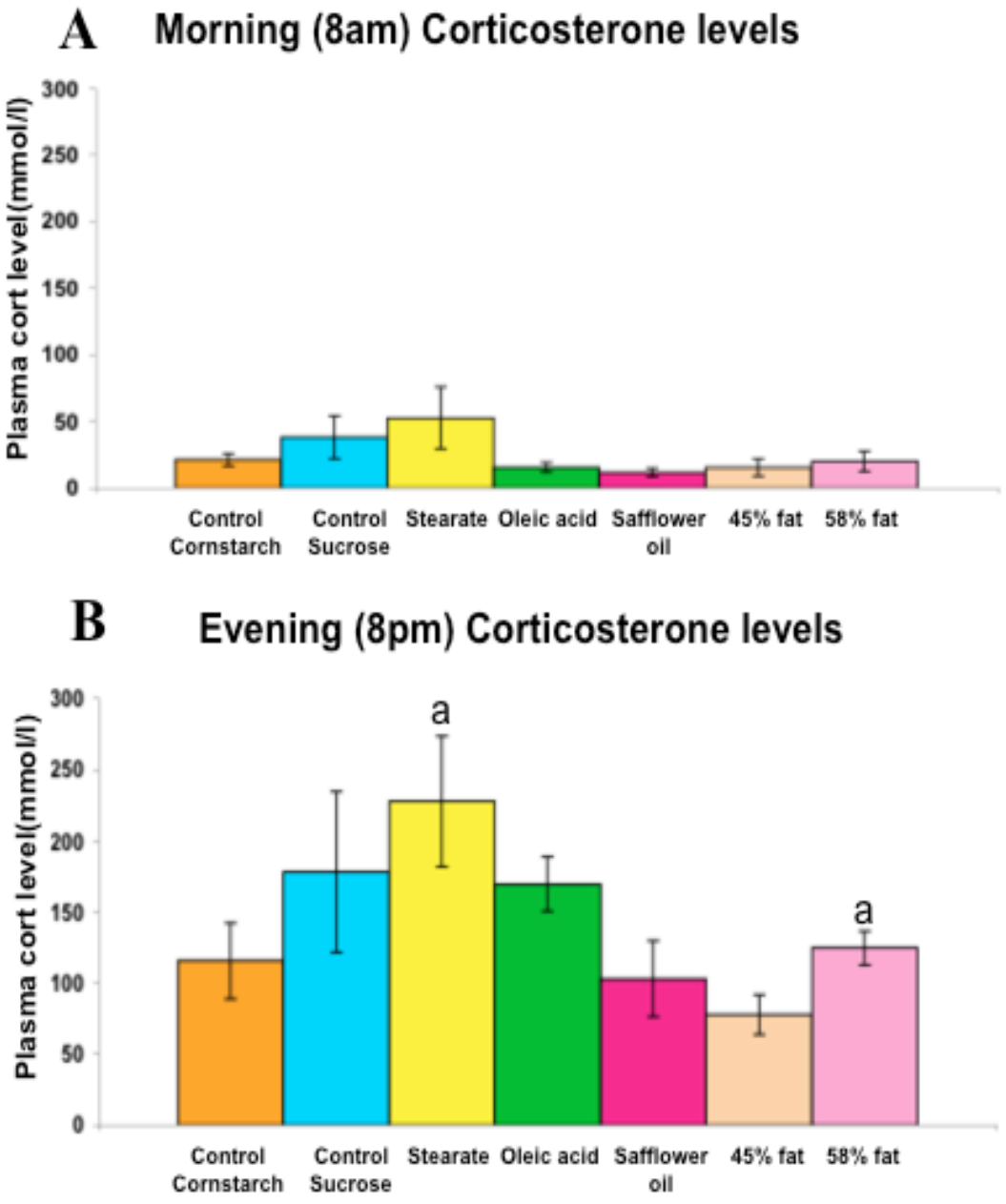

Figure 3.14 Consequence of high fat diet on plasma corticosterone level

Plasma corticosterone levels in the morning (8am; A) and in the evening (8pm; B) in mice fed control cornstarch (■), control sucrose (■), stearate (▲), high oleic acid (■), safflower oil (▲), 45% fat (●) and 58% fat (●) diets. Data are expressed as mean ± SE (n=6), a= significantly different from each other, $P < 0.05$, compared each other (one way ANOVA followed by Dunnett post hoc test).

a trend for lower plasma Cort levels was observed in the 45% fat fed group compared to the control sucrose group (Fig 3.14B). Among the high fat diet groups, the stearate group had 2-fold higher peak plasma Cort levels than the 58% fat group (Fig 3.14B).

3.2.1.3 Effect of *ad libitum* feeding HF upon 11β-HSD1 mRNA and activity levels in adipose tissue

To assess the effects of the HF diets on 11β-HSD1, northern analysis was used to measure levels of 11β-HSD1 mRNA in Mes and Sc fat depots as well as liver. In addition, 11β-HSD1 enzyme activity was measured in homogenates of adipose tissues and liver (by Lynne Ramage).

In both Sc and Mes fat, 11β-HSD1 mRNA levels did not differ in mice fed control cornstarch or control sucrose diet (Fig 3.15A). In Sc fat, 11β-HSD1 mRNA levels were significant higher in the stearate group compared to the control cornstarch group (Fig 3.15A). In contrast, all the other HF diet groups showed a marked reduction in 11β-HSD1 mRNA levels compared to the control cornstarch group (Fig. 3.15A). This effect paralleled similar changes in 11β-HSD1 activity levels (Fig. 3.15B). In Mes fat, compared to the control cornstarch group, no differences were observed in mice fed stearate, oleic acid or safflower oil diets (Fig. 3.16). However, both the 45% fat and 58% fat groups showed a significant decrease in 11β-HSD1 mRNA expression in Mes fat (Fig. 3.16A). In Mes fat, 11β-HSD1 activity was significantly increased in the control sucrose and the stearate diet groups, compared to the control cornstarch group (Fig. 3.16B). Despite the reduction in 11β-HSD1 mRNA levels, no difference in 11β-HSD1 activity was observed in the 45% fat group, nor did 11β-HSD1 activity, differ in the oleic acid or safflower oil groups (Fig. 3.16B). However, the 58% fat group showed a significant reduction in 11β-HSD1 activity in Mes fat compare to the control cornstarch group (Fig. 3.16B).

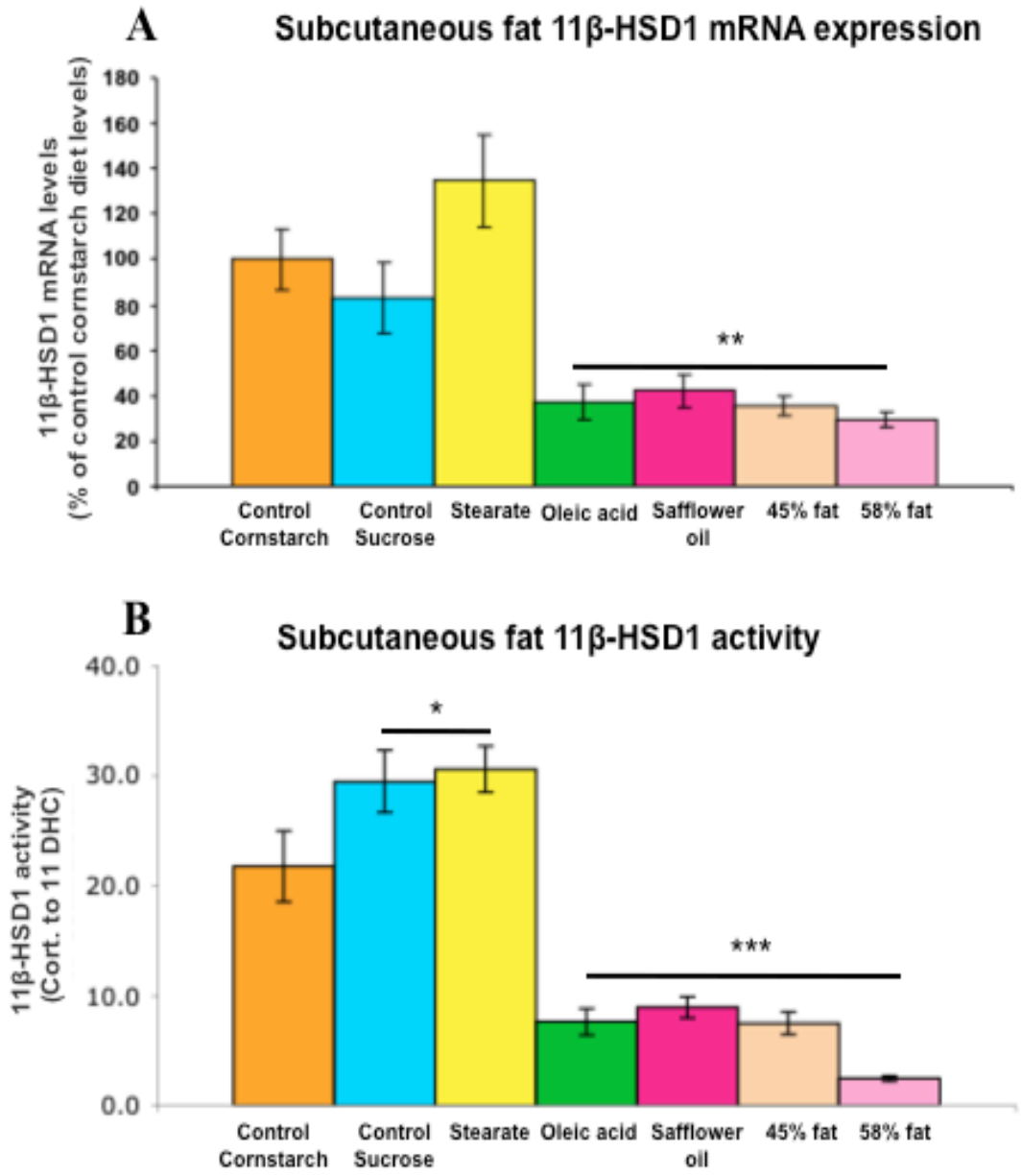

Figure 3.15 Consequence of high fat diet on subcutaneous fat 11β-HSD1 mRNA expression and activity levels

Subcutaneous fat (A) 11β-HSD1 mRNA expression and (B) 11β-HSD1 activity in mice fed control cornstarch (■), control sucrose (■), stearate (▲), high oleic acid (■), safflower oil (▲), 45% fat (●) and 58% fat (●) diets. Data are expressed as mean ± SE (n=6) *P<0.05, **<0.01, ***P < 0.001, compared to control cornstarch diet (one way ANOVA followed by Dunnett post hoc test).

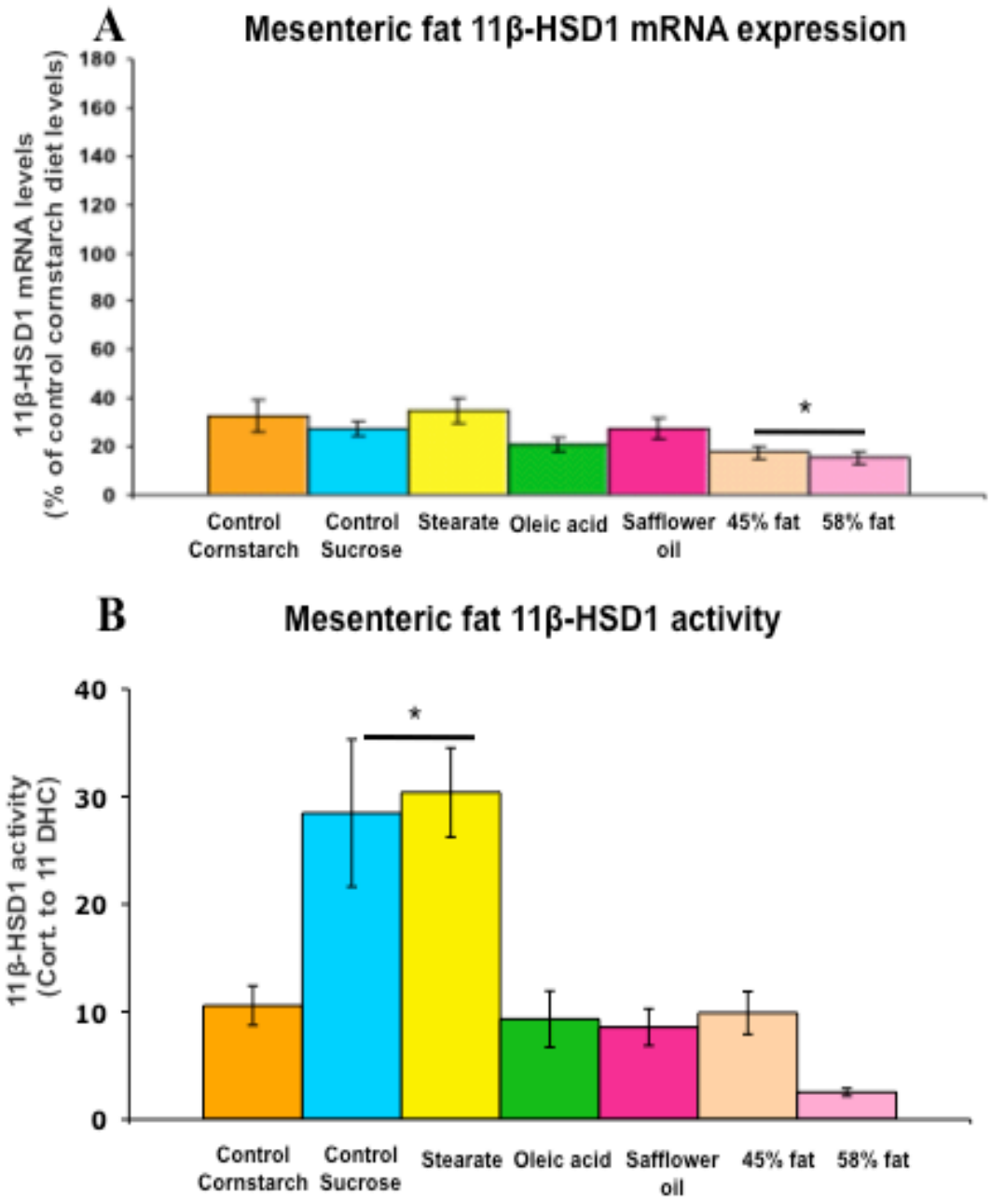

Figure 3.16 Consequence of high fat diet on mesenteric fat 11β-HSD1 mRNA expression and activity levels

Mesenteric fat (A) 11β-HSD1 mRNA expression and (B) activity in mice fed control cornstarch (■), control sucrose (■), stearate (▲), high oleic acid (■), safflower oil (▲), 45% fat (●) and 58% fat (●) diets. Data are expressed as mean ± SE (n=6) *P<0.05 compared to control cornstarch diet (one way ANOVA followed by Dunnett post hoc test).

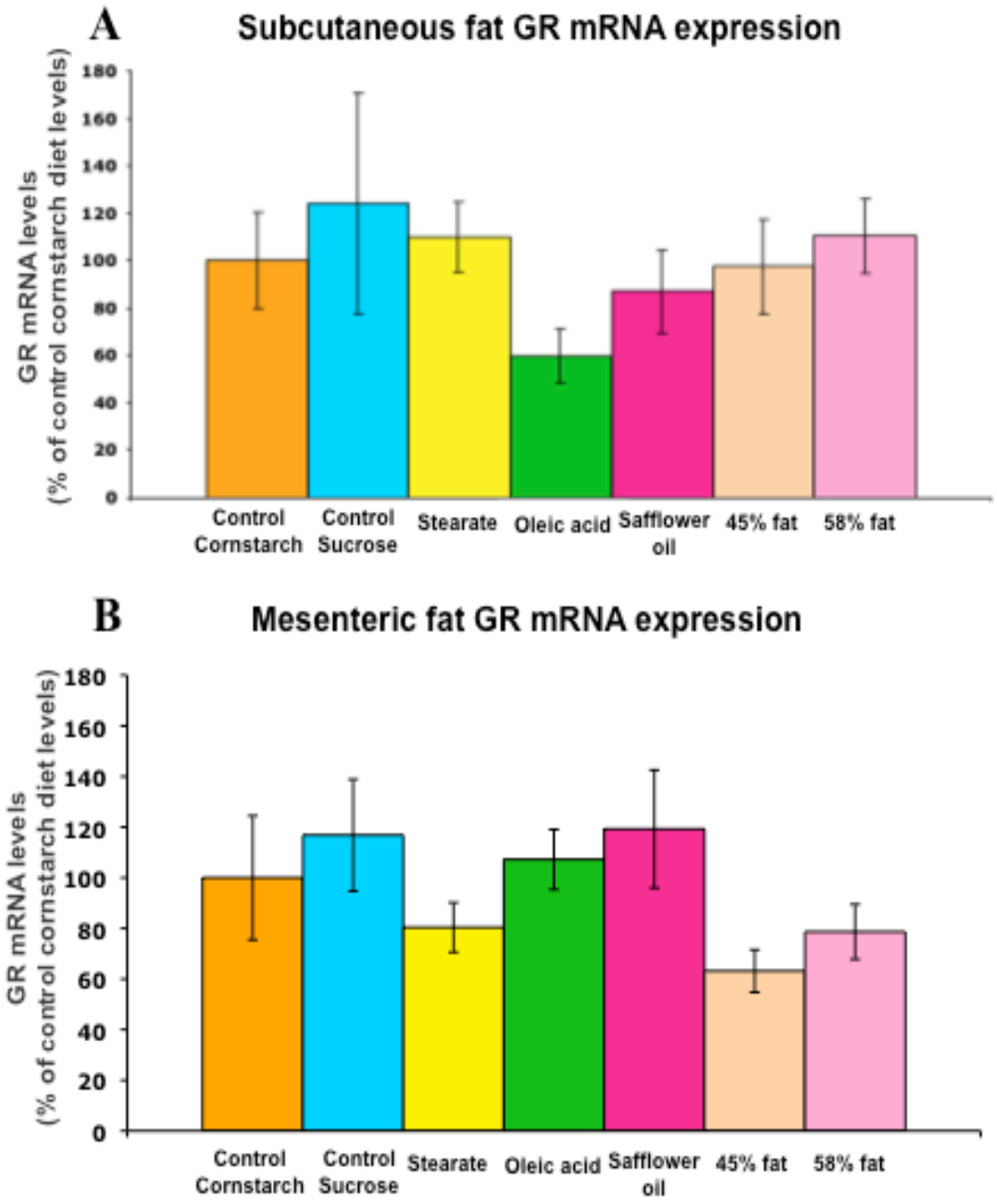

Figure 3.17 Consequence of high fat diet on subcutaneous fat and mesenteric fat GR mRNA expression

(A) Subcutaneous fat and (B) mesenteric fat GR mRNA expression in mice fed control cornstarch (■), control sucrose (■), stearate (▲), high oleic acid (■), safflower oil (▲), 45% fat (●) and 58% fat (●) diets. Data are expressed as mean ± SE (n=6). There were no significant differences between groups.

3.2.1.4 Effect of *ad libitum* feeding HF upon GR mRNA levels in adipose tissues

A major determinant of glucocorticoid action is glucocorticoid receptor density. GR mRNA levels in Sc, Mes fat were measured using real-time PCR. There was no significant effect of diet on GR mRNA levels in any of the tissues examined (Fig. 3.17).

3.3 Discussion

3.3.1 Study 1

Over the study period (28 days), all diet groups were adjusted to the same number of calories consumed by the group that consumed the least calories in the first week of the study; the control group. Under these pair-fed conditions, diets enriched in different dietary fats caused a differential variation in body weight and fat distribution in mice. The stearate group showed an unexpected weight loss from day 21, whereas the high oleic acid group maintained a high body weight throughout the study. Furthermore, the stearate group killed in the morning exhibited significantly smaller Sc, Mes and Epi fat depot weights but normal liver weight. Whilst the mice killed in the evening showed the same trend for lighter mes fat compared to control, thus not significant while their livers were significantly heavier compared with control-fed mice. This dramatic increase in liver weight followed soon after feeding (diet was provided at ~3pm) and may reflect "binge" eating in the stearate group. This is supported by plasma corticosterone levels, which were 10 fold markedly elevated in the stearate group in the morning compared to control mice. However, this difference was diminished in the evening, consistent with the high morning levels being due to fasting. Plasma insulin levels in the stearate group were low in the morning, but high in the evening; 7 fold increased over levels in control mice. The high evening insulin levels could be a postprandial effect and low morning insulin levels could result from fasting, as the stearate-fed mice consumed their food very rapidly compared to other groups (data not shown).

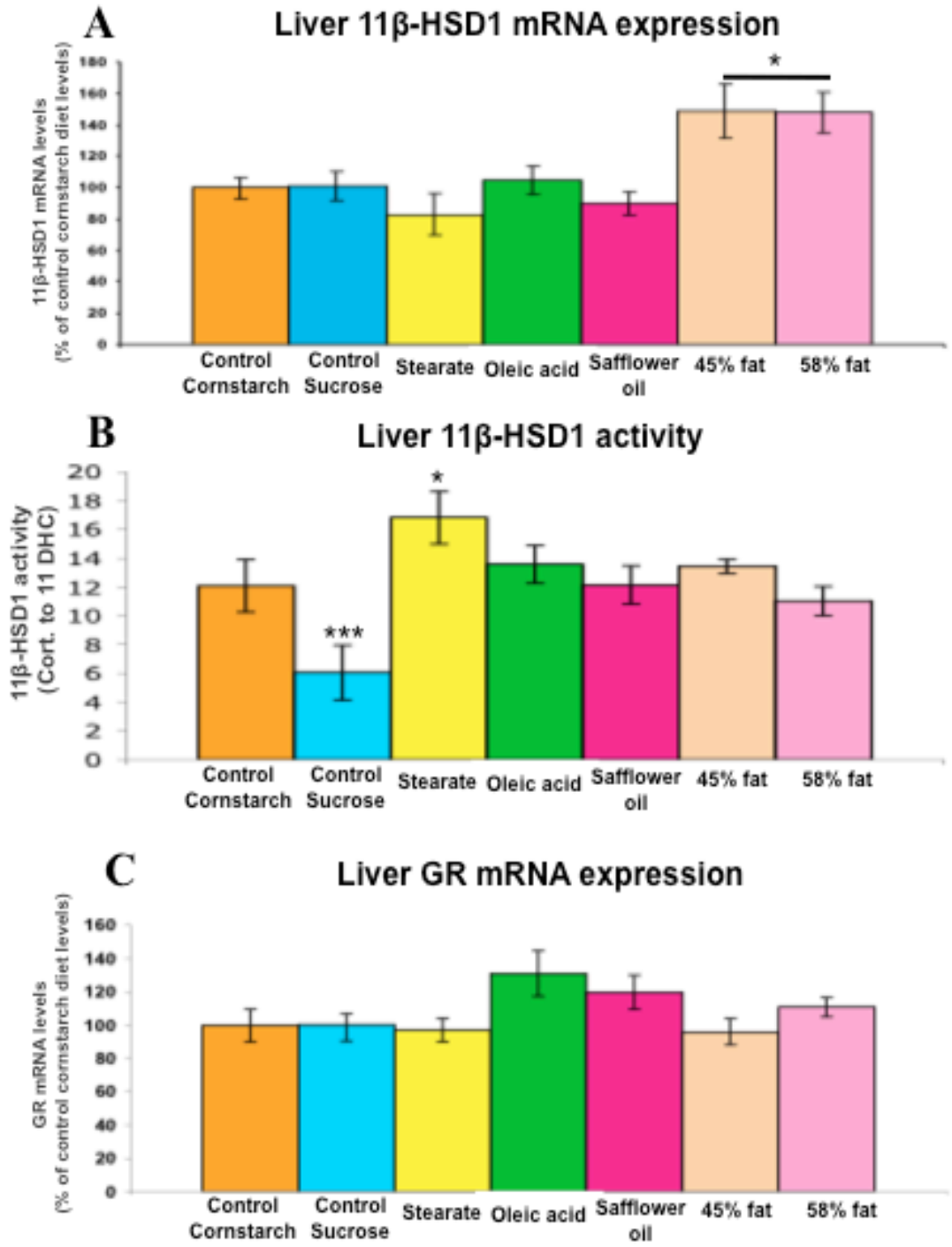

Figure 3.18 Consequence of high fat diet on liver 11β-HSD1 and GR mRNA expression.

Liver (A) 11β-HSD1 mRNA levels, (B) 11β-HSD1 activity and (C) GR mRNA levels in mice fed cornstarch (■), control sucrose (■), stearate (▲), high oleic acid (■), safflower oil (▲), 45% fat (●) and 58% fat (●) diets. Data are expressed as mean ± SE (n=6), ***P < 0.001, compared to control diet. *P<0.05, **P < 0.01, compared to control diet (one way ANOVA followed by Dunnett post test).

Dietary fat intake altered 11β-HSD1 activity and mRNA levels in adipose tissue. Compared to the control group, stearate-fed mice killed in the morning showed a significant increase in 11β-HSD1 activity in all three fat depots (subcutaneous, epididymal and mesenteric). If this is not an effect of the constituents of the diet, this up-regulation of 11β-HSD1 activity in the stearate group could be the result of overnight fasting as it was not so marked in stearate-fed mice in the evening. A trend to down-regulation of adipose 11β-HSD1 activity in mice fed diets enriched in oleic acid and safflower oil was found in all three fat depots but this did not reach significance. These groups also showed a trend toward higher weight gain, suggesting that the HF-mediated down regulation of 11β-HSD1 previously reported could be related to weight gain. In contrast to 11β-HSD1, GR mRNA levels in Sc and Mes fat of stearate-fed mice killed in the morning were decreased compared to control mice. An inverse relationship between 11β-HSD1 and GRmRNA has been described in human adipose tissue (Michailidou, Jensen et al. 2007). However, an inverse relationship between adipose GR and 11β-HSD1 mRNA levels was not observed in adipose tissue of mice fed high oleic acid or safflower oil diets.

In addition to adipose tissues, the pattern of GR and 11β-HSD1 mRNA expression in the liver was also examined. With the exception of the stearate group, the different diets regulated 11β-HSD1 mRNA levels in the opposite direction to that observed in the adipose tissue, as previously observed (Morton, Ramage et al. 2004). However, none of the differences in 11β-HSD1 or GR mRNA levels between diet groups in the morning achieved significance. In contrast, a 3-fold increase in 11β-HSD1 mRNA expressions was observed in the stearate group killed in the evening. This marked increase could result from binge eating. More interestingly, the trend to lower levels of 11β-HSD1 and GR mRNA in liver in the evening, seen in the control, high oleic and safflower oil enriched diet groups was reversed in the group fed stearate. To fully understand these effects, it will be important to determine the effect of stearate diet on metabolism, as stearate diet was the only diet to alter liver weight (but only PM) suggesting an acute effect on liver function. In addition, malabsorption of stearate diet is also a consideration as stearate-fed mice exhibited significant gut hypertrophy (data not shown). This could indicate an altered

absorptive state in order to extract nutrients from a highly saturated fat diet-enriched meal.

This study was designed to test which constituents of a HF diet are responsible for down-regulation of adipose 11β-HSD1. Accordingly, three diets differing in their fatty acid composition – saturated, mono-unsaturated and poly-unsaturated fat were compared. Although none produced the expected down-regulation, a trend towards down-regulation of adipose 11β-HSD1 was observed in the high oleic acid and safflower oil-fed group. This suggested that mono-saturated or poly-saturated fat might be responsible, but none of the effects were significant, so no conclusions are possible.

Data in this study have shown a complex interaction between circulating glucocorticoid, peripheral 11β-HSD1 activity and GR mRNA expression. However, Study 1 differed from previous studies in two important respects: (1) these high fat diets contained just 45% compared to the previous study which contained 58% (Morton, Ramage et al. 2004). (2) The calorie intake within the study was restricted to be the same as the control diet. In addition, the mixed fat content within the original 58% fat diet might also be responsible for the down-regulation of 11β-HSD1 activity. Therefore, it was necessary to conduct a second study which allowed *ad libitum* access to all diets and which included additional HF diets.

3.3.2 Study 2

To investigate some of the questions arising from the Study 1; the *ad libitum* feeding study was carried out. Three additional diet groups were also included, the 58% mixed-fat diet and the control cornstarch diet used in the original HF study, and a 45% mixed-fat diet. Recent studies have suggested that diets enriched in sucrose suppress HPA axis activity (Pecoraro, Reyes et al. 2004) and also affect peripheral GC action (Dallman, Pecoraro et al. 2005).

Whilst the control cornstarch and the control sucrose diet produced comparable effects on the majority of the parameters measured, they differed in their effects on 11β-HSD1 activity, but not mRNA levels. Enzyme activity was higher in the sucrose diet group in Sc fat and Mes fat, with the opposite pattern being seen in

liver to that found in adipose tissue. Consistent with previous studies (Morton, Ramage et al. 2004; Drake, Livingstone et al. 2005), 58% mixed-fat down-regulated adipose tissue 11β-HSD1. The results were apparent in this study after 4 weeks following either 45% or 58% mixed-fat diet, although the magnitude of the effect was smaller with 45% mixed-fat diet than 58% mixed-fat diet. This suggests that the % of fat within the diet, whilst influencing the outcome but itself was not causing the down-regulation. However, the composition of the diet could be an important factor, Oleic acid produced similar effects to the 58% mixed-fat diet, suggesting that the mono-unsaturated fat may be the major factor constituent causing the down-regulation. However, safflower oil enriched diet also down-regulated 11β-HSD1, so the question of exactly which component within diet actually regulated 11β-HSD1 still remains to be answered.

As in study 1, the most pronounced dietary effects were observed in the sterate-fed group. Stearate diet resulted in weight loss (Fig. 3.11) and an improved glucose homeostasis. These results are not consistent with other's observations that saturated fats cause insulin resistance (Chavez and Summers 2003). In addition, the stearate diet caused significant elevations in 11β-HSD1 activities in both Sc and Mes fat depots although 11β-HSD1 mRNA levels were elevated only in Sc fat and not Mes fat. A similar variance between 11β-HSD1 activity and mRNA levels were also found between intact preadipocytes visceral adipose tissue and in human visceral adipose tissue (Goedecke, Wake et al. 2006; De Sousa Peixoto, Turban et al. 2008), suggesting there might be regulation of enzyme activity at the post-transcriptional level. In addition, this discrepancy could be depot-specific as visceral fat has been shown to be less sensitive towards the effect of insulin.

The effect of stearate goes against the hypothesis that high adipose HSD1 levels always associate with obesity and insulin resistance (Seckl, Morton et al. 2004). The weight loss in the stearate fed-group suggests that this result observed with pair feeding (Study 1) was not entirely due to insufficient food (fasting) but included another mechanism (e.g. malabsorption) (Sampath and Ntambi 2005). In humans, it was found that stearate is less effectivly absorbed by the gut (Sampath and Ntambi 2005). It is possible that the stearate diet used here was not completely absorbed and mice were in a state of caloric restriction. A fecal assay for signs of

malabsorption may be necessary to investigate whether this is a cause of weight loss in the sterate group. This would be consistent with the significant weight loss, the low levels of plasma insulin and the elevated plasma corticosterone levels. Furthermore stearate has been shown to be a poor substrate for TG synthesis (Sampath and Ntambi 2005). In future investigations, an alternative short chain saturated fat such as palmitate could be used.

In this study, the 58% fat diet resulted in the biggest weight gain. Interestingly, a comparable body weight gain was observed in the oleic acid fed group containing just 45% calories as fat. In addition, both the 58% fat and oleic acid groups expressed an altered glucose homeostasis suggestive of insulin resistance, accompanied by low circulating corticosterone levels and significantly reduced 11β-HSD1 mRNA and activity in Sc fat. Thus, the 45% oleic acid diet caused many of the same effects as the 45% and 58% mixed-fat diets.

In the future, it would be of interest to measure the level of PPARγ mRNA expression within these HF diet groups as PPARγ has been shown to regulate both adipocyte differentiation and adipocyte 11β-HSD1 levels (Beaven and Tontonoz 2006; Sharma and Staels 2007). Further investigations could be carried out by including essential fatty acids (omega 3 and 6) in the diets as both omega 3 and 6 polyunsaturated fatty acids have been shown to have beneficial effects upon insulin sensitivity and they reduce cardiovascular risk (Harbige 2003, Lee et al.,2006). In addition, the effect of sucrose and other carbohydrates would be worth investigating given the role in obesity of carbohydrates like corn syrup, fructose and foods that are highly processed and energy-dense (Fried and Rao 2003). These studies would allow us a better understanding of the impact of different diets on the development of obesity and metabolic syndrome.

4 The effects of adipose GR density on peripheral glucocorticoid action

In order to test the hypothesis that adipose GR density is an important determinant of adipose acquisition in diet-induced obesity, it is necessary to specifically alter GR density in adipose tissues. To do this, transgenic mice were created in which the adipocyte-specific aP2 enhancer/promoter drives expression of rat glucocorticoid receptor. The "sense" rat GR cDNA is ~80% identical to mouse GR cDNA and a similar "sense" rat GR cDNA has previously been used to overexpress GR, in β cells under the control of the insulin promoter (Delaunay, Khan et al. 1997) and in thymus under the control of the p56 proximal Lck promoter (Pazirandeh, Xue et al. 2002). In addition, transgenic mice in which the aP2 enhancer/promoter was used to drive an "antisense" rat GR cDNA (to reduce adipose GR levels) were characterized.

4.1 Results

4.1.1 Construction and validation of an aP2-GR "sense" transgene

The aP2-GR construct was assembled by ligating restriction fragments from a plasmid encoding the 5.4kb aP2 enhancer/promoter (Ross, Graves et al. 1993) (provided by B. Spiegelman) to the rat GR cDNA as described in Methods section (Chapter 2, section 2.2.13). The final construct structure is shown in Fig. 4.1A. As the GR cDNA was assembled from several fragments it was necessary to verify that it was functional. For transfection, modification of the construct was needed, as the aP2 promoter only functions in adipocytes which are not readily transfectable. Accordingly, the aP2 promoter region was replaced by the SV40 viral promoter/enhancer which is active in many cell types creating the plasmid pJan3 (Capter 2, section 2.2.14). B103 neuroblastoma cell (Schubert et al, 1974) have negliglible levels of GR mRNA but glucocorticoid responsiveness is restored by transfection with exogenous GR (Freeman, Munn et al. 2004). In order to test whether the rat GR cDNA within the construct was functional, B103 cell were co-transfected with pJan3 or pRS-hGR (Giguere, Hollenberg et al. 1986) encoding

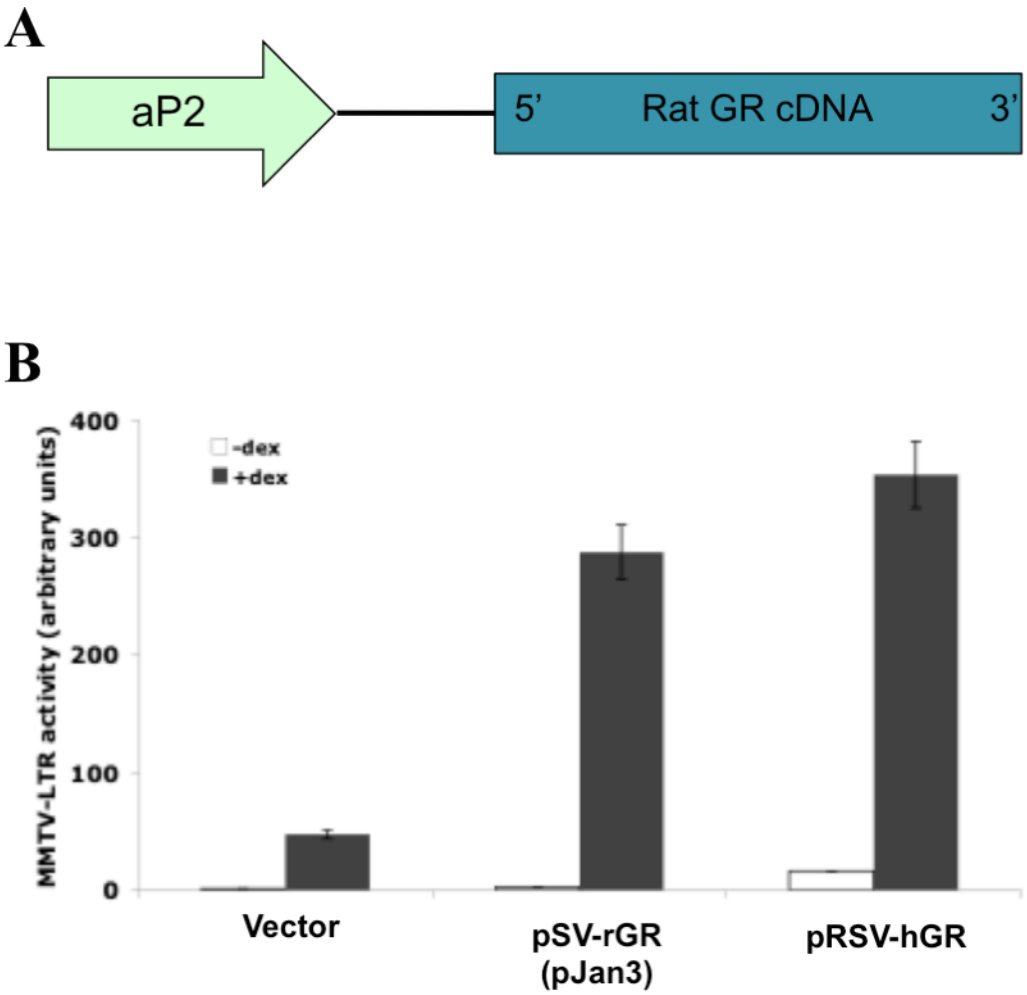

Figure 4.1 Construct used to generate aP2-GR "sense" transgenic mice

(A) The final construct structure of the aP2-GR "sense" transgene, consists of the aP2 promoter driving rat GR cDNA. (B) Transfection result validating the rat GR cDNA used within the construct. The aP2 promoter was exchanged for an SV-40 promoter to generate pSV-rGR (pJan3) (Chapter 2, section 2.2.14). Transfection of pSV-rGR (pJan3) into B103 cells showed that it resulted in a greater dexamethasone induction than vector only. pSV-GR gave a similar level of response to pRSV-hGR (encoding human GR), which was used a positive control. Data are mean ± SEM of 3 independent transfections for each construct.

human GR as a positive control and MMTV LTR-luc, a glucocorticoid responsive promoter linked to a luciferase reporter gene (Lefebvre, Berard et al. 1991). Transfected cells were cultured in the presence or absence of 10^{-6}M dexamethasone. In the presence of dexamethasone, transfection of pJan3 resulted in a 30-fold increase in MMTV LTR activity, similar to the increase in promoter activity in cells transfected with pRS-hGR (Fig. 4.1B). This demonstrated that the rat GR cDNA used in the construct is functional.

4.2 Generation and breeding of aP2-GR transgenic mice

4.2.1 Generation of aP-2GR mice

The purified transgene fragment was microinjected into the pronuclei of fertilized C57BL/6 x CBA/C3H F_1 embryos by the re-derivation service, University of Edinburgh. After microinjection, a total of 55 mice were obtained from 5 pregnant females. The presence of the transgene was detected via PCR on genomic DNA extracted from tails (Fig 4.2). 5 founder mice were identified; 3 male (numbers 15, 32, 33) and 2 female (numbers 17 and 26).

Founder (G_0) mice were backcrossed to C67BL/6J mice to establish lines. Of the five founders, only four (15, 17, 26 and 32) produced offspring. However, line 26 only managed to produce transgenic offspring for the first generation and the transgene was not transmitted to the second generation. Line 15 did not produce any transgenic offspring when crossed to C57BL/6, so lines 17 and 32 were selected for breeding. Of the two selected lines, line 32 consistently gave a stronger intensity transgene PCR product (Fig. 4.2 and data not shown). To distinguish between the two lines, line 17 and 32 were renamed B-FSG and D-FSG, respectively.

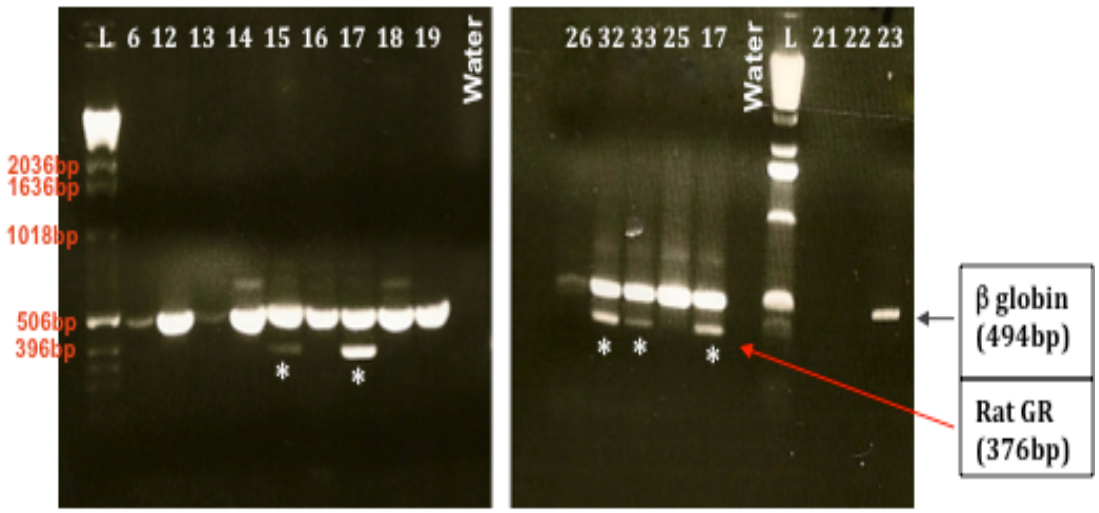

Figure 4.2 Representative gels showing PCR genotyping of founder aP2-GR transgenic mice

Founder transgenic mice carrying the rat aP2-GR transgene were identified by PCR using primers which specifically amplify a 376 bp fragment of rat GR. A 494 bp PCR product of β-globin was also amplified as a positive control for the presence of DNA. "L" represents lanes loaded with 1kb ladder and "Water" were negative controls containing water instead of DNA. PCR reactions on DNA from mice no. 6, 12, 13, 14, 15, 16, 17, 18, 19, 26, 32, 33, 25, 17, 21 22 and 23 were loaded in the indicated lanes. * Indicates the presence of a product from rat GR in mice mice no. 15, 17, 32 and 33, which carry the transgene

.

4.3 Characterization of B- and D-FSG mouse lines

4.4 Transgene expression in adipose tissue

4.4.1 Rat GR mRNA is expressed in both B- and D-FSG mice

To determine whether the rat GR transgene is expressed in adipose tissue, custom real-time PCR assays were developed by Dr. Elaine Marshall. Mouse and rat GR cDNA show 80% homology (http://nrr.georgetown.edu/grr/seqcomp.html). To distinguish between rat and mouse mRNA, a gene region unique to mouse GR was selected for the mouse GR primer-probe set and a gene region specific for the rat GR was selected.

Levels of rat GR mRNA and mouse GR mRNA were measured using real-time PCR in adipose tissues (subcutaneous, gonadal and mesenteric depots) and liver of transgenic (tg) mice (F_1 backcross to C57BL/6J) and their non-transgenic (non-tg) littermates. Liver GR mRNA levels served as a control, as aP2 is not normally expressed in liver and therefore the transgene was not predicted to be expressed in liver.

In both sexes of D-FSG tg mice (F_1 generation), rat GR mRNA was present in all adipose tissues; subcutaneous (Sc), gonadal (Gon) and mesenteric (Mes), compared to the low levels detected in non-tg mice (probably due to cross-reactivity of the rat assay with mouse GR mRNA) (Fig. 4.3A and Fig. 4.4A). No differences were seen in hepatic GR mRNA levels between tg and non-tg mice with either the rat-specific or the mouse-specific real time PCR assay (Fig. 4.3 and Fig. 4.4), and mRNA detected by the rat GR assay in liver is likely to be due to cross-reactivity with mouse GR mRNA.

In B-FSG, only female mice were examined. In tg mice (F_1 generation) when compared to non-tg littermates, rat GR mRNA was clearly present in Sc, Gon and Mes adipose tissues with very low level or none rat GR mRNA found in brown adipose tissue (BAT) or liver (Fig. 4.5A). In addition, levels of endogenous mouse GR mRNA did not differ in adipose tissue or liver between tg and non-tg mice (Fig. 4.5B).

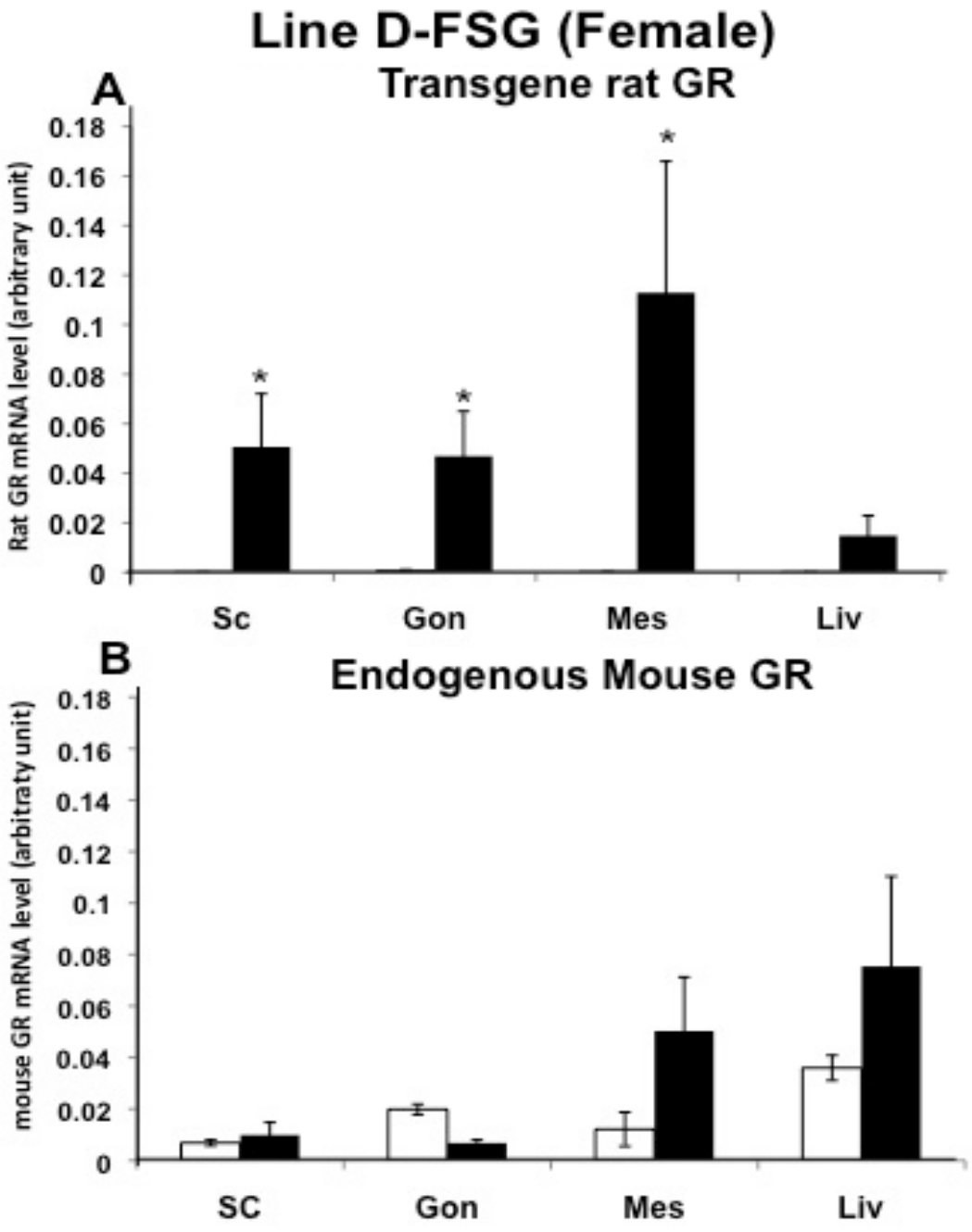

Figure 4.3 Transgenic aP2-GR mice express rat GR mRNA specifically in white adipose tissue of 4-5 month old female mice (line D-FSG)
Levels of (A) rat GR mRNA and (B) endogenous mouse GR mRNA were measured in non-tg (white) and tg (black) 4-5 month old female D-FSG (F_1 generation) mice (n=4/group). GR mRNA levels are expressed relative to the internal control cyclophilin A and are reported in arbitrary units (AU). Data are mean ± SEM; and were analysed by 2-way ANOVA; *P<0.05 Data generated by Dr. Elaine Marshall.
Abbreviations: SC; subcutaneous, Gon; gonadal, Mes; mesenteric and Liv; liver

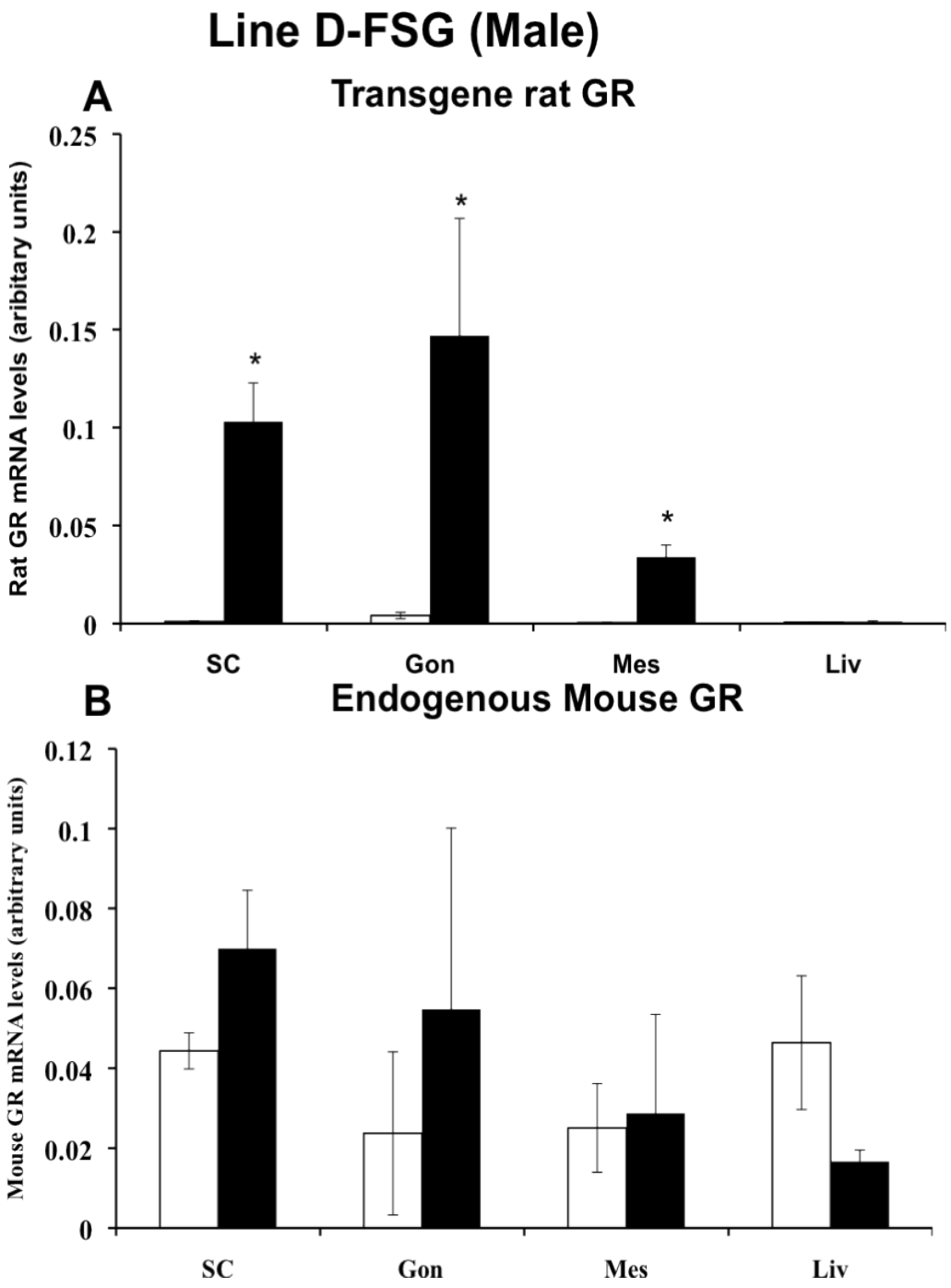

Figure 4.4 Transgenic aP2-GR mice express rat GR mRNA specifically in white adipose tissue of 4-5 month old male mice (line D-FSG)

Levels of (A) rat GR mRNA and (B) endogenous mouse GR mRNA were measured in non-tg (white) and tg (black) 4-5 month old male D-FSG (F_1 generation) mice (n=4/group). GR mRNA levels are expressed relative to the internal control cyclophilin A and are reported in arbitrary units (AU). Data are mean ± SEM; and were analysed by 2-way ANOVA; *P<0.05 Data generated by Dr. Elaine Marshall. Abbreviations: SC; subcutaneous, Gon; gonadal, Mes; mesenteric and Liv; liver

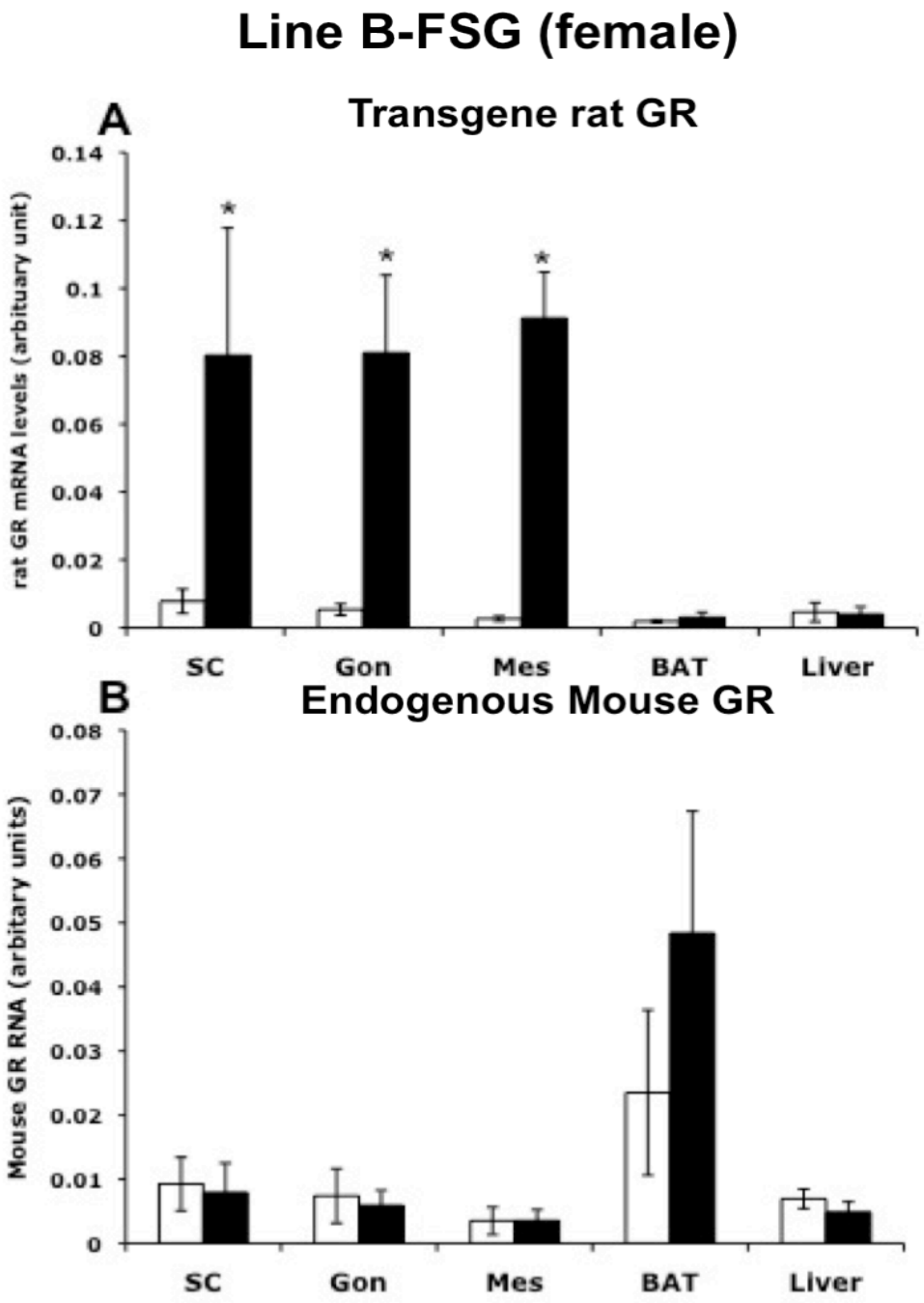

Figure 4.5 Transgenic aP2-GR mice express rat GR mRNA specifically in white adipose tissue of 4-5 month old female mice (line B-FSG)

Levels of (A) rat GR mRNA and (B) endogenous mouse GR mRNA were measured in non-tg (white) and tg (black) 4-5 month old female B-FSG (F_1 generation) mice (n=4/group). GR mRNA levels are expressed relative to the internal control cyclophilin A and are reported in arbitrary units (AU). Data are means ± SEM; and were analysed by 2-way ANOVA; *P<0.05 Data generated by Dr. Elaine Marshall. Abbreviations: SC; subcutaneous, Gon; gonadal, Mes; mesenteric and Liver; liver

4.4.2 Increased adipose tissue weight in transgenic female D-FSG mice

To assess any difference between tg and non-tg littermates in adipose tissue weight and distribution, various adipose tissues; subcutaneous (Sc), gonadal (Gon), mesenteric (Mes), perirenal (Per), retroperitoneal (RP) and brown adipose tissue (BAT) from B and D-FSG mice were dissected and weighed. In addition, liver (Liv) and kidney (Kid) were also dissected and weighed as control tissues where no effect of transgene is predicted within these organs.

In male D-FSG (F_1 generation; 5-6 month old) mice no difference in body weight was found between genotypes (non-tg, 35.4±1.3g vs tg, 34.26±1.8g, n=4/group)., In addition there were no differences in weights of adipose tissues kidney or liver (Fig. 4.6A). However, in female D-FSG (F_1 generation; 5-6 month old) mice, the body weight of the tg mice was 11% heavier than their non-tg littermates (non-tg, 22.0±0.6g *vs* tg, 24.8±0.9g, n=5/group) and the mice showed a significant increase in weight of white adipose tissues (Sc, Gon and Mes) with no difference in weight of BAT, kidney or liver, compared to non-tg littermates (Fig 4.6B).

In female, B-FSG (F_1 generation, 4-5 month old) mice, there was no difference in tissue weight (Sc, Gon, Mes, Per, RP, BAT, kidney and liver) after correction to liver weight which showed no difference between non-tg and tg mice (non-tg, 1.02±0.05g *vs* tg, 1.01±0.04g, n=4/group) (Fig 4.7).

4.5 Effect of transgene on response to HF diet

As the preliminary characterization showed adipose-specific expression of the rat GR transgene and demonstrated the predicted increase in adipose tissue weight, although only in female of the higher copy number line (D-FSG), the effects of transgene upon response to diet-induced obesity were investigated in D-FSG mice.

4.5.1 Experimental design

A 6 month high fat diet study was carried out during which blood pressure and metabolic parameters (glucose and insulin tolerance, plasma glucose and triglyceride level) were measured (Fig. 4.8).

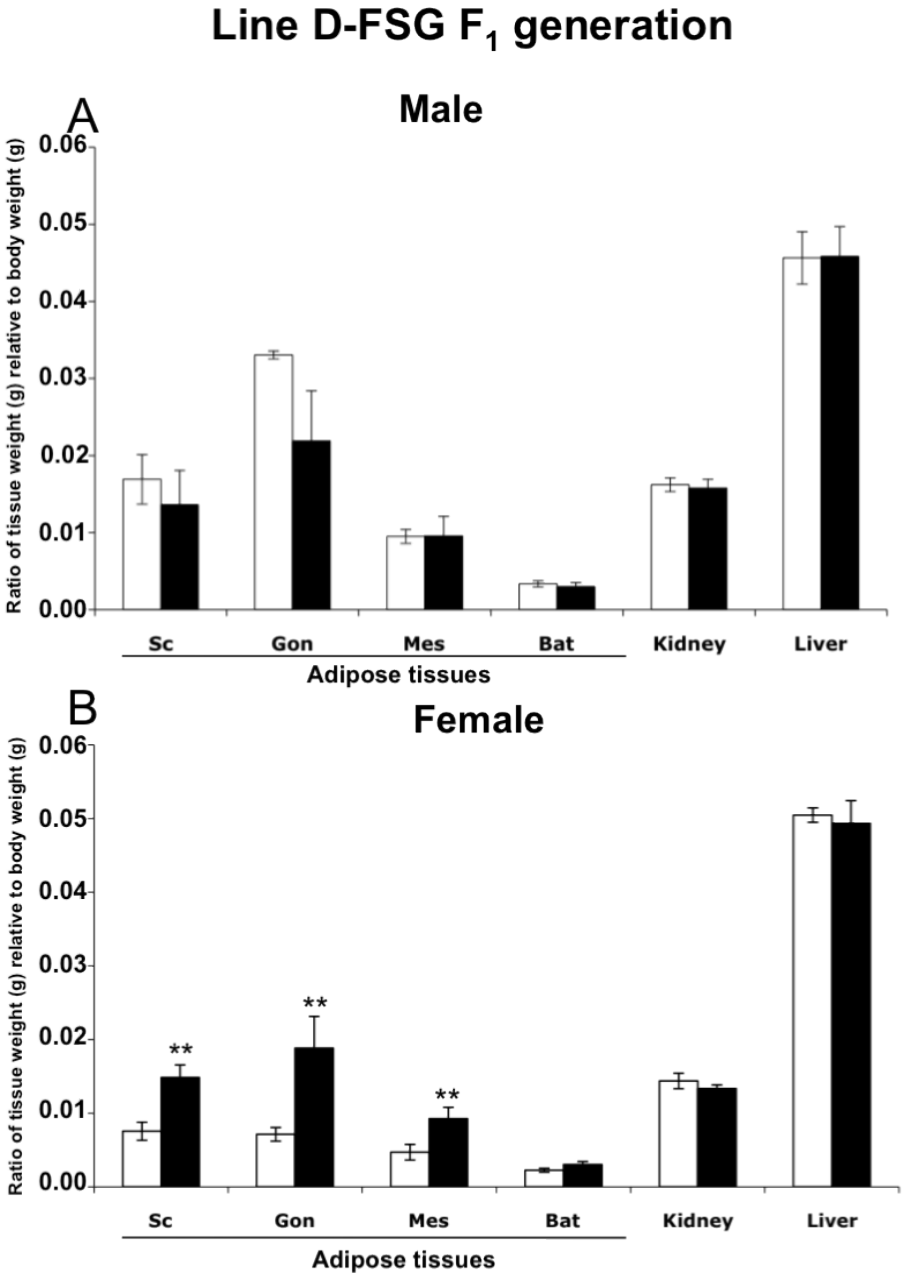

Figure 4.6 Sex-specific effect of aP2-GR transgene on adipose tissue weight in line D-FSG mice

Weights of adipose tissues and organs of non-tg (white) and tg (black) 4-5 month old (A) male (n=4/group) and (B) female (n=5/group) D-FSG (F_1 generation) mice. Data are expressed as tissue weight (g) relative to body weight (g). Body weight was not different between genotype in male D-FSG mice (non-tg, 35.4±1.3g *vs* tg, 34.3±1.8g) but was significantly difference in female D-FSG mice (non-tg, 22.0±0.6g *vs* tg, 24.8±0.9g; t-test; $p<0.05$). Data are means ± SEM and were analysed by 2way-ANOVA; **$p<0.01$, compared to non-tg littermates.

Abbreviations: SC; subcutaneous, Gon; gonadal, Mes; mesenteric, Bat; brown adipose tissue.

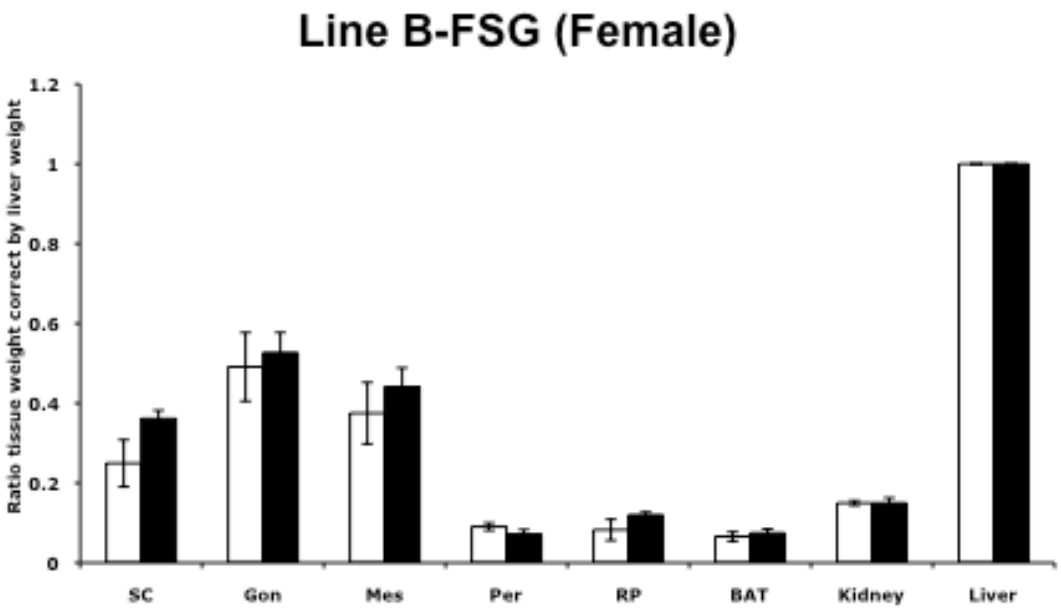

Figure 4.7 No significant effect of aP2-GR transgene on adipose tissue weights of line B-FSG mice

Weights of adipose tissues and organs from non-tg (white) and tg (black) female 4-5 month old B-FSG (F_1 generation) mice. Data are expressed as ratio of adipose tissue or kidney weight relative to liver weight (arbitrarily set to 1 for each group) and are means ± SEM (non-tg n=6, tg n=4). Liver weight was not different between genotypes (non-tg, 1.02±0.05g *vs* tg, 1.01±0.04g). Data were analysed by 2-way ANOVA; no statistically significant differences in adipose tissue or kidney weights were found between genotypes.

Adipose tissues were: subcutaneous (Sc), gonadal (Gon), mesenteric (Mes), perirenal (Per), retroperitoneal (RP) and brown adipose tissue (BAT).

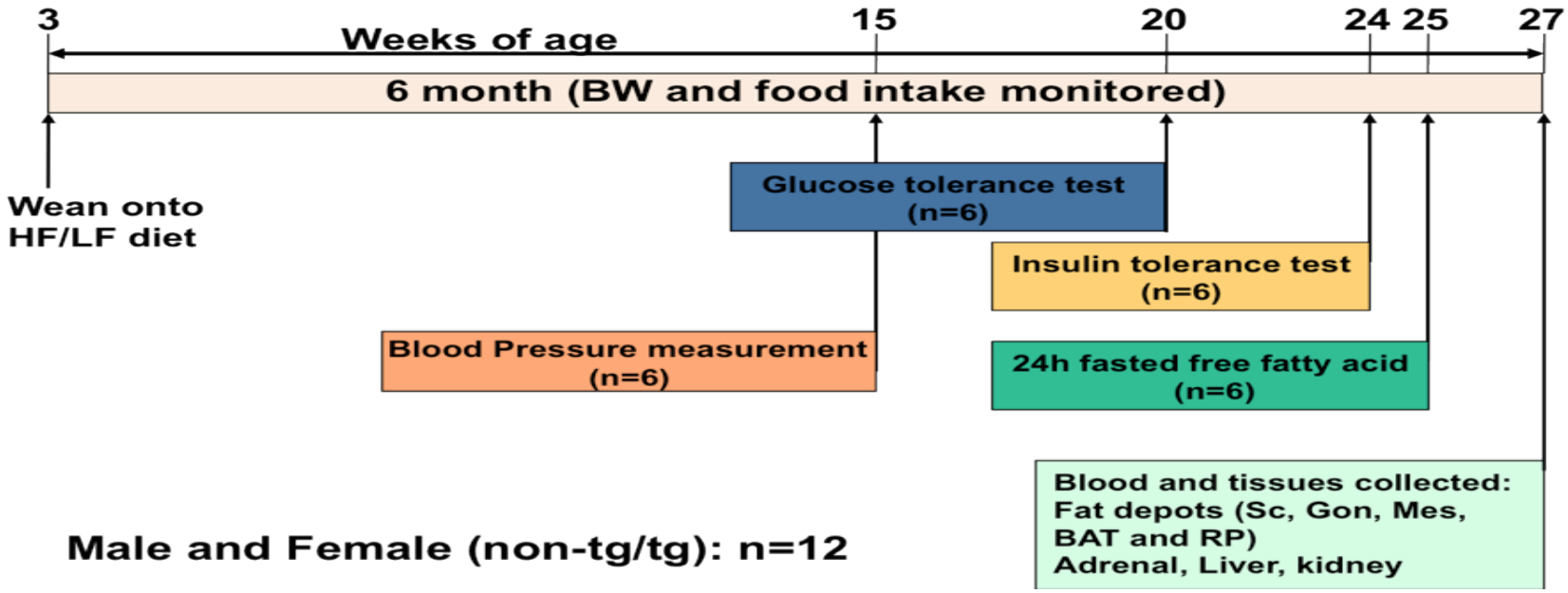

Figure 4.8 Scheme showing experimental design

Male and female tg and non-tg littermates (F_2 generation; n=12/group) were weaned onto either high fat (HF) (58% kcal as fat) or low fat (LF) cornstarch-enriched diet (11% kcal as fat) and were allowed *ab libitium* access to food throughout the study. Body weight and food intake was monitored and recorded weekly. In a subgroup of mice (n=6/group), blood pressure was measured weekly for 10 weeks. At 20 weeks of the study, glucose tolerance tests were carried out (n=6/group) and at 24 weeks, insulin tolerance tests were carried out (n=6/group). One week prior to the end of the study, mice were fasted for 24h and blood was taken for assay of plasma free fatty acid and triglyceride levels. At the end of the study (27 weeks), mice (n=6) were culled in the morning (7:00 – 9:00 am), blood was collected for plasma corticosterone assay, adipose tissues (Sc, Gon, Mes, RP and BAT), tail (to confirm genotype), kidney, adrenal, liver were taken and stored at -80C for later analysis. In order to examine fat cell size, the remaining mice (n=6) within the group were culled and adipose tissues and liver placed in formalin for histology.

4.5.2 D-FSG Transgenic mice showed normal weight gain and tissue weights on HF diet

High fat (HF) diet increased body weight gain in both male and female mice as expected (Fig. 4.9). HF diet increased mean body weight by ~50% for both genotypes, in both sexes (Fig. 4.9). However, no effect of transgene was observed in HF or LF fed diet groups at the end of the study (Fig. 4.9). Furthermore, no difference in food intake (g) was observed between genotypes, for either diet or sex (data not shown).

There was no effect of transgene on adipose tissue or organ weights in either male or female mice, although there was a significant effect of diet on adipose tissue weight in all groups (Fig. 4.10). In addition, no difference in adrenal weight was found in HF or LF diet-fed group of either sex (Fig. 4.11).

4.5.3 Blood pressure in D-FSG mice was unaffected by transgene

To investigate the effect of high fat diet on blood pressure in D-FSG mice, blood pressure was measured from week 15 of the study; weekly blood pressure measurements were monitored for the following 10 weeks. No difference in blood pressure was observed between genotypes for either sex and either diet, although HF-fed mice exhibited higher blood pressure in both sexes compared to mice fed LF diet (Fig. 4.12 and 4.13).

4.5.4 HF diet caused insulin resistance in both tg and non-tg D-FSG mice

To investigate the effect of transgene on glucose homeostasis, glucose tolerance tests and insulin tolerance tests were carried out. Fasting plasma glucose levels were measured prior to glucose or insulin injection at time 0 and repeated 15, 30, 60 and 120 min after the injection. In glucose tolerance tests (GTT), glucose intolerance was observed only in male HF-fed mice but no differences between genotypes were seen in plasma glucose levels of either sex on either diet (Fig. 4.14). Consistent with the GTT results, insulin tolerance tests (ITT) showed no difference

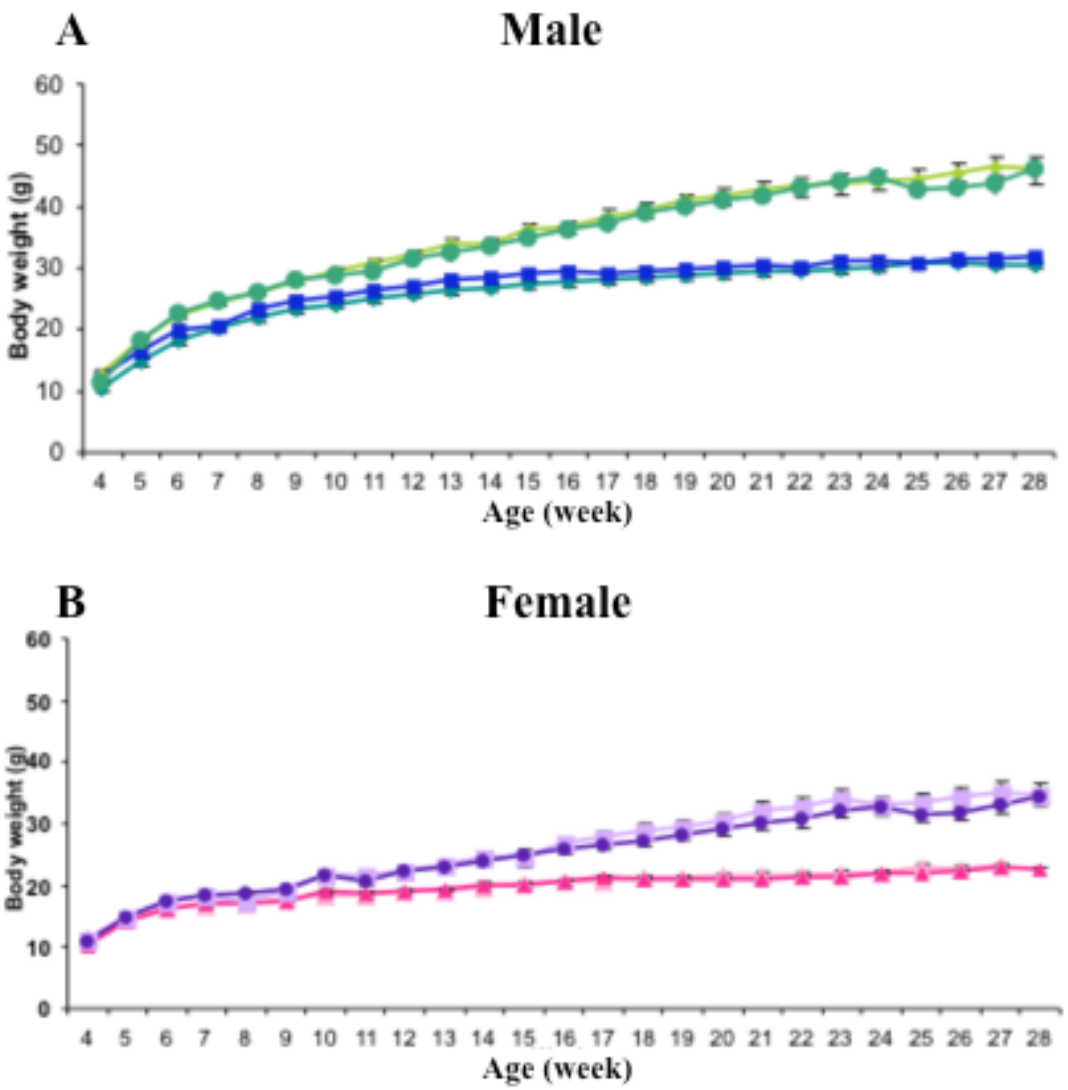

Figure 4.9 D-FSG transgenic mice showed normal weight gain on both HF and LF diets

Longitudinal body weight of D-FSG mice (F_2 generation) tg or non-tg mice fed either LF diet or HF diet, (A) male and (B) female mice. Mice were fed experimental diet from weaning (at 3 weeks of age) to the end of the study (28 weeks of age). Data are means ± SEM, n=12/group. 2-way repeated measure ANOVA showed a significant effect of diet from wk 13 of the study in both sexes ($P<0.001$), but no effect of genotype in either sexes.
Key: Male non-tg LF (■), male tg LF (■), male non-tg HF (■), male tg HF (■), female non-tg LF (■), female tg LF(■), female non-tg HF (■) and female tg HF(■).

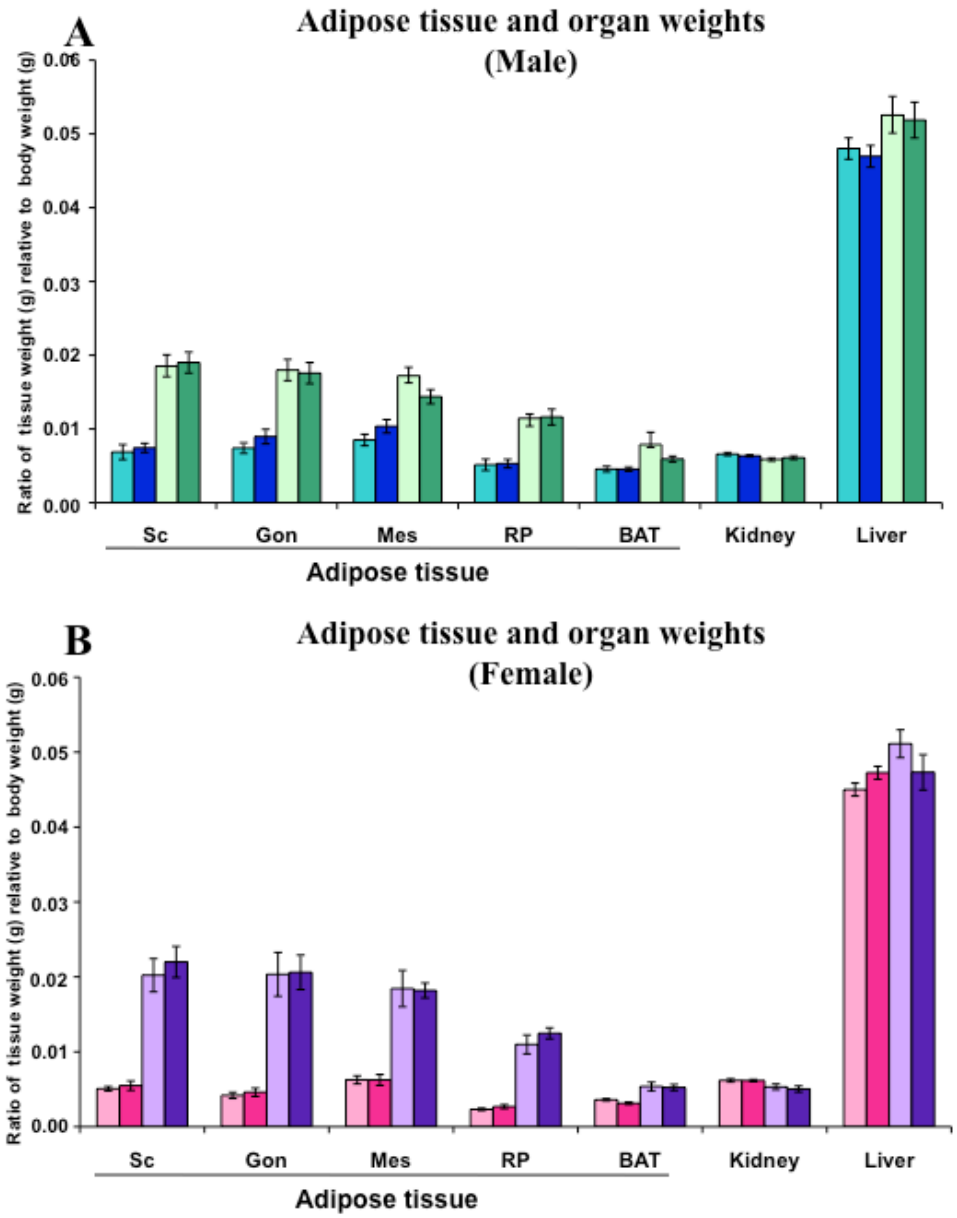

Figure 4.10 Effect of diet on adipose tissue and organ weights in D-FSG transgenic mice

Adipose tissue and organ weights of D-FSG (F_2 generation) tg and non-tg mice fed LF diet or HF diet (A) male and (B) female. Data are expressed as % of body weight and are mean ± SEM. 2 way ANOVA showed a significant effect of diet in both males and females ($p<0.0001$) but no significant effect of genotype in either sex.
Adipose tissues were: subcutaneous (Sc), gonadal (Gon), mesenteric (Mes), retroperitoneal (RP) and brown adipose tissue (BAT)
Key: Male non-tg LF (■), male tg LF(■), male non-tg HF (■), male tg HF (■), female non-tg LF (■), female tg LF(■), female non-tg HF (■) and female tg HF(■).

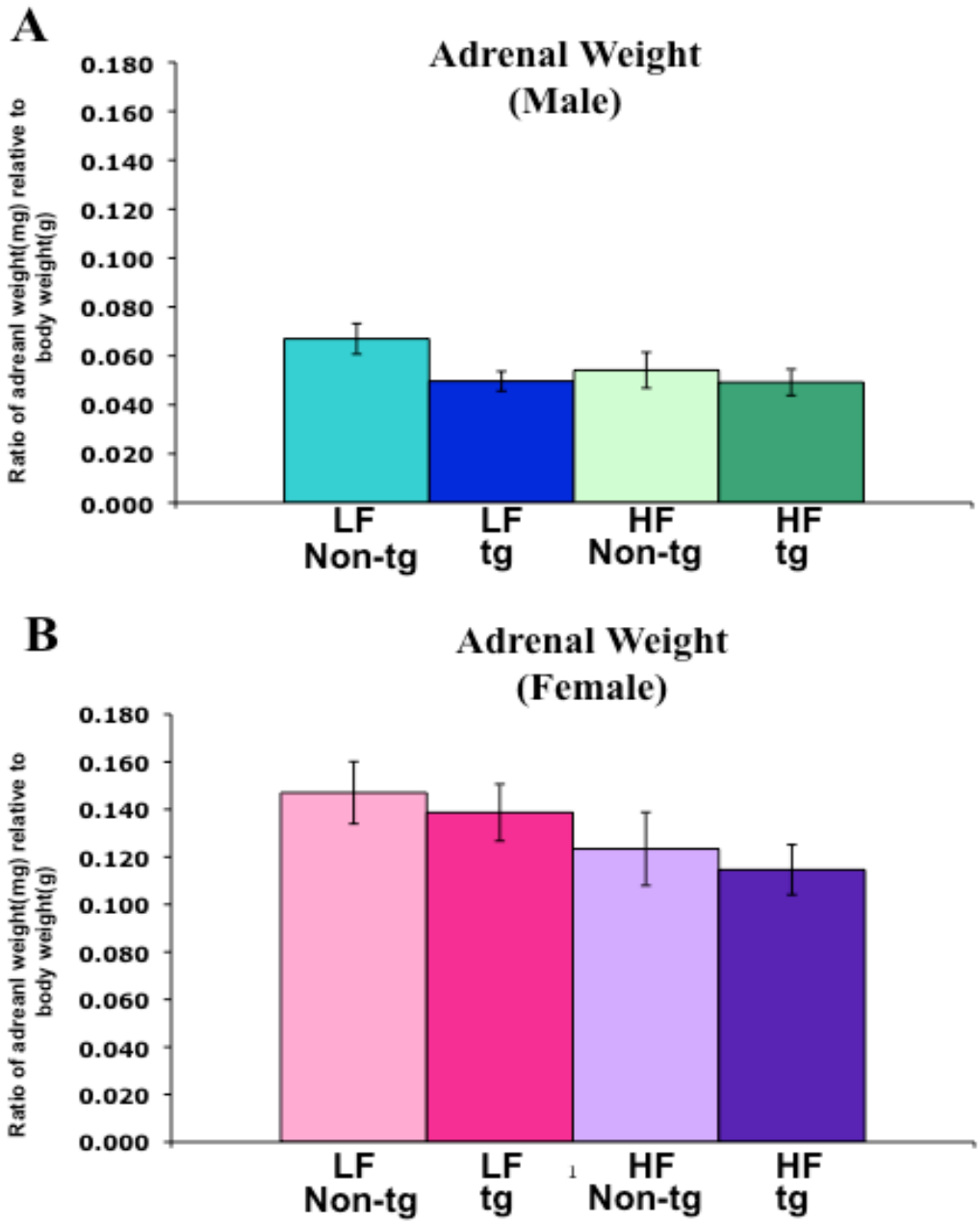

Figure 4.11 No effect of diet or transgene on adrenal weight in D-FSG transgenic mice

Adrenal weights of D-FSG (F_2 generation) non-tg and tg mice fed LF diet or HF diet. (A) male and (B) female mice. Data are mean tissue weights ± SEM (n=6/group). 2-way ANOVA showed no significant effect of diet or genotype in either male or female mice.

Key: Male non-tg LF (■), male tg LF (■), male non-tg HF (■), male tg HF (■), female non-tg LF (■), female tg LF (■), female non-tg HF (■) and female tg HF(■).

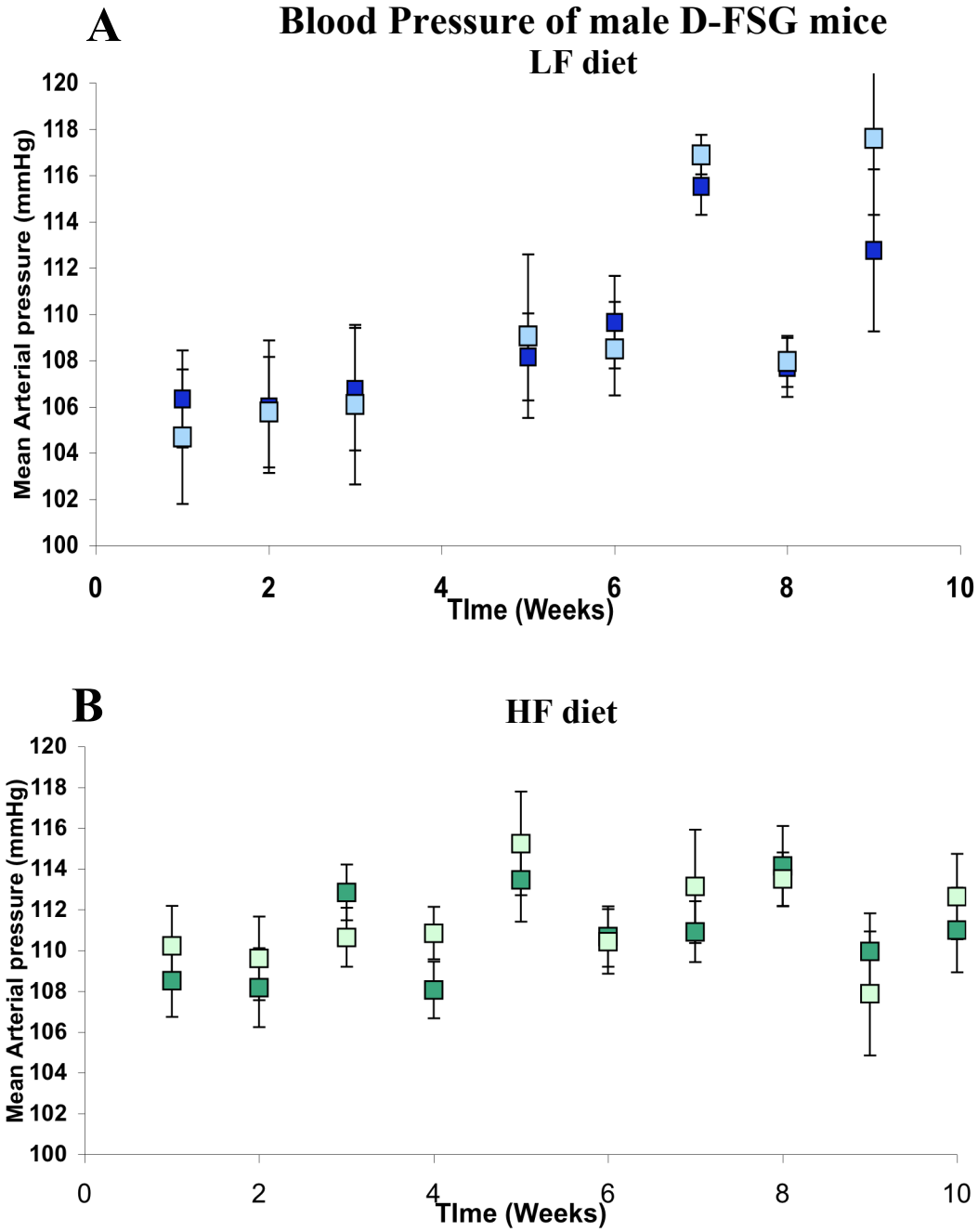

Figure 4.12 No effect of genotype on blood pressure in male D-FSG transgenic mice fed either LF or HF diet

Weekly blood pressure measurements of male D-FSG (F_2 generation) non-tg and tg mice fed (A) LF diet or (B) HF diet. Data are expressed as mean arterial pressure ± SEM, (n=6/group) Repeated measures 2 way ANOVA showed no effect of genotype or diet.
Key: Non-tg LF (■), tg LF (■), non-tg HF (■) and tg HF (■)

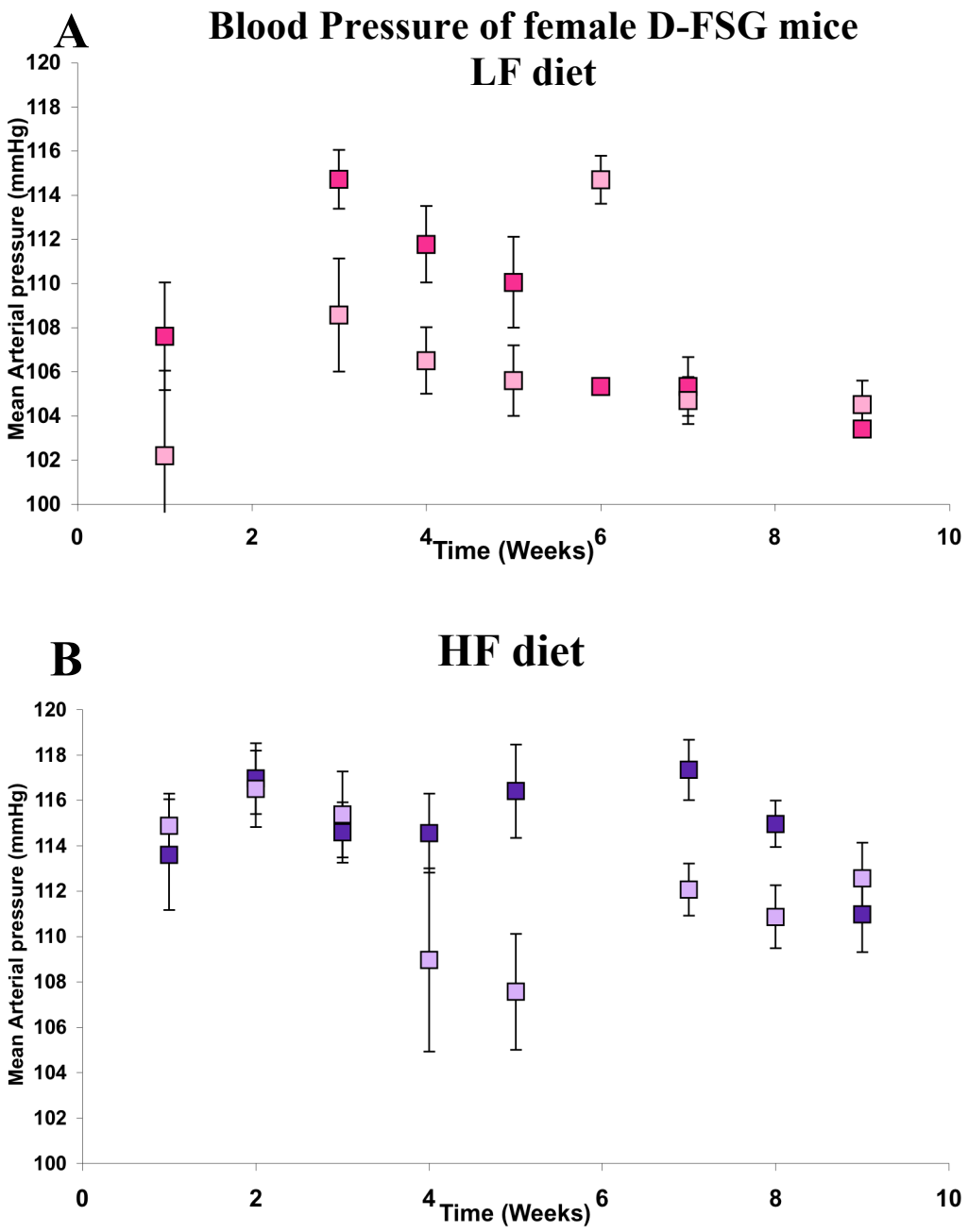

Figure 4.13 No effect of genotype on blood pressure in female D-FSG transgenic mice fed either LF or HF diet

Weekly blood pressure measurements of female D-FSG (F_2 generation) non-tg and tg mice fed (A) LF diet or (B) HF diet. Data are expressed as mean arterial pressure ± SEM, n=6/group. Repeated measure 2 way ANOVA showed no statistically significant difference between genotypes.
Key: Non-tg LF (■), tg LF (■), non-tg HF (■) and tg HF(■).

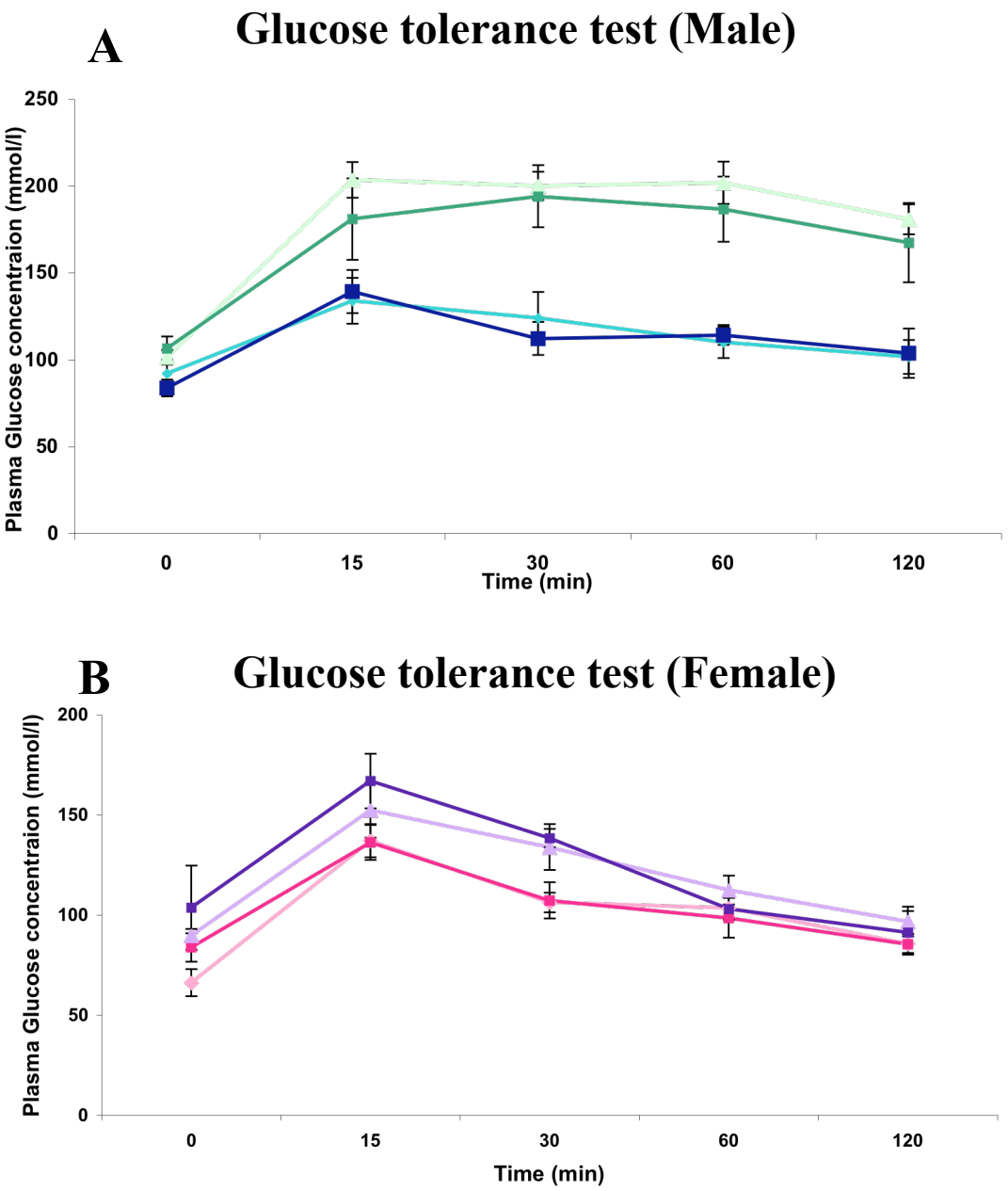

Figure 4.14 Glucose tolerance test in D-FSG mice

Glucose tolerance test (GTT) in 25-27 week old D-FSG (F_2 generation) mice fed LF or HF diet; (A) male and (B) female. For GTT, mice were fasted for 6h; blood was collected 0 and 15, 30, 60 and 120 min after intraperitoneal injection of glucose (2mg/g body weight) at time 0. Data are expressed as mean plasma glucose level ± SEM**,** n=6/group. Data were analysed by repeated measures 2 way ANOVA and showed no statistically significant differences between genotypes.
Key: Male non-tg LF (■), male tg LF (■), male non-tg HF (■), male tg HF (■), female non-tg LF (■), female tg LF (■), female non-tg HF (■) and female tg HF(■).

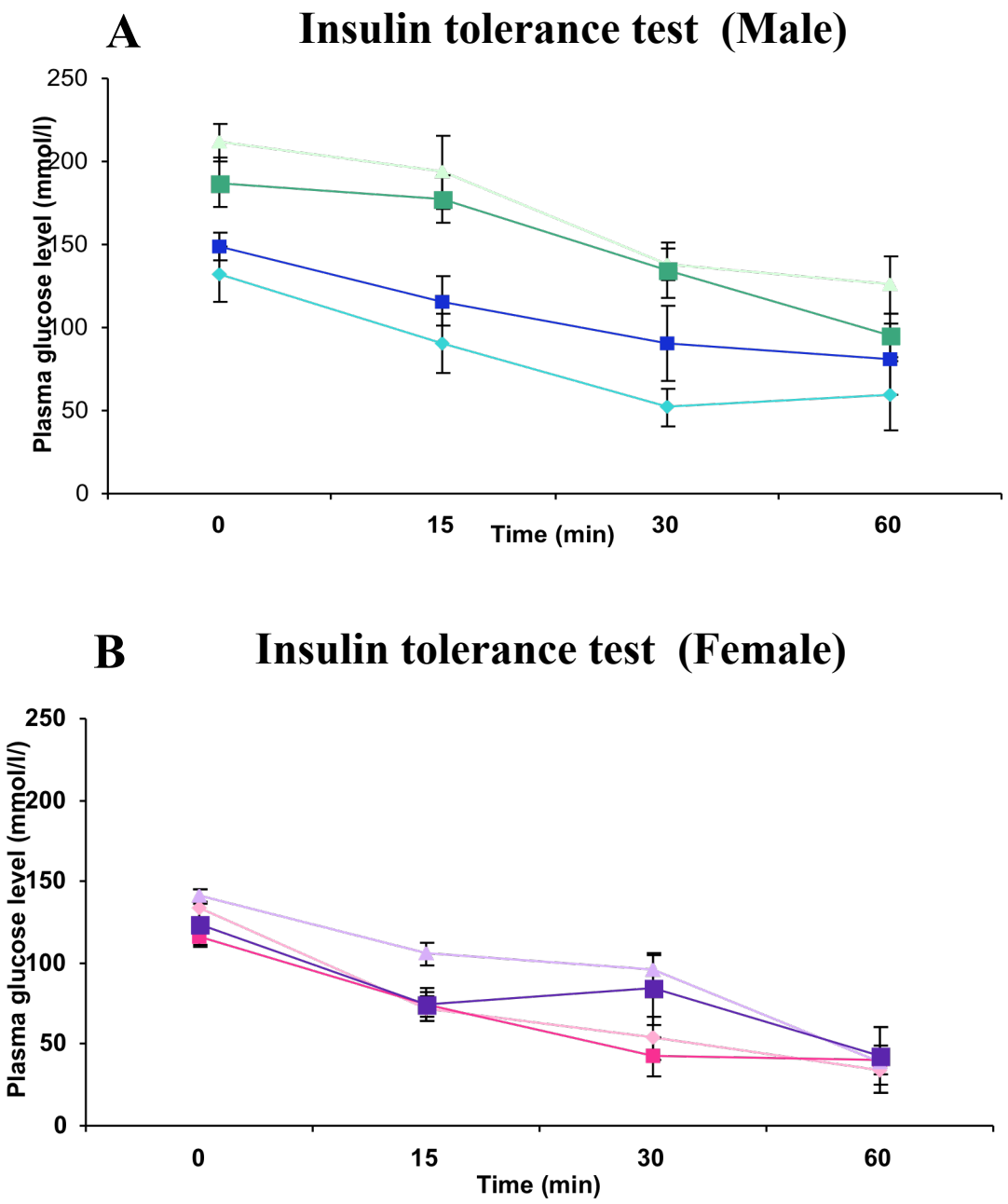

Figure 4.15 Insulin tolerance test in D-FSG transgenic mice

Insulin tolerance test (ITT) in 25-27 week old D-FSG (F_2 generation) mice fed LF or HF diet (A) male and (B) female mice. For ITT, mice were fasted for 6h; blood was collected 0, 15, 30 and 60 min after intraperitoneal injection of insulin (male; 1.5mU/g BW, female; 1mU/g BW) at time 0. Data are expressed as mean plasma glucose level ± SEM, n=6/group. Data were analysed by repeated measures 2-way ANOVA and showed no statistically significant differences between genotypes.
Key: Male non-tg LF (■), male tg LF (■), male non-tg HF (■), male tg HF (■), female non-tg LF (■), female tg LF (■), female non-tg HF (■) and female tg HF (■).

between genotypes in plasma glucose levels following insulin injection, in either sex or on either diet (Fig. 4.15).

4.5.5 Effect of transgene upon liver triglyceride levels in D-FSG mice

To investigate the effect of transgene on the level of triglyceride, liver triglyceride was measured. In males, liver triglyceride levels did not differ between tg and non-tg mice. However, both HF diet groups exhibited a 2-fold increase in liver triglyceride levels compared to LF diet groups (Fig. 4.16A). No difference was found in female liver triglyceride level between genotypes on either diet (Fig. 4.16B).

4.5.6 Adipocyte size in D-FSG mice was unaffected by transgene

To investigate the effect of transgene on adipocyte size, fixed and embedded Sc adipose tissue was sectioned (5μm sections) and stained with hematoxylin and eosin. No gross difference in adipocyte size was apparent between genotypes in both sexes (Fig. 4.17 and 4.18). However, increased adipocyte sizes were found in both sexes of HF fed mice compared to LF-fed mice (Fig. 4.17 and 4.18).

4.5.7 Unaltered body weight in 1 year old transgenic female B- and D-FSG mice

Due to the lack of phenotype in the dietary study carried out on the F_2 D-FSG mice, the adipose tissue phenotype was reinvestigated in 1 year old F_1 generation female mice of both lines. Adipose tissues; subcutaneous (Sc), gonadal (Gon), Mesenteric (Mes), retroperitoneal (RP) and brown adipose tissue (BAT), liver (Liv) and kidney (Kid) were collected and weighed. In 1 year old B-FSG F_1 generation female mice, no body weight or adipose tissue weight difference was found between tg and non-tg littermates (Fig. 4.19 A and B). In 1 year old D-FSG females, although no difference in body weight was found between genotypes (non-tg, 28.68±2.3 vs tg,

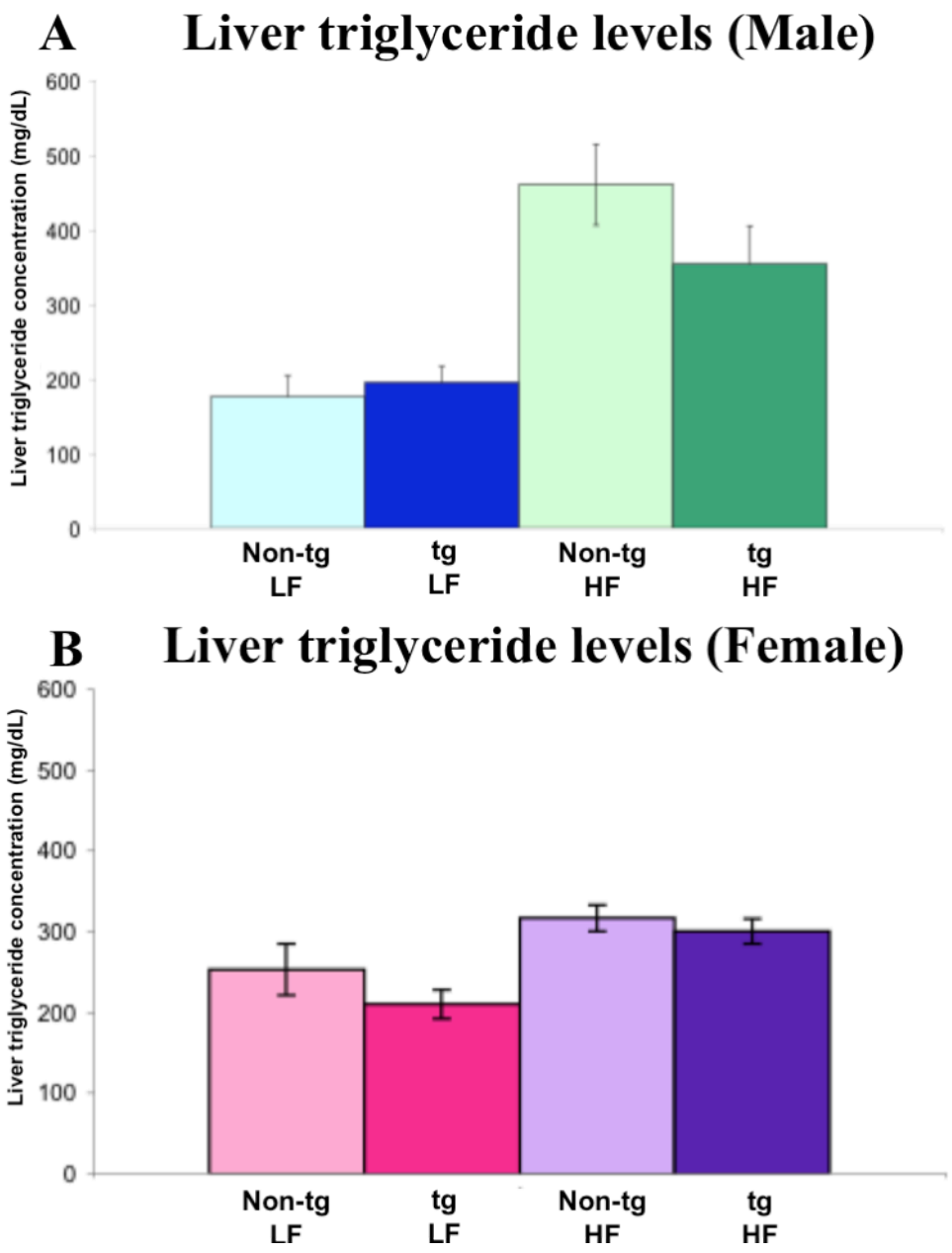

Figure 4.16 Unaltered liver triglyceride levels in D-FSG transgenic mice

Liver triglyceride levels in 28 week old D-FSG (F_2 generation) non-tg and tg mice fed LF diet or HF diet. (A) male and (B) female mice. Data are expressed as mean ± SEM; n=6/group**.** ANOVA showed significant effect of diet in males ($p<0.001$) but no significant effect of genotype in either sex.
Key: Male non-tg LF (■), male tg LF (■), male non-tg HF (■), male tg HF (■), female non-tg LF (■), female tg LF(■), female non-tg HF (■) and female tg HF(■).

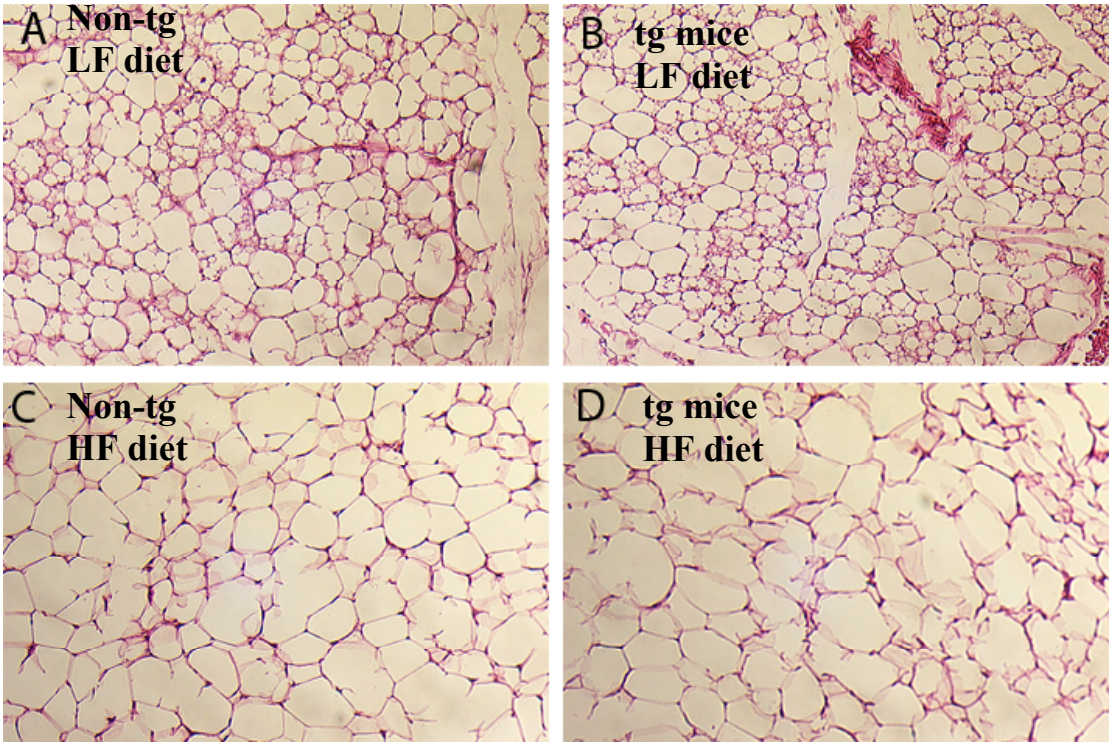

Figure 4.17 Similar adipocyte histology between non-tg and tg male D-FSG mice

Representative images of H and E stained sections of Sc adipose tissues from 29 week old male D-FSG (F_2 generation) mice. (A) LF diet, non-tg, (B) LF diet, tg (C) HF diet, non-tg and (D) HF diet, tg mice. All images were captured at x10 magnification.

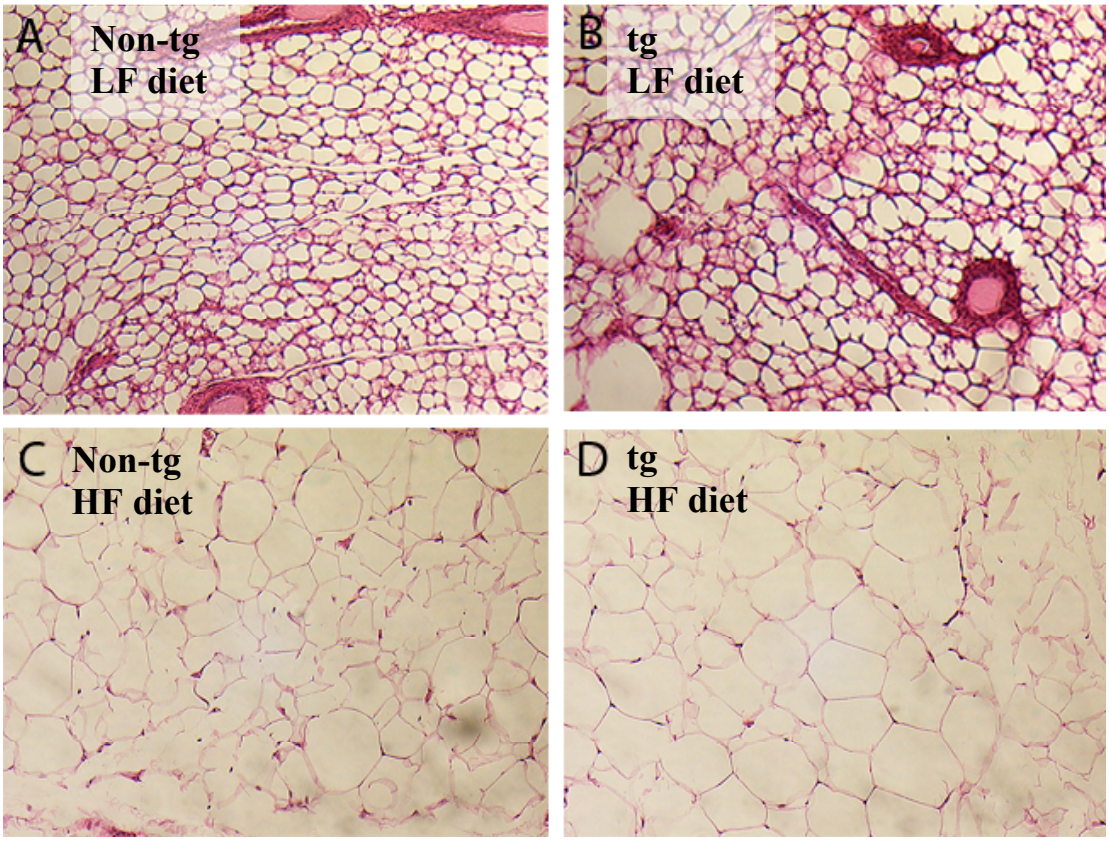

Figure 4.18 Similar adipocyte histology between non-tg and tg female D-FSG mice

Representative images of H and E stained sections of Sc adipose tissues from 29 week old female D-FSG (F_2 generation) mice. (A) LF diet, non-tg, (B) LF diet, tg (C) HF diet, non-tg and (D) HF diet, tg mice. All images were captured at x10 magnification.

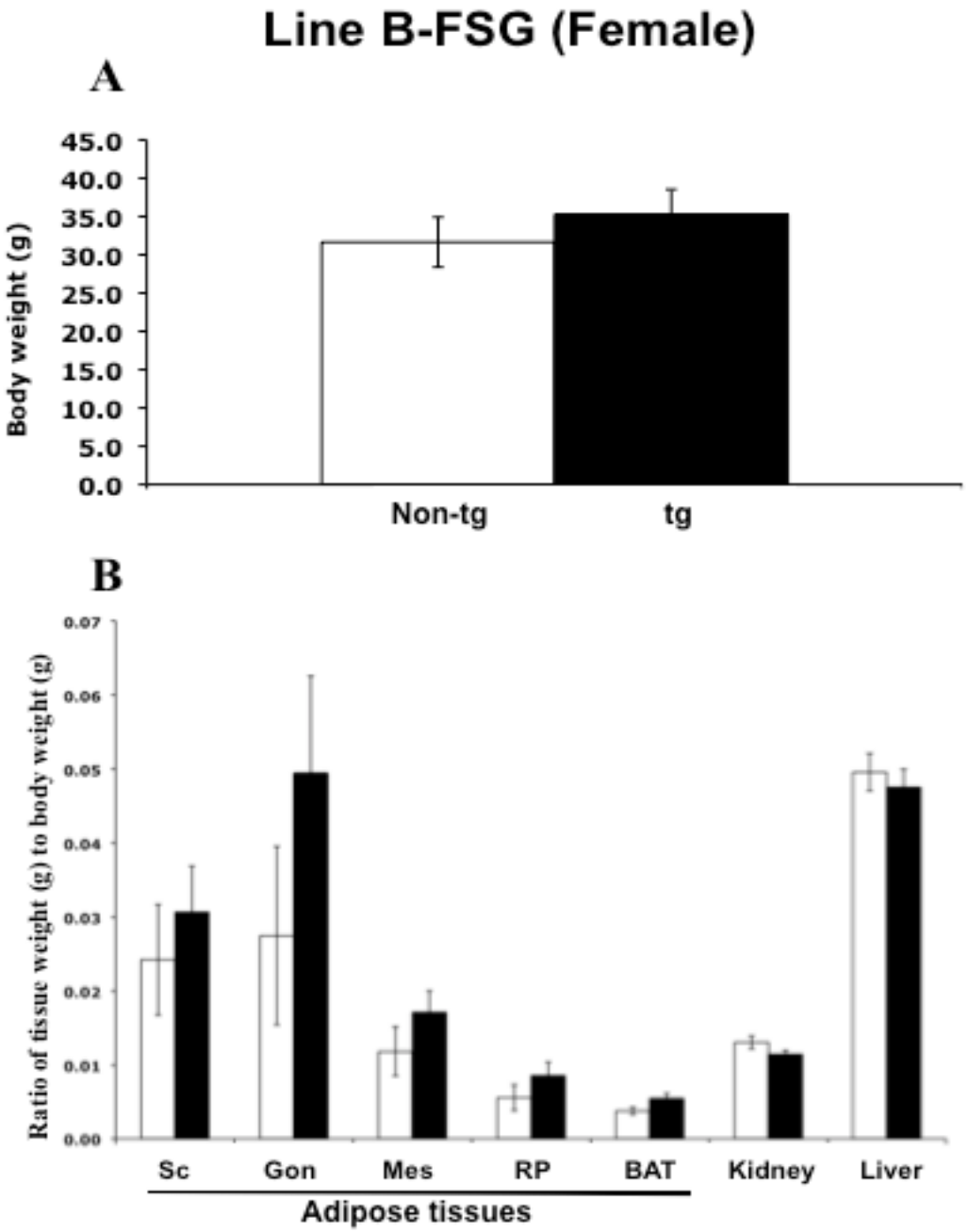

Figure 4.19 No effect of aP2-GR transgene on body weight and tissue weight of 1 year old female B-FSG mice

(A) Body weight and (B) adipose tissue and organ weights of non-tg (white) and tg (black) female 1 year old female B-FSG (F_1 generation) mice (non-tg, n=4; tg, n=5). Data are means ± SEM; no significant differences were found in body weight or tissue weights.
Abbreviations: SC; subcutaneous, Gon; gonadal, Mes; mesenteric, RP; retroperitoneal, BAT; brown adipose tissue

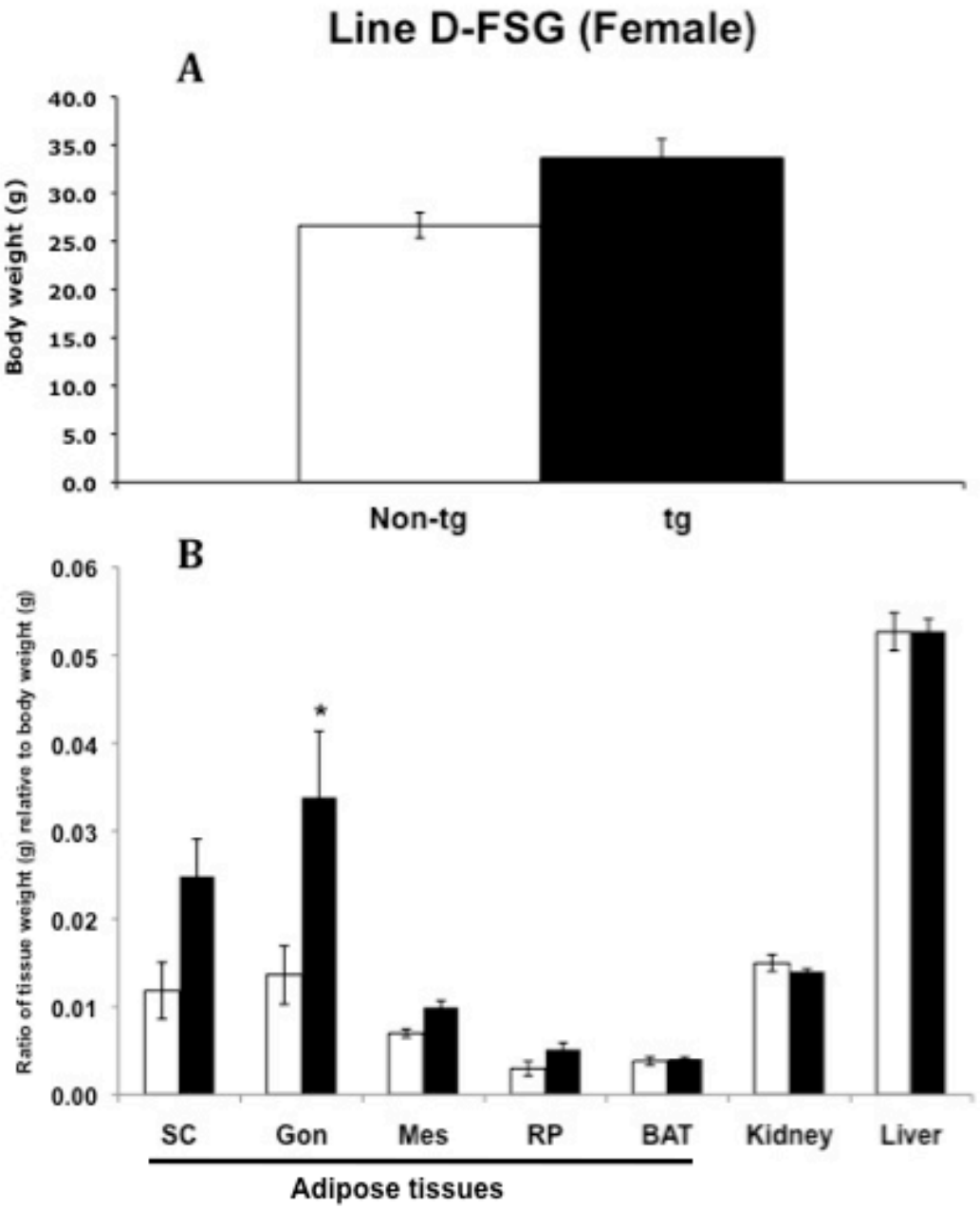

Figure 4.20 Significant effect of aP2-GR transgene on adipose tissue weight but not on body weight of 1 year old female D-FSG mice

(A) Body weight and (B) weight of adipose tissues and organs of non-tg (white) and tg (black) 1 year old female D-FSG (F_1 generation) mice (non-tg, n=6; tg, n=5). Data are means ± SEM; analysed by (A) t-test, (B) 2-way ANOVA; *$p<0.05$, compared to non-tg littermate.
Abbreviations: SC; subcutaneous, Gon; gonadal, Mes; mesenteric, RP; retroperitoneal, BAT; brown adipose tissue

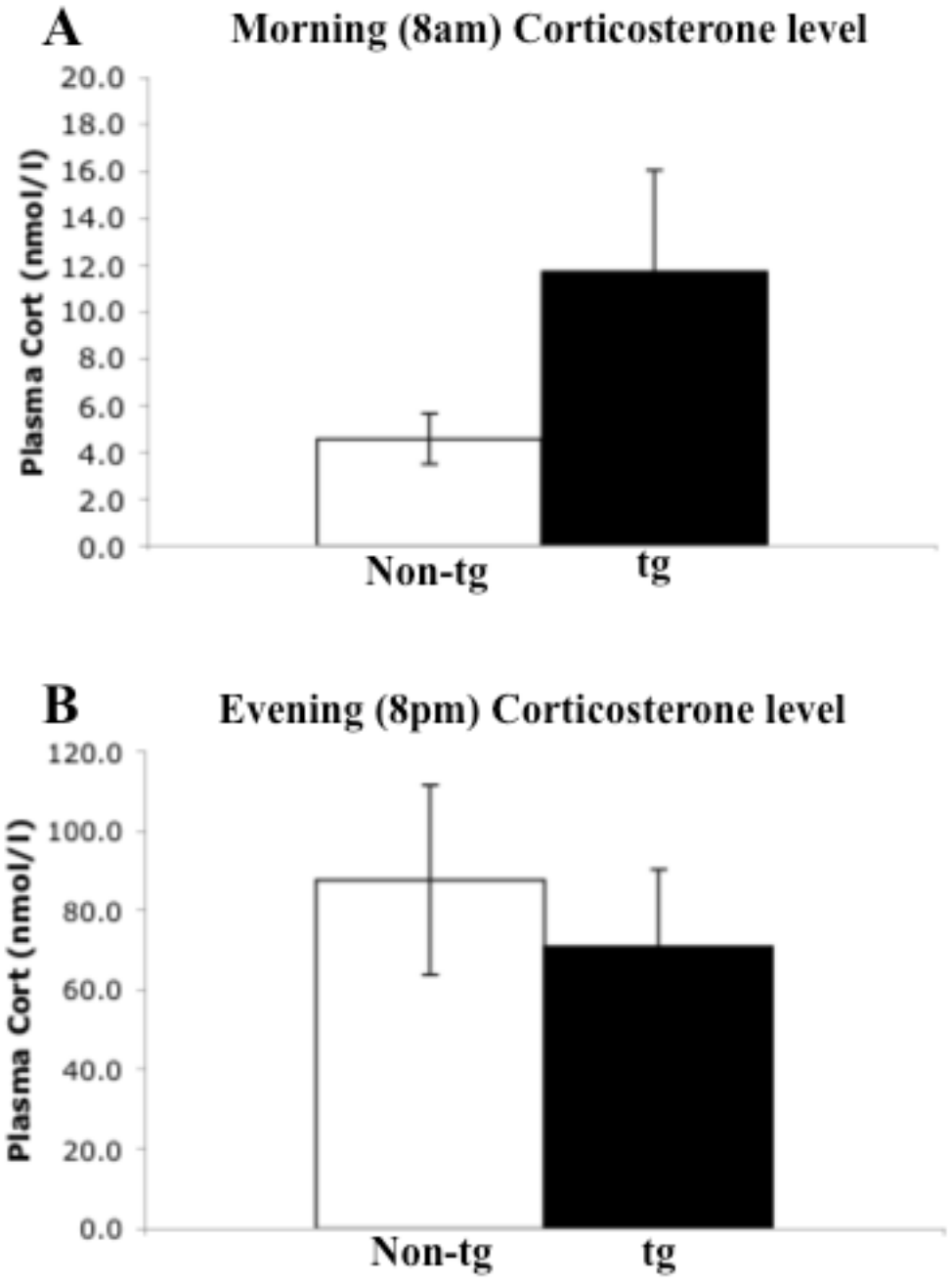

Figure 4.21 aP2-GR transgene did not affect plasma corticosterone levels in female D-FSG mice

Plasma corticosterone (Cort) levels in 1 year old female non-tg (white) and tg (black) D-FSG (F_1 generation) mice in the (A) morning (8am) and (B) evening (8pm). Data are means ± SEM (non-tg n=6, tg n=5); t-tests showed no statistically significant differences between genotypes.

33.64±2.0g; non-tg n=6, tg n=5) a significant increase was found in gonadal adipose tissue weight (Fig. 4.20).

4.5.8 Unaltered plasma corticosterone levels in female D-FSG mice

In addition to body weight, both morning and evening plasma corticosterone were measured in 1 year old F_1 generation D-FSG female mice. However, no difference was found between tg and non-tg littermates at both morning and evening. (Fig. 4.21)

4.5.9 Variable rat GR expression was found in adipose tissue of both B- and D-FSG mice using a competitive RT-PCR assay

Because real-time PCR had shown rat GR is expressed specifically in adipose tissues and a significant increase in adipose tissues was found in transgenic F_1 generation D-FSG females, the 24 week HF diet study was carried out on the F_2 generation of D-FSG transgenic mice as describe above. However, no phenotype was found in this study. In order to address the problem, competitive RT-PCR was carried out to semi-quantitatively assess transgene RNA expression relative to endogenous mouse GR mRNA. Adipose RNA was reverse transcribed and PCR was carried out using primers that amplify both rat GR and mouse GR cDNA. In order to distinguish rat GR from mouse GR, the PCR product was then digested using enzyme Pst1, predicted to only digest the rat GR DNA (Fig 4.22).

GR mRNA in various adipose tissues depots of both B- and D-FSG at different generations was measured using competitive RT-PCR (Table 4.1). Variable GR expression was found in individual transgenic mice of either sex and amongst different generations (Table 4.1)

4.5.10 RNAse Protection assay

To determine the level of rat GR expression as well as total GR mRNA (rat and mouse) within adipose tissues, RNAse protection assays (RPA) were used. To

detect rat GR mRNA in mouse tissues a rat probe was used, which shares 80% identical sequence to mouse GR mRNA. RNAse T_1 (cleaves at every mismatched G nucleotide in double stranded RNA) and RNAse A/T_1 (cleaves at every mismatched C, T and G nucleotide) were used to measure total GR mRNA levels (endogenous mouse GR and rat transgene GR mRNA) or transgene-derived rat GR mRNA, respectively. In addition, an actin probe was included as an internal control for RNA levels.

RNA was extracted from adipose tissues of both B- and D-FSG mice of both sexes from generation F_2-F_8. A total of 19 RPAs were performed to try to establish whether rat GR was expressed in adipose tissues of B/D-FSG mice and the assays that were carried are summarized in Table 4.2. Two representative RPAs are shown in Fig. 4.23 and 4.24, respectively. In the experiment shown in Fig. 4.23, RNA extracted from Mes fat of B-FSG female mice was hybridized to the rat GR probe then digested using RNase A/T_1. This resulted in protection of only rat GR mRNA. Upon 6h exposure, only a faint GR protected band was observed in the lane where rat liver RNA was used as a positive control (Fig. 4.23, lane 6), whereas actin protected fragments were observed in all the samples loaded indicating that the RNA was not degraded. Overnight exposure of the gel allowed better detection of the RNase A/T_1 protected GR fragment (Fig. 4.23B). Again, although rat GR mRNA was detected in the positive control (rat liver RNA), no rat GR mRNA was detected in Mes fat in B-FSG mice (Fig. 4.23). In the representative experiment shown in fig. 4.24, RNA extracted from Sc, Gon, BAT and liver of D-FSG (F_1 generation) female mice was hybridized to rat GR cDNA and then digested with either RNase T_1 (to detect total GR mRNA) or RNase A/T_1 (to detect rat GR mRNA). No difference in total GR mRNA levels within adipose tissues was observed between genotypes nor was any rat GR mRNA detected within BAT or liver (Fig. 4.24C).

4.5.11 Western Blot

In order to investigate any alteration in GR protein levels in the transgenic mice, western blotting was used. An antibody which identifies both rat and mouse GR was selected. A representative western blot is shown in Fig. 4.25. GR protein

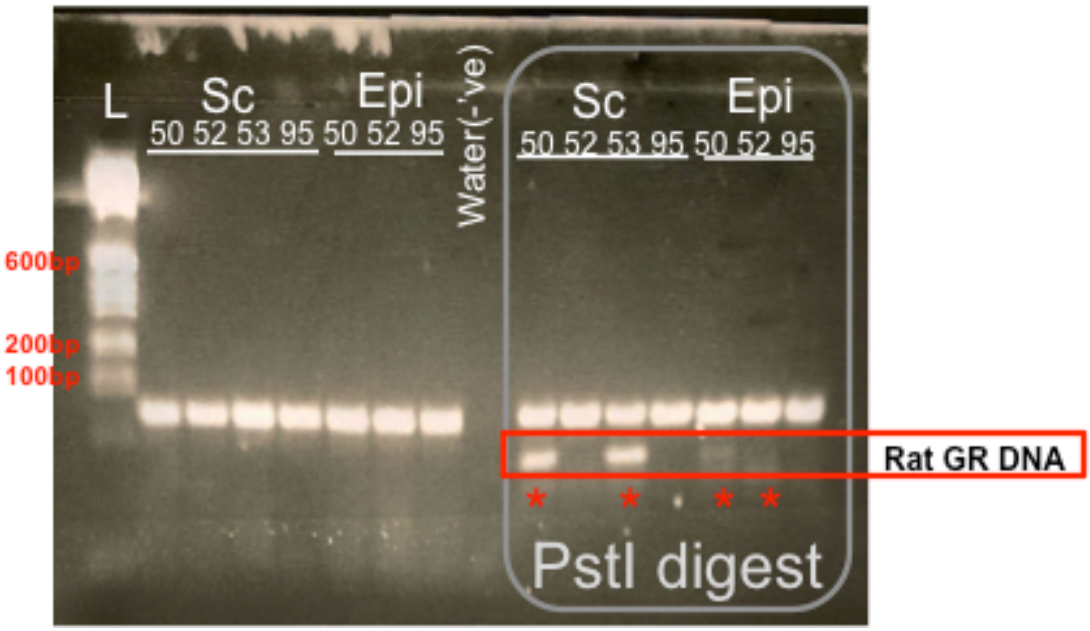

Figure 4.22 PCR assay to detect experiment of rat GR mRNA

Representative image of competitive RT-PCR reactions carried out on cDNA from Sc and Gon fat depots from male D-FSG (F_1 generation) mice. The primers amplify both endogenous mouse and transgenic rat cDNA. On the left, undigested PCR product (96bp) arising from both mouse and rat GR mRNA. Only the rat PCR product contains a PstI site. On the right, PstI digested PCR product with the lower band sitting at 46bp representing the rat GR DNA product and the upper 96bp product, the undigested mouse PCR product. Numbers above the lanes are ID of individual mice; * indicates the mice which are transgenic.
Key: L; 100bp ladder

B-FSG transgenic mice

Mouse ID	SEX	Generation	SC	Gon	Mes
33	Female	F_1	++	-	+
36	Female	F_1	+	+	++
B23	Male	F_3	-	ND	ND
B24	Male	F_3	-	ND	ND
B25	Male	F_3	-	ND	ND
B6	Female	F_3	-	ND	+
B71	Female	F_6	-	ND	ND
B73	Female	F_6	-	ND	ND

D-FSG transgenic mice

Mouse ID	SEX	Generation	SC	Gon	Mes
72	Male	F_1	+	++	+
75	Male	F_1	++	++	+
93	Male	F_1	-	-	-
94	Male	F_1	++	-	+++
50	Female	F_1	++	-	ND
53	Female	F_1	++	-	ND
D7	Female	F_2	+++	++	ND
D56	Male	F_4	+	-	-
D67	Female	F_5	++	ND	ND

Table 4.1 Summary table of PstI digests to detect rat GR mRNA expression in aP2-GR transgenic mice

Summary table of competitive RT-PCR on cDNA extracted from adipose tissues of various generations of B-FSG and D-FSG transgenic mice. Pst1 digestion was used after RT-PCR to distinguish rat GR from mouse GR. +++, rat GR product shows stronger intensity than mouse GR product; ++, rat GR product shows similar intensity to mouse GR product; +, rat GR product shows less intensity than mouse GR product; -, rat GR product is negligible or absent; ND, not done.

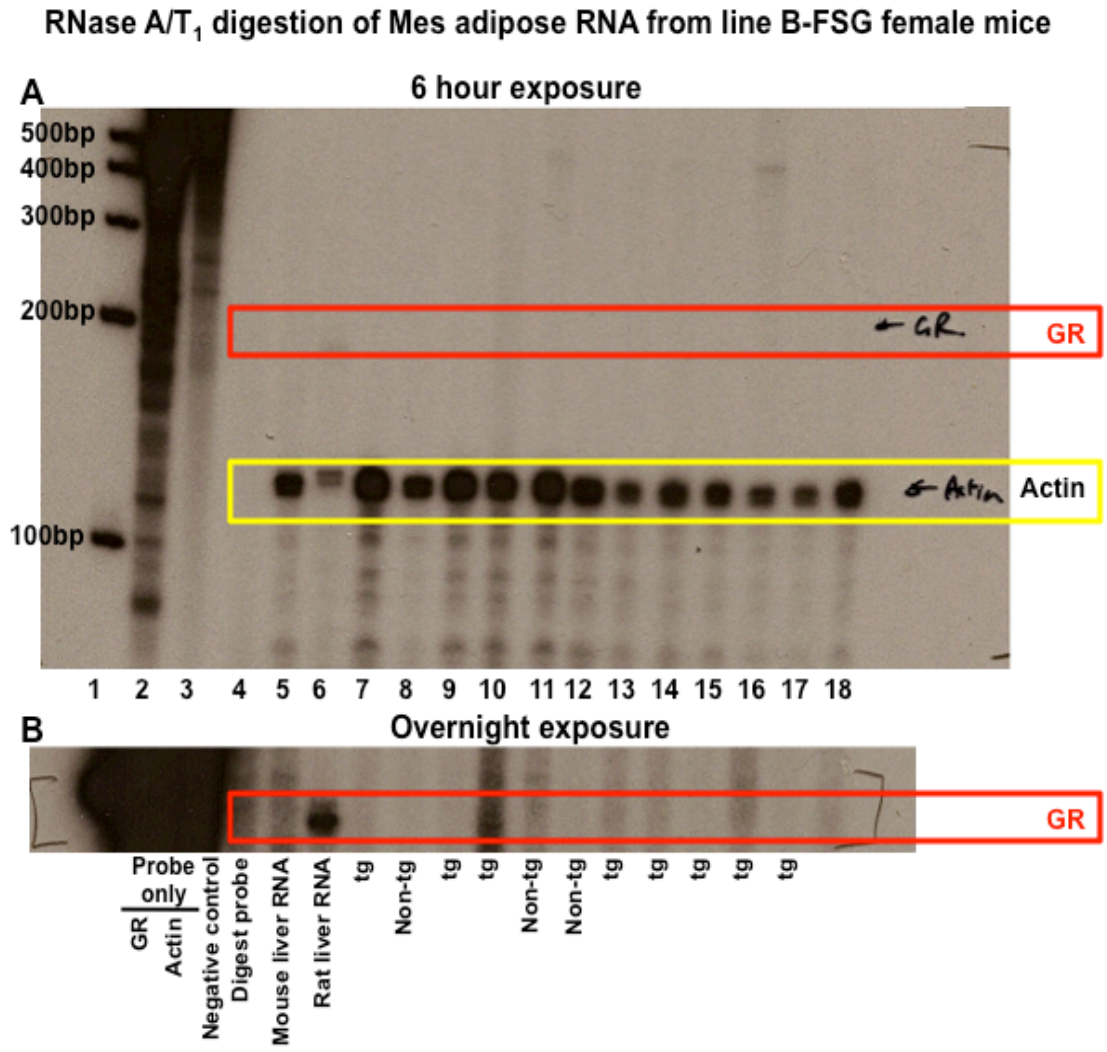

Figure 4.23 RNase protection assay (RPA) to measure rat GR mRNA levels in line B-FSG transgenic mice

Representative autoradiograph showing rat GR mRNA levels in Mes fat of female line B-FSG (F_1 generation) transgenic mice measured using RNase protection assay with RNase A/T_1 digestion. Lanes 8-18 represent different mice with each lane loaded with a reaction containing 8μg RNA. (A) 6 hour exposure of GR protected fragment and the internal control actin protected fragment. (B) overnight exposure of the same gel to show GR fragment. No expression of rat GRmRNA was detected in any of the RNA samples from tg or non-tg mice. Lane 1 contained labeled markers, lanes 2 and 3 contained probe but no RNAse and lane 4 contained yeast tRNA instead of mouse RNA plus both rat GR and actin probes, which serves as the negative control.

levels in Sc fat of D-FSG F_1 generation female mice were analysed and tubulin was used as internal control for the amount of protein being loaded. No differences in Sc GR protein levels were observed when tg were compared with non-tg littermates and the internal control (tubulin) indicated comparable levels of protein were loaded among all samples (Fig. 4.25B). Liver GR levels were also analysed using western blot and no difference was observed between tg and non-tg littermates (data not shown).

4.6 Construction and characterization of transgenic mice expressing an aP2-antisense GR transgene

aP2-anti-sense GR mice were generated by Elaine Marshall. These mice contained a transgene comprising the aP2 enhancer/promoter (as used for aP2-rat GR mice, described above) driving a portion of GR cDNA in the anti-sense direction. The region of rat GR cDNA chosen has been previously shown to reduce GR expression in transgenic mice (Pepin, Pothier et al. 1992; King, Vacchio et al. 1995). One line with the highest copy number was selected for further study. For ease of identification the line was named GR-5. Preliminary characterization showed female F_1 generation transgenic GR-5 mice had reduced WAT mass (data not shown). However no phenotype was seen in male mice (data not shown). GR-5 (F_2 generation) transgenic mice were subject to the same HF diet experimental protocol as D-FSG mice.

4.6.1 Female GR-5 transgenic mice showed lower body weight gain upon HF diet

Body weights were measured throughout the study. In female mice fed LF diet, no difference in body weight was observed between genotypes (Fig. 4.26). However, HF-fed female tg mice showed reduced body weight gain compared to their non-tg littermates (Fig. 4.26). From week 19 onward the mean body weight of female GR-5 mice fed HF diet was significantly less than their non-tg HF-fed littermates (Fig. 4.26). At the end of the study, HF fed female GR-5 mice were 18% lighter than their non-tg HF fed littermates. Interestingly, in males no difference in

body weight was observed between genotypes in mice fed either HF or LF diet (Fig. 4.26).

4.6.2 Difference in adipose tissue weight upon HF diet in both sexes of GR-5 transgenic mice

To assess any differences in adipose tissue distribution and weight between genotypes, adipose tissues (Sc, Gon and Mes) were dissected and weighed. In addition, kidney and liver were dissected and weighed as before. No differences between genotypes in adipose tissue and organ weights were found in LF-fed mice in both sexes (Fig. 4.27). However, following HF diet, a significant increase in Sc and Gon fat was found in GR-5 tg males compare to non-tg littermates (Fig. 4.27A). On the other hand, GR-5 tg female mice only shown a significant reduction in Sc fat weight (Fig. 4.27B and 4.28).

4.6.3 No effect of transgene on glucose homeostasis

To investigate the effect of transgene on glucose homeostasis, glucose tolerance test were carried out. Fasting plasma glucose levels were measured as describe in section 4.4.4. In glucose tolerance tests (GTT), glucose intolerance was observed only in male HF-fed mice but no differences between genotypes were seen in plasma glucose levels of either sex on either diet (Fig. 4.29). Although a slightly improved glucose response was observed in tg female mice in both diet group, this was not significant when calculated using area under curve (LF non-tg, 42940±1988 vs. LF tg 38710±758.8 g; non-tg n=6, tg n=6; HF non-tg, 55540±3171 vs. HF tg 45620±4642 g; non-tg n=6, tg n=5).

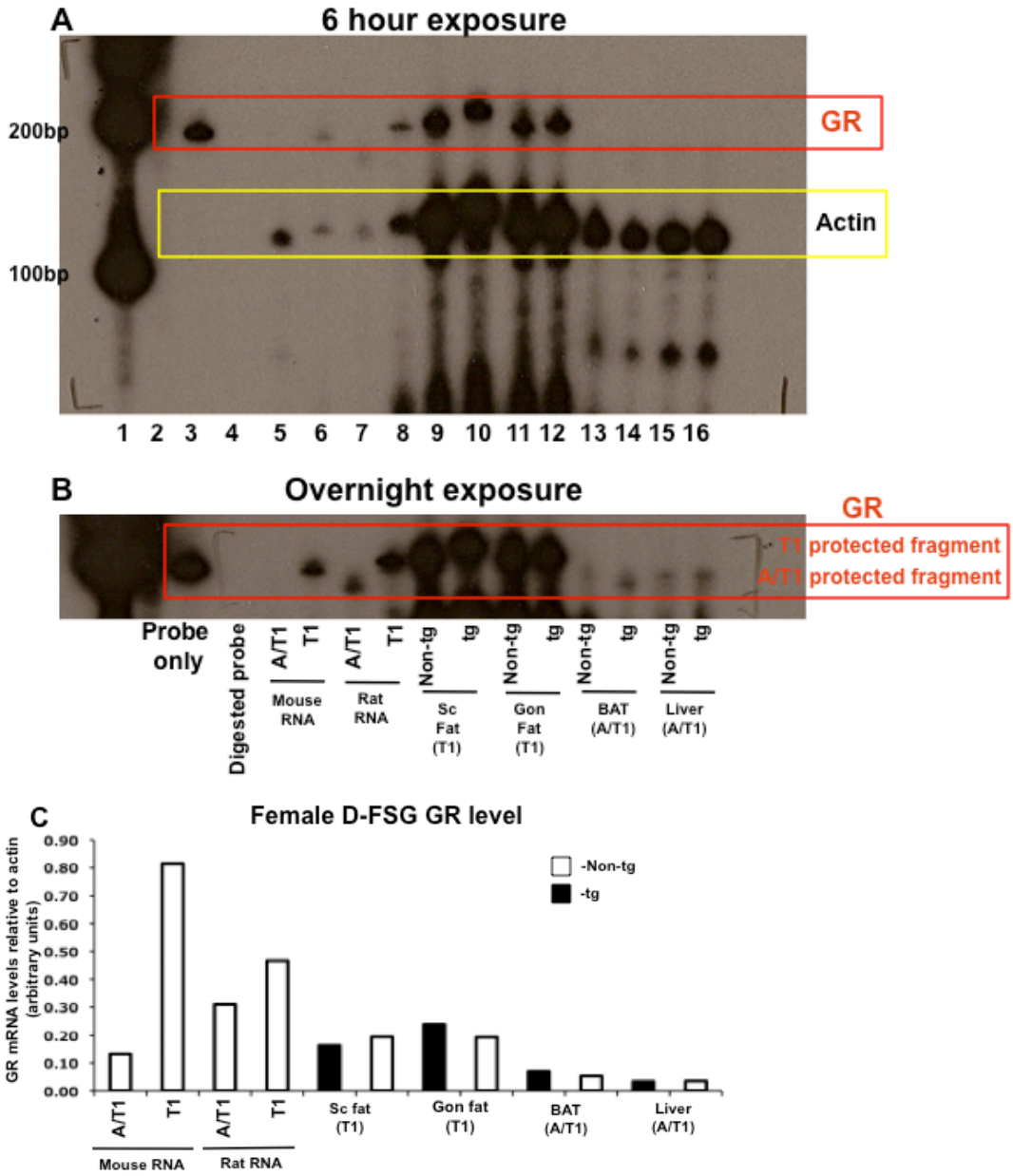

Figure 4.24 RNase protection assay (RPA) to measure rat GR mRNA levels in line D-FSG transgenic mice

Representative autoradiograph showing RPA to measure rat GR mRNA levels (RNase A/T_1) and total GR mRNA levels (endogenous mouse and rat GR; RNase T_1) in adipose tissue of female D-FSG (F_5 generation) mice. (A) 6h exposure showing GR and actin (internal control) protected fragments and (B) overnight exposure of the same gel to show GR protected fragment. Lane 1 contains labelled RNA marker, lanes 2 and 3 contain reaction with probe (GR or actin) but no RNase, lane 4 contains yeast tRNA instead of mouse RNA as negative control, lanes 7 and 8 contain rat liver RNA as positive control. Lanes 9-16 contain RNA from adipose tissues of a single tg and non-tg mouse digested with RNase T_1 as indicated. All reactions contained 8µg RNA. (C) Quantitative of GR/actin products from gel shown above.

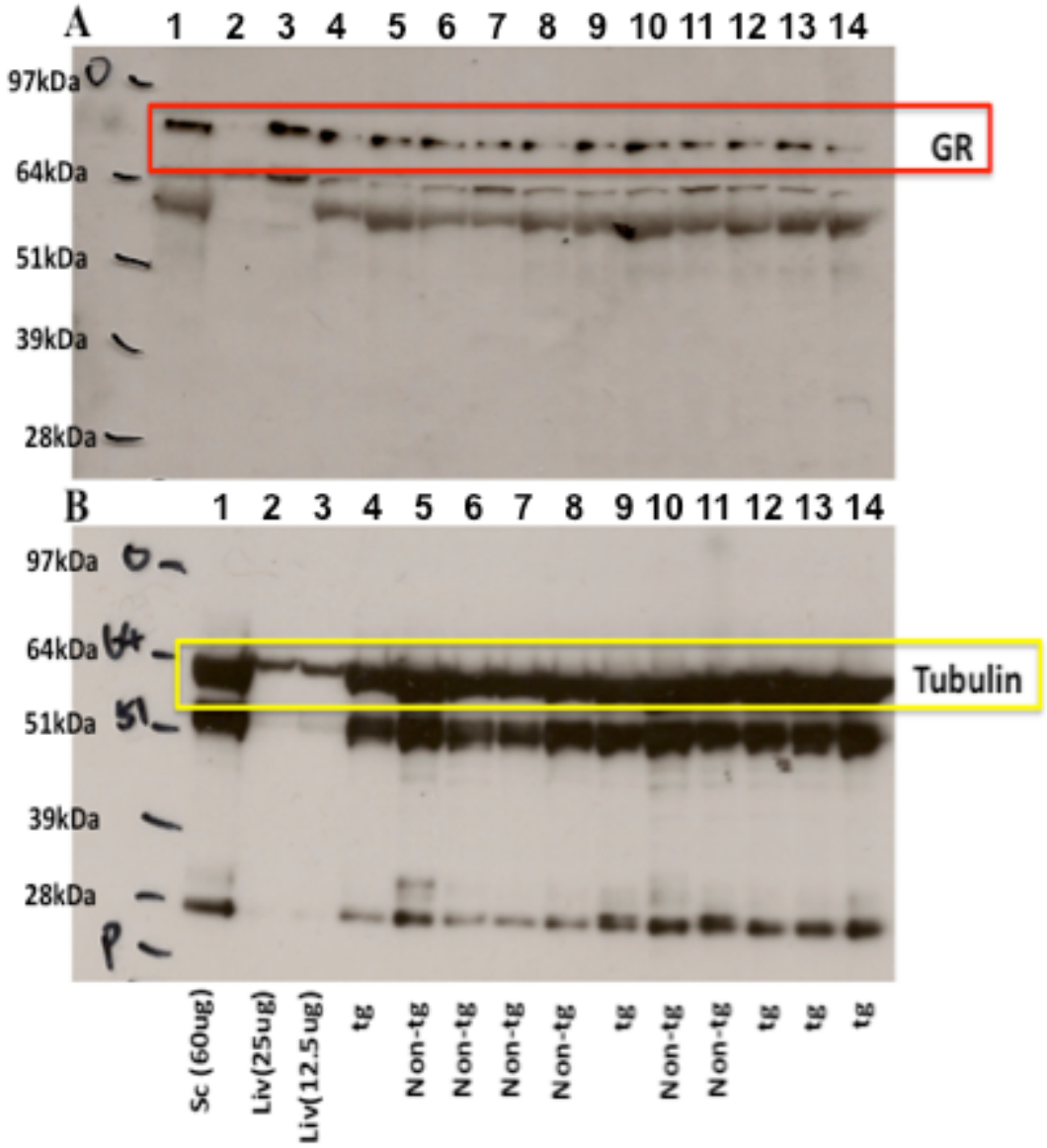

Figure 4.25 Western blot to measure GR protein levels in Sc adipose tissue of female D-FSG (F_1 generation) mice

Representative western blots showing (A) GR and (B) tubulin levels in Sc adipose tissue of tg and non-tg D-FSG females (F_1 generation). 30µg of protein was loaded in each lane except for lane 1 (60µg protein from Sc adipose tissue of non-tg mice). Lanes 2 and 3 contained 25µg and 12.5µg protein respectively, from liver of non-tg mice. The molecular weight marker is shown on the left of the blot.

B-FSG transgenic mice

Sex	Generation	Sc	Gon	Epi	T_1	A/T_1
Male	F_3	1	ND	1	✔	✔
Female	F_3	2	2	2	✔	
Female	F_4	2	ND	2	✔	✔
Female	F_5	1	ND	1	✔	✔
Female	F_6	3	ND	3	✔	✔

D-FSG transgenic mice

Sex	Generation	Sc	Gon	Mes	T_1	A/T_1
Female	F_1	6	6	ND	✔	
Female	F_2	ND	ND	14	✔	✔
Male	F_4	1	ND	1	✔	
Female	F_5	1	1	1	✔	✔
Male	F_6	ND	ND	3	✔	✔
Female	F_6	ND	ND	6	✔	✔
Male	F_7	ND	ND	5	✔	✔
Female	F_8	ND	ND	1	✔	✔

Table 4.2 Summary table of RPA assay

Summary table of RNA protection assays carried out on RNA extracted from adipose tissues of various generations of B-FSG and D-FSG transgenic mice, using T_1 digestion to measure total GR mRNA level and A/T_1 to measure rat GR mRNA level. The number within the table indicates the number of mice that were examined. ✔; type of digestion carried out, ND; not done.

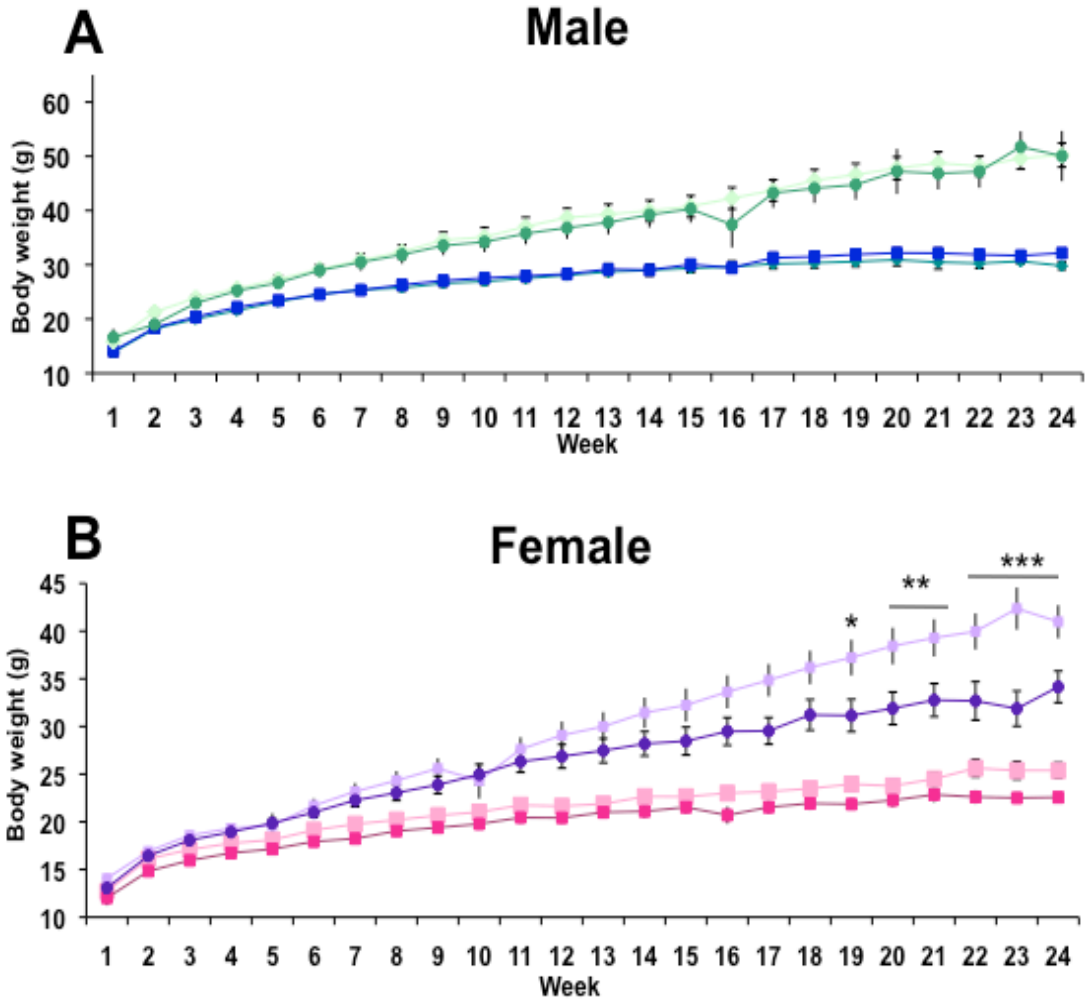

Figure 4.26 Female GR-5 transgenic mice showed reduced weight gain upon HF diet

Longitudinal body weight of tg or non-tg GR-5 mice (F_2 generation) fed either low fat (LF) or high fat (HF) diet. Mice were fed experimental diet from weaning (at 3 weeks of age) to the end of the study (24 weeks of age). Data are mean body weight of each group ± SEM, n=12/group. 2-way repeated measures ANOVA showed a significant effect of diet in both sexes ($p<0.001$) and a significant effect of genotype in HF-fed female mice from week 19 onward (*$P<0.05$, ** $P<0.01$, ***$P<0.001$)

Keys Male non-tg LF(■), male tg LF(■), male non-tg HF (■), male tg HF (■), female non-tg LF (■), female tg LF(■), female non-tg HF (■) and female tg HF(■).

4.7 Discussion

The transfection studies demonstrated that the cloned rat GR cDNA fragment is functional. It was not possible to confirm that the aP2 enhancer/promoter fragment was active due to a lack of a suitable cell line. However, similar aP2 enhancer/promoter constructs have been reliably used to express transgenes in adipose tissue of mice (Moitra, Mason et al. 1998; Shimomura, Hammer et al. 1998; Masuzaki, Paterson et al. 2001; Kershaw, Morton et al. 2005) Preliminary examination of F_1 tg mice by real-time PCR showed adipose-specific expression of rat GR mRNA and also showed a significant increase in adipose tissue weight in female D-FSG mice at 4-5 months of age. Due to time and animal constraints, the initial phenotyping of D- and B-FSG lines was carried out on the second (F_2) generation. Between the two transgenic lines, D-FSG was selected for a 24 week high fat diet study, but no difference in body weight gain, adipose tissue weights, blood pressure, plasma lipid or liver triglyceride levels was found on either LF or HF diet in either sex. However, in 1 year old F_1 chow-fed female mice, the difference in adipose tissues was maintained, at least in gonadal fat. The reason for the loss of adipose phenotype at F_2 is unclear. Competitive (semi-quantitative) PCR using various adipose tissue depots from tg mice from different generations showed rat GR mRNA was expressed in both transgenic lines with very low levels of transgene expression in adipose tissue of B-FSG mice and clearly detectable levels in D-FSG mice. However, the PCR assay also showed great variability between mice within each line and between adipose depots within individual mice, with no expression in some adipose tissue of some tg mice. In addition, little or no transgene expression was detected by RPA in adipose depots of any generation, nor did total GR mRNA (endogenous mouse plus transgenic rat) differ between tg and non-tg mice. It is also possible that mouse GR mRNA was down–regulated to compensate for transgene-derived GR mRNA. However, no evidence was obtained to support this.

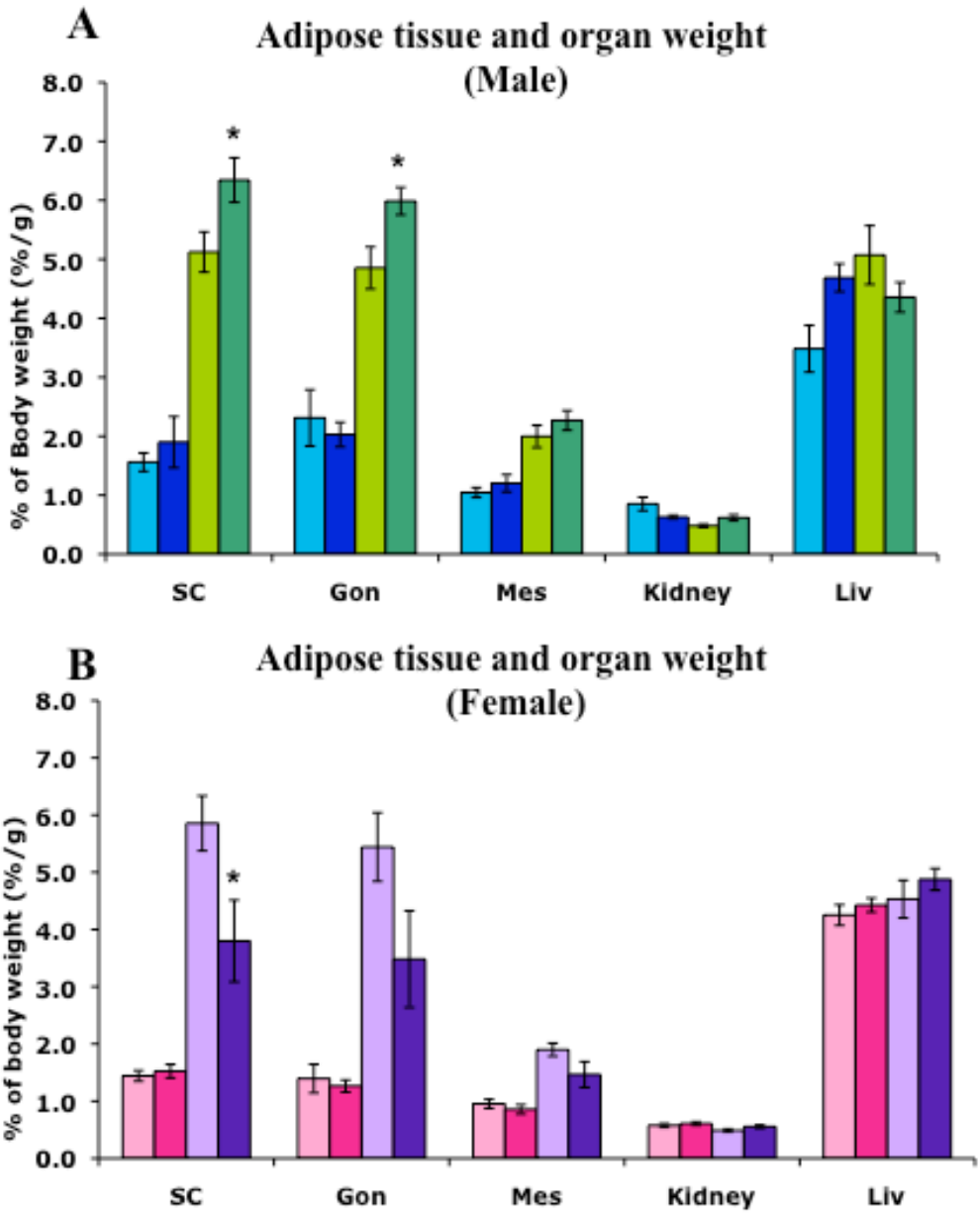

Figure 4.27 Following HF diet, female GR-5 mice show reduced adipose tissue mass compared to non-tg littermates

Adipose tissue and organ weights of GR-5 (F_2 generation) mice fed LF diet or HF diet. (A) male and (B) female mice. Data are expressed as % of body weight of each group and are mean ± SEM, n=12/group. 2-way ANOVA and shown no significant difference in the LF diet and significant in HF diet when compare to non-tg control (*P<0.05)

Tissues and organs are subcutaneous (Sc), gonadal (Gon), Mesenteric (Mes) adipose and liver (liv).

Key: Male non-tg LF(■), male tg LF(■), male non-tg HF (■), male tg HF (■), female non-tg LF (■), female tg LF(■), female non-tg HF (■) and female tg HF(■).

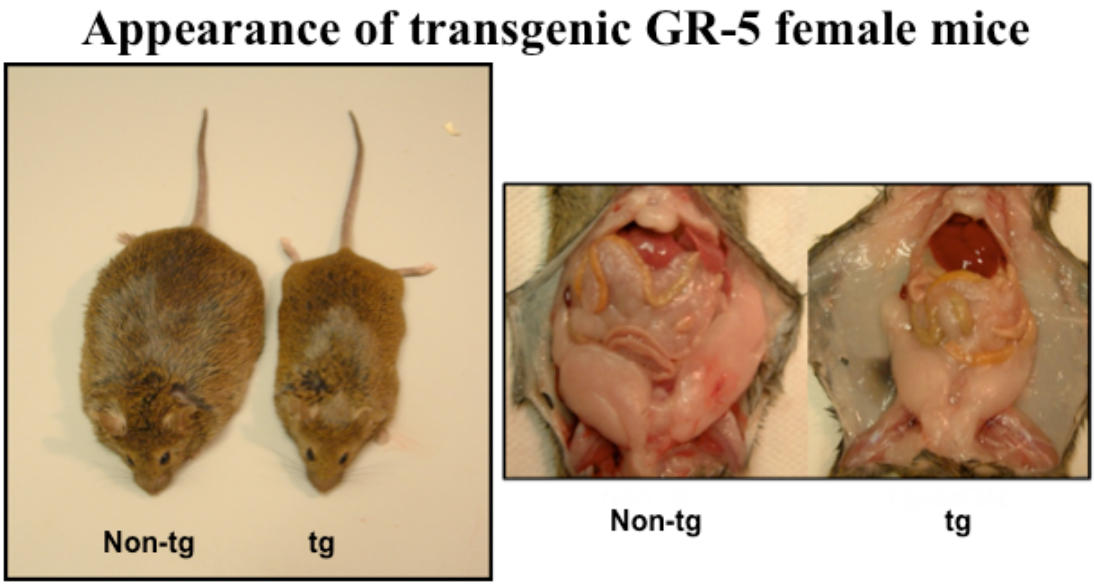

Figure 4.28 Reduction in body weight and adipose accumulation in female GR-5 transgenic mouse upon HF diet

Appearance of HF fed female GR-5 (F_2 generation) mice with non-tg littermate shown on the left of both pictures and tg shown on the right.

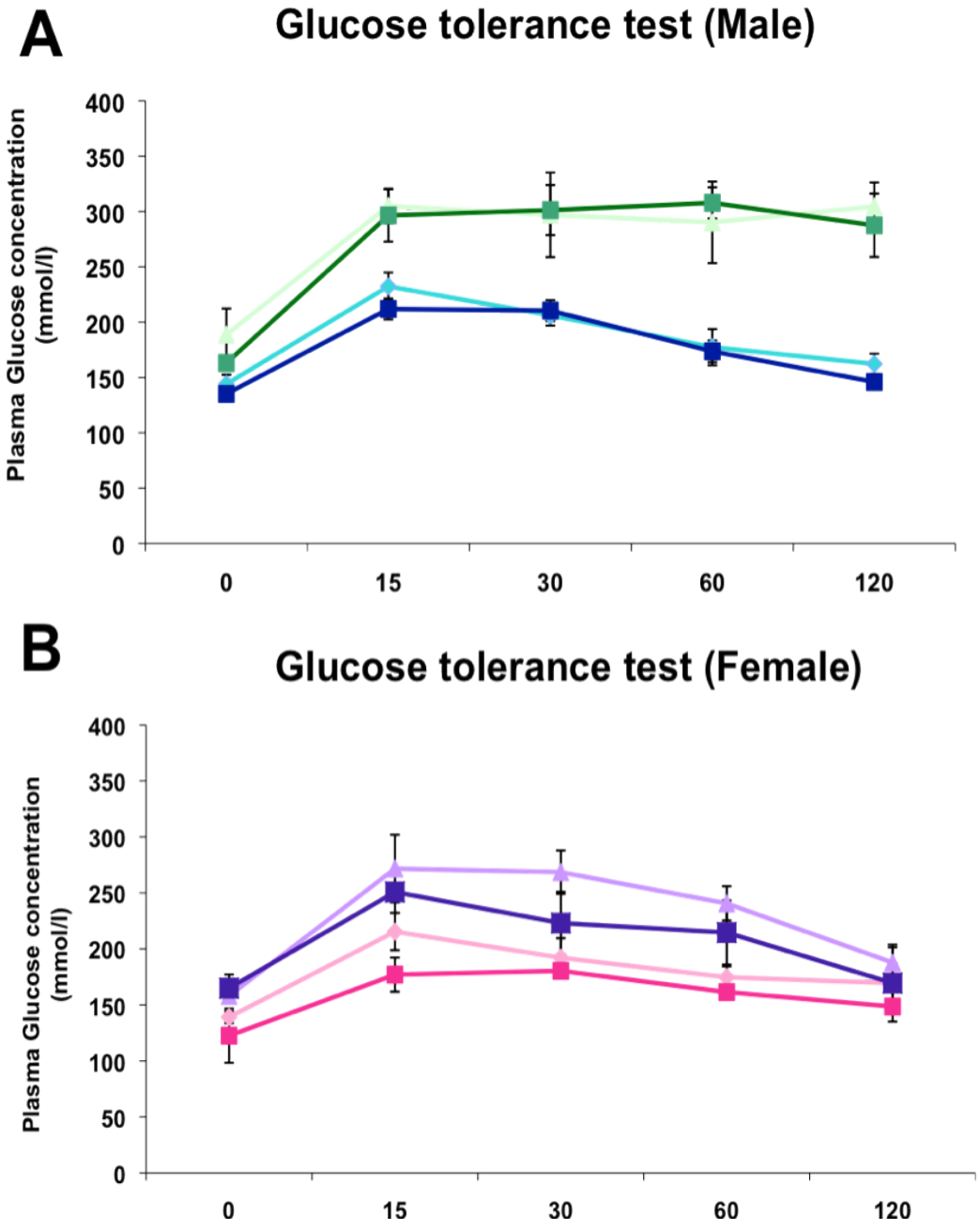

Figure 4.29 Unaltered glucose tolerance in GR-5 transgenic mice

Glucose tolerance tests (GTT) in 26-27 week old GR-5 (F_2 generation) non-tg (straight line) and tg (dashed lines) mice fed LF (square symbols) or HF (triangular symbols) diets; (A) male and (B) female. For GTT, mice were fasted for 6h; blood was 0 and 15, 30, 60 and 120 min after intraperitoneal injection of glucose (2mg/g body weight) at time 0. Data are expressed as mean plasma glucose level ± SEM**.** N=6/group. Data were analysed by repeated measures 2 way-ANOVA.

Key: Male non-tg LF(■), male tg LF(■), male non-tg HF (■), male tg HF (■), female non-tg LF (■), female tg LF(■), female non-tg HF (■) and female tg HF(■).

Unfortunately, these assays could not be performed on F_1 female D-FSG tg mice due to insufficient tissues. It is possible that transgene expression decreased between F_1 and subsequent generations, although this could not be confirmed. With hindsight, greater quantitative analysis should have been performed on the F_1 and F_2 generations before carrying out the HF diet study, to establish whether total adipose tissue GR mRNA was elevated in tg mice.

In parallel, aP2-anti-sense GR mice (GR-5 line) were produced by Dr. Elaine Marshall and preliminary characterization was carried out on the F_1 generation of these mice. Similar to the aP2-GR tg mice, adipose tissue weights differed only in female mice, with lower adipose tissue weight in the tg mice compared to the non-tg. As with the D-FSG mice line, a 24 week high fat diet study was carried on the F_2 GR-5 mice. From week 19 of the study onward, HF-fed tg female mice showed a significant reduction in body weight gain, but such a reduction was not observed in the LF-fed female group; only a trend to lower body weight was observed. This could be due to the low fat content of the LF diet (compared to the chow diet fed to F_1 mice) which might not produce a difference in body weight as observed in the chow-fed mice group. To investigate whether the reduction in body weight of the HF-fed tg female mice was due to reduced food intake, this should be measured. Unfortunately in this experiment, such measurement was not possible as the majority of the mice shredded the food and scattered it within the cages. A future experiment should either use metabolic cages or a computerised system to measure daily food and water consumption. The reduction in body weight of the HF-fed tg mice compared to controls could also be explained by increased metabolic rate. Measurement of both core body temperature and oxygen consumption would be needed to test this.

To establish whether GR protein expression was reduced within adipose tissues of tg mice, western blotting was carried out. To date, the results have been inconclusive as variable measurements have been obtained from the same tissue samples, which could be due to protein escape during loading, incomplete protein transfer or incorrect extraction method. Further investigation is required to establish whether there is a reduction in GR levels in adipose tissues of GR-5 mice.

To investigate whether the HPA axis is normal in tg mice, adrenal weight of the mice as well as plasma corticosterone levels in both morning and evening should be measured. In addition, measurement of levels of plasma corticosterone during HF diet should be investigated. Measurement of glucocorticoids and their metabolites in urine collected from the metabolic cages would give an insight into the clearance rate of plasma corticosterone in these mice.

Quantitative (real-time) PCR should be carried out on adipose tissues obtained from the dietary study to examine levels of mRNA encoding leptin which plays an important role in appetite regulation; adiponectin, which is involved in regulation of fatty acid metabolism, uncoupling protein 1 (UCP1) which is involved in thermal regulation as well as other metabolically important genes.

As glucocorticoids affect lipolysis within adipose tissue, fasting free fatty acid levels as well as liver triglyceride levels should also be measured to look at the effect of adipose GR on free fatty acid trafficking. In addition, Sc as well as Mes adipose tissue could be stained with oil red O to investigate any change in fat cell size in tg mice fed high fat diet.

In the HF diet study, male tg mice did not display any difference in body weight gain throughout the study under both diet conditions. However, both Sc and Gon fat depots were found to be heavier in the HF fed-tg mice compared to controls. The mechanism causing this remains unclear, especially given the opposite effect to that seen in females. However, such difference could be due to the sex-specific difference in glucocorticoid biology, with female having larger adrenal glands and exhibiting higher level of basal corticosterone (Le Mevel, Abitbol et al. 1979). Similar sex-specific phenotypes have been observed in GR+/- mice (Michailidou Z. (2008) PhD thesis, University of Edinburgh). They exhibit a hyperactive HPA axis, with enlarge adrenal glands and elevated evening plasma corticosterone levels in both sexes. However, only male mice exhibit an elevated corticosterone response after acute stress (Michailidou Z. (2008) PhD thesis, University of Edinburgh). Alternatively, gonadal steroids also play an important role in energy balance and potently influence body composition (reviewed in Lovejoy and Sainsbury 2009). The phenotype difference observed here could due to an interaction of GR with sex-specific adipose functions.

In the meantime, more detailed characterization of the GR-5 mice transgene expression pattern is also needed. Although initial real-time data from B-FSG and D-FSG had shown no rat GR mRNA expression were found in kidney and liver. However recent studies have shown aP2 gene expression in brain, lung and macrophages (Liu, Quinet et al. 2007). To test whether the phenotype observed is the consequence of transgene expression in adipose tissues, in situ hybridization could be carried out on brain tissue to look for transgene expression. To ensure expression observed in adipose tissues was solely from the adipocyte, adipose tissues from transgenic mice could be collagenase digested and separated into adipocyte and stromal vascular layers, and transgene expression measured in each fraction using real-time PCR. In addition, to test that the phenotype was not due to transgene expression in macrophages or other immune cells, a bone marrow transplant experiment could be carried out. To deplete bone marrow cells, GR-5 and WT (C57BL/6 mice) recipient mice would be lethally irradiated, then bone marrow cells reconstituted, form either GR-5 or WT mice. Establishing whether the obesity-resistant phenotype followed the host genotype or the recipient genotype would determine whether transgene expression in the adipocyte or the macrophage is responsible for the observed phenotype. Nevertheless, the preliminary data reported here suggests that GR density in adipocyte may be an important determinant of adipose tissue accumulation in mice.

5 Discussion

Substantial evidence links disturbances of fatty acid metabolism and glucocorticoid (GC) action in obesity. Tissue GC actions are determined by GR density and ligand availability, the latter depending upon circulating plasma corticosterone levels and activity of an intracellular enzyme-11β-HSD1. In this thesis I have investigated the interaction of environmental and genetic manipulations upon adipose GC action and its impact upon obesity.

Data from the dietary fatty acid-enrichment studies showed unaltered adipose and liver GR mRNA expression levels among all diet groups. However, differential effects on adipose 11β-HSD1 mRNA levels were observed between the stearate fed-group and all the other groups, especially the oleic acid group. This was more apparent in study 2, where the diets were fed *ad libitium* and the oleic acid fed group showed the same pattern of down-regulation of adipose 11β-HSD1 expression along with a marked increase in body weight as observed in the 58% mixed fat diet and also the 45% mixed fat diet, although to a lesser extent. Thus, unsaturated fat (when predominates in the mixed fat diet), down-regulated adipose 11β-HSD1. However, the reverse pattern was seen in the stearate fed-group whether pair-fed or fed *ad libitium* with increased adipose 11β-HSD1 mRNA levels and activity, accompanied by elevated plasma corticosterone levels and reduced fasting plasma insulin levels. This suggested that adipose 11β-HSD1 mRNA levels might be highly regulated by GC, since the substrate for 11β-HSD1 (11-dehydro-corticosterone) is elevated upon activation of HPA axis (e.g. following stress) (Harris, Kotelevtsev et al. 2001), potentially producing a "feed-forward" regulation of GC action in adipose tissue. This hypothesis is further validated in POMC null mice (which lack endogenous GC), where corticosterone replacement resulted in increased adipose 11β-HSD1 mRNA levels but unaltered hepatic 11β-HSD1 mRNA levels (Michailidou, Coll et al. 2007). This is also apparent in C57BL/6 wild type mice in which high physiological GC levels (but not supraphysiological) caused an increase in adipose 11β-HSD1 mRNA (Michailidou, Coll et al. 2007). Similarly, GC administration in rat increased adipose 11β-HSD1 mRNA and activity (Balachandran, Guan et al. 2008). However, such an increase was not observed *in*

vitro in GC-treated differentiated 3T3-L1 adipocytes, which model many aspects of adipocyte biology (Balachandran, Guan et al. 2008). Furthermore, these studies were conducted in a relativly short period of time (1 week); in contrast mice fed high fat diet for a longer period present a chronically activated HPA axis yet still show a down regulation of 11β-HSD1 (Morton, Ramage et al. 2004), though this is not seen in rat (Drake, Livingstone et al. 2005). Thus, other factors than simply plasma GC levels or 11β-HSD1 substrate levels are likely to be involved in the up-regulation of adipose 11β-HSD1 seen in the stearate-fed mice and regulation of adipose GC action may be complex.

An unexpected observation from the two control diet groups in Study 2 was an elevation in mesenteric fat and liver 11β-HSD1 activity levels but unaltered 11β-HSD1 mRNA levels between the groups. This suggests that the control sucrose diet might be triggering a different pathway to the control cornstarch diet, which acts post-transcriptionally to modulate 11β-HSD1 activity. 11β-HSD1 reductase activity depends on the availability of the nicotinamide co-factor NADPH, which is produced within the endoplasmic reticulum lumen by the enzyme hexose-6-phosphate dehydrogenase (H6PDH), physically associated with 11β-HSD1 (Atanasov, Nashev et al. 2008). Recent studies have shown that mutations in H6PDH cause cortisone reductase deficiency (CRD), in which activation of cortisone to cortisol (11β-reductase activity) does not occur (Draper, Walker et al. 2003; Lavery, Walker et al. 2008). Initial studies had suggested that alterations in the 11β-HSD1 gene could contribute to CRD (Draper, Walker et al. 2003). However, recent re-analysis showed that deficiency in 11β-reductase activity in CRD is entirely due to H6PDH mutations without alteration in 11β-HSD1 (Lavery, Walker et al. 2008). However, H6PDH expression and activity levels were not examined in the studies reported in this thesis, thus it is not known whether the control diet differentially regulated H6PDH. This should be examined in the future. Interestingly, a study in rats has shown an effect of sucrose on H6PDH expression and consequently on GC metabolism (London, Lala et al. 2007). Rats with access to the drinking sucrose solution showed increased 11β-HSD1 and H6PDH mRNA levels in mesenteric fat and a reduction in liver 11β-HSD1 mRNA levels, the latter accompanied by an increase in H6PDH mRNA levels (London, Lala et al. 2007). This suggests dietary

sugar (e.g. sucrose or possibly fructose or glucose) influences 11β-HSD1 mRNA levels. In the study reported here, the control diet enriched in sucrose did not alter 11β-HSD1 mRNA levels in liver and mesenteric fat compared to control diet enriched in cornstarch but it did increase mesenteric and subcutaneous fat 11β-HSD1 enzyme activity and the reverse in liver. Because the activity assays carried out here were homogenate assays, H6PDH is assumed not to play an important role in the assay (NADP is added as co-substrate with corticosterone in 11β-dehydrogenase activity assays carried out with homogenates). It is also possible that 11β-HSD1 interaction with H6PDH might alter post-translational modification of 11β-HSD1, (e.g. glycosylation) which might regulate activity.

The results presented in this thesis suggest that GC receptor density may be an important determinant of adipose tissue accumulation in obesity, although possibly only in females. Although the experiments designed to increase GR density in adipose initially produced promising data, the subsequent high fat diet experiment failed to show any effect. The follow-up investigation revealed variation in adipose tissue GR density within a single tg mouse and between mice from different generations. However, female mice with decreased adipose GR density appeared to be protected from obesity. Further work is needed to establish the mechanisms but the finding is of great interest.

Mice with globally reduced GR gene density ($GR^{+/-}$) showed an entirely normal response to high fat diet, in terms of weight gain, adiposity, lean mass and insulin/glucose homeostasis (Michailidou, Carter et al. 2008). They also showed a compensatory increase in HPA axis activity with increased plasma corticosterone levels (Michailidou, Carter et al. 2008). In contrast, mice with globally reduced GR density, due to expression of an antisense-rat GR transgene under the control of a human neurofilament gene promoter (a very similar anti-sense GR construct to the one used in this thesis) were obese and also had elevated circulating corticosterone levels; these mice therefore had disturbances in both HPA axis regulation and metabolism (Pepin, Pothier et al. 1992; Richard, Chapdelaine et al. 1993). The differences between the anti-sense GR mice and the $GR^{+/-}$ mice may be due to a variable expression of the anti-sense transgene, particularly in the brain, where the promoter was designed to target. Although GR-5 mice carry a very similar anti-sense

GR transgene, expression was designed to be restricted to adipose tissue. These mice show an opposite, lean phenotype, suggesting that the effect on HPA axis and ligand availability in the global anti-sense GR mice may over-ride the local effects of reduced GR density in adipose tissue. It is also possible that GR density in adipose tissue was little affected by the transgene in the global anti-sense transgenic mice. In addition, the data reported here suggest that adipose GR density is only limiting in the accumulation of adipose tissue in female mice on a high fat diet. It is also important to note that the female mice tested here were young, as they were weaned onto the high fat diet. Unfortunately, due to time constraints, it was not possible to measure 11β-HSD1 mRNA levels in adipose tissue of GR-5 mice and it will be important to investigate this in the future. However, evidence from male $GR^{+/-}$ mice has shown unaltered levels of 11β-HSD1 mRNA in all adipose tissues (Michailidou, Carter et al. 2008). Reduced GC levels in adipose tissue, through transgenic expression of 11β-HSD2 in adipose tissue (aP2-11β-HSD2 mice), produced a phenotype with similarities to GR-5 mice in resistance to weight gain, reduced fat accumulation and increased energy expenditure (Kershaw, Morton et al. 2005). However, energy expenditure of GR-5 has not been investigated in the experiments reported here and will be needed in future studies. Taking into account that only male aP2-11β-HSD2 mice were reported (Kershaw, Morton et al. 2005). However, both sexes of aP2-11β-HSD1 transgenic mice are obese and hyperphagic, with elevated adipose corticosterone levels but normal plasma concentrations (Masuzaki, Paterson et al. 2001). In our study, only female mice exhibited the reduction in weight, suggesting that differential regulation of adipose GC action in males and females, with GC levels more important in males and GR density more important in females, where circulating GC levels are higher (Kitay 1961). Taken together, the findings suggest that the effect of GR density on adipose GC action can be over-ridden by increased 11β-HSD1 activity levels which affect adiposity in both sexes.

Obviously, there are limitations to the experiments reported here. The dietary fat manipulation experiments were only carried out in males yet the effect of altered GR density was only seen in females. The dietary studies (Study 1 and 2) were carried out on males to avoid complications due to the effects of the oestrus cycle in females. Sex steroids, especially oestrogen, may play an important role in adipose

tissue accumulation. It is well know that prior to menopause, women carry more fat in peripheral depots (gynoid distribution) whereas men carry more fat viscerally (android distribution). However, after menopause women accumulate more fat in the abdominal and visceral regions, becoming more android-like in the fat distribution (Shi, Seeley et al. 2009). The effect of GR density in the studies reported here related to relatively young and reproductively viable females, which may mirror the situation in humans prior to menopause and suggest that oestrogen may be an important factor in the phenotype. Sex hormones have a considerable influence on adipose accumulation and the development of metabolic syndrome. Various mouse models have shown that sex steroids have significant influences on fat distribution. Visceral obesity and insulin resistance were observed in estrogen receptor-α knockout mice (αERKO) and in ArKO mice deficient in aromatase, the enzyme that produces estradiol from its precursor testosterone (Heine, Taylor et al. 2000; Jones, Thorburn et al. 2000). On the other hand, mice that lack the nuclear receptor co-repressor, Receptor Interacting Protein 140 (RIP140) in which estrogen action is increased, shown a lean phenotype and resistance to diet-induced obesity (Leonardsson, Steel et al. 2004). It is possible that GC and oestrogen pathways interact in a sex-specific manner to regulate obesity. At one level, the effect of sex upon the HPA axis is well known, with a higher level of HPA activity in females. It is also possible that these pathways interact locally, within adipose tissue to regulate adipocyte function and adipose tissue metabolism. This will be an important area to investigate in the future and the studies described in this thesis may contribute to that.

6 Publications from thesis

Tak Yung Man, Lynne Ramage, Adnan Gokcel, Zoi Michailidou, Christopher J. Kenyon, Karen Chapman, Jonathan R. Seckl and Nicholas M. Morton. Dietary Regulation of Peripheral Glucocorticoid Action: Comparison of Saturated and Unsaturated Fats
Abstracts, British Endocrine Society, Glasgow 2006

Tak Yung Man, Lynne Ramage, Adnan Gokcel, Zoi Michailidou, Christopher J. Kenyon, Karen Chapman, Jonathan R. Seckl and Nicholas M. Morton. Saturated Fat Elevates and Unsaturated Fats Down-Regulate Adipose Tissue Glucocorticoid Re-amplification
Abstracts, The Endocrine Society, Boston 2006

Elaine Marshall and **Tak Yung Man**, Nicholas Morton, Christopher Kenyon, Jonathan Seckl and Karen Chapman. Manipulation of the adipocyte glucocorticoid receptor in the development of obesity, hypertension and the metabolic syndrome *Abstract*, Centre for cardiovascular Sciences annual symposium day, Edinburgh UK 2006

Tak Yung Man, Elaine Marshall, Christopher J. Kenyon, Nicholas M. Morton, Jonathan R. Seckl and Karen Chapman. Effect of GR density in adipose tissue in the development of metabolic syndrome and obesity *Abstract*, Centre for cardiovascular Sciences annual symposium day, Edinburgh UK 2007

Elaine Marshall, **Tak Yung Man**, Karen Chapman, Chris Kenyon, Nik M. Morton and Jonathan Seckl. Adipose GR knockdown protects against obesity in female mice Oral, presentation British Endocrine Society, Harrogate 2008

Appendix A: Address of suppliers

Amersham Pharmacia Biotech UK Ltd
Amersham Place, Little Chalfont, Bucks, UK, HP7 9NA

Anachem Ltd
Anachem House, 20 Charles Street, Luton Bedfordshire, UK, LU2 0EB

Applied Biosystems
7 Kingsland Grange, Woolston, Warrington, Cheshire, UK WA1 4SR

BDH Merck Ltd
VWR International LTD, Merck House, Poole, Dorset, UK, BH15 1TD

Becton, Dickinson and company
The Danby Building, Edmund Halley Road, Oxford Science Park, Oxford, Oxfordshire, UK, OX4 4DQ

Bio-Rad Laboratories Ltd
Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire, UK, HP2 7DX

Cambrex Corporation
One Meadowlands Plaza, East Rutherford, NJ 07073 USA

Cambridge BioScience Ltd
24-25 Signet Court, Newmarket Road, Cambridge, UK, CB5 8LA

Fisher Scientific UK Ltd
Bishop Meadow Road, Loughborough, Leicestershire, UK, LE11 5RG

Hayman Ltd
Eastways Park, Witham, Essex, UK, CM8 3YE

Invitrogen Life Technologies
Ichinnan Bussiness Park, 3 Fountain drive, Paisley, UK, PA4 9RF

Promega Corporation
Delta House, Southampton Science Park, Southampton, Hampshire, UK, SO16 7NS

PerkinElmer
Chalfont Road, Seer Green, Beaconsfield, Bucks, UK, HP9 2FX

Qiagen Ltd
Qiagen house, Fleming way, Crawley, West Sussex, UK, RH10 9NQ

Research Diets, Inc.
20 Jules Lane, New Brunswick, NJ, USA, 08901

Roche Diagnostic Ltd
Charles Avenue, Burgess Hill, West Sussex, UK RH15 9RY

Sigma-Aldrich Company Ltd
The Old Brickyard, New Road, Gillingham, Dorset, UK, SP8 4XT

Santa Cruz Biotechnology, Inc.
2145 Delaware Avenue, Santa Cruz, California, 95060, U.S.A.

Thermo Fisher Scientific Inc.
81 Wyman Street, Waltham, MA, USA 02454

7 Reference

Adams, M., O. C. Meijer, et al. (2003). "Homodimerization of the glucocorticoid receptor is not essential for response element binding: activation of the phenylethanolamine N-methyltransferase gene by dimerization-defective mutants." Mol Endocrinol **17**(12): 2583-2592.

Agarwal, A. K., C. Monder, et al. (1989). "Cloning and expression of rat cDNA encoding corticosteroid 11 beta-dehydrogenase." J Biol Chem **264**(32): 18939-43.

Agarwal, A. K., F. M. Rogerson, et al. (1995). "Analysis of the human gene encoding the kidney isozyme of 11 beta-hydroxysteroid dehydrogenase." J Steroid Biochem Mol Biol **55**(5-6): 473-9.

Akabayashi, A., Y. Watanabe, et al. (1994). "Hypothalamic neuropeptide Y, its gene expression and receptor activity: relation to circulating corticosterone in adrenalectomized rats." Brain Res **665**(2): 201-12.

Akompong, T., R. L. Spencer, et al. (1993). "Glucocorticoids inhibit soluble phospholipase C activity and cytosolic guanine nucleotide regulatory protein-alpha i immunoreactivity in spleen." Endocrinology **133**(5): 1963-70.

Albeck, D. S., N. B. Hastings, et al. (1994). "Effects of adrenalectomy and type I or type II glucocorticoid receptor activation on AVP and CRH mRNA in the rat hypothalamus." Brain Res Mol Brain Res **26**(1-2): 129-34.

Angulo, J. A. and B. S. McEwen (1994). "Molecular aspects of neuropeptide regulation and function in the corpus striatum and nucleus accumbens." Brain Res Brain Res Rev **19**(1): 1-28.

Antoni, F. A., M. C. Holmes, et al. (1985). "Pituitary binding of vasopressin is altered by experimental manipulations of the hypothalamo-pituitary-adrenocortical axis in normal as well as homozygous (di/di) Brattleboro rats." Endocrinology **117**(4): 1293-9.

Atanasov, A. G., L. G. Nashev, et al. (2008). "Direct protein-protein interaction of 11β-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase in the endoplasmic reticulum lumen." Biochim Biophys Acta **1783**(8): 1536-1543.

Balachandran, A., H. Guan, et al. (2008). "Insulin and dexamethasone dynamically regulate adipocyte 11beta-hydroxysteroid dehydrogenase type 1." Endocrinology **149**(8): 4069-79.

Barish, G. D. (2006). "Peroxisome proliferator-activated receptors and liver X receptors in atherosclerosis and immunity." J Nutr **136**(3): 690-4.

Bauer, A., F. Tronche, et al. (1999). "The glucocorticoid receptor is required for stress erythropoiesis." Genes & Development **13**(22): 2996-3002.

Bayo, P., A. Sanchis, et al. (2008). "Glucocorticoid receptor is required for skin barrier competence." Endocrinology **149**(3): 1377-88.

Beaven, S. W. and P. Tontonoz (2006). "Nuclear receptors in lipid metabolism: targeting the heart of dyslipidemia." Annu Rev Med **57**: 313-29.

Beckford, U., M. C. Holmes, et al. (1983). "Biphasic inhibition of bioactive hypothalamic corticotrophin releasing factor secretion in vitro by

corticosterone and prevention of the second phase by various steroids." J Endocrinol **97**(3): 339-46.
Benediktsson, R., A. A. Calder, et al. (1997). "Placental 11 beta-hydroxysteroid dehydrogenase: a key regulator of fetal glucocorticoid exposure." Clin Endocrinol (Oxf) **46**(2): 161-6.
Bertalan, R., A. Patocs, et al. (2009). "The protective effect of the ER22/23EK polymorphism against an excessive weight gain during pregnancy." Gynecol Endocrinol: 1-4.
Bhattacharyya, S., D. E. Brown, et al. (2007). "Macrophage glucocorticoid receptors regulate Toll-like receptor 4-mediated inflammatory responses by selective inhibition of p38 MAP kinase." Blood **109**(10): 4313-9.
Bhattacharyya, S., D. E. Brown, et al. (2007). "Macrophage glucocorticoid receptors regulate Toll-like receptor-4-mediated inflammatory responses by selective inhibition of p38 MAP kinase." Blood **109**: 4313-4319.
Björntorp, P. and R. Rosmond (2000). "Obesity and cortisol." Nutrition **16**(10): 924-936.
Blanchard, D. C., R. L. Spencer, et al. (1995). "Visible burrow system as a model of chronic social stress: behavioral and neuroendocrine correlates." Psychoneuroendocrinology **20**(2): 117-34.
Bohn, M. C., B. McEwen, et al. (1984). "Development and characterization of glucocorticoid receptors in rat superior cervical ganglion." Brain Res **316**(2): 211-8.
Bookout, A. L., Y. Jeong, et al. (2006). "Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network." Cell **126**(4): 789-99.
Boyle, M. P., J. A. Brewer, et al. (2005). "Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior." Proc Natl Acad Sci U S A **102**(2): 473-8.
Boyle, M. P., B. J. Kolber, et al. (2006). "Forebrain glucocorticoid receptors modulate anxiety-associated locomotor activation and adrenal responsiveness." J Neurosci **26**(7): 1971-8.
Bradbury, M. J., S. F. Akana, et al. (1991). "Regulation of basal ACTH secretion by corticosterone is mediated by both type I (MR) and type II (GR) receptors in rat brain." J Steroid Biochem Mol Biol **40**(1-3): 133-42.
Bradley, U., M. Spence, et al. (2009). "Low-Fat versus Low-Carbohydrate Weight Reduction Diets: Effects on Weight Loss, Insulin Resistance and Cardiovascular Risk A Randomised Control Trial." Diabetes.
Brewer, J. A., O. Kanagawa, et al. (2002). "Thymocyte apoptosis induced by T cell activation is mediated by glucocorticoids in vivo." J Immunol **169**(4): 1837-43.
Brewer, J. A., B. Khor, et al. (2003). "T-cell glucocorticoid receptor is required to suppress COX-2-mediated lethal immune activation." Nat Med **9**(10): 1318-22.
Brouwer, J. P., B. C. Appelhof, et al. (2006). "Prediction of treatment response by HPA-axis and glucocorticoid receptor polymorphisms in major depression." Psychoneuroendocrinology **31**(10): 1154-63.

Brown, R. W., K. E. Chapman, et al. (1996). "Purification of 11 beta-hydroxysteroid dehydrogenase type 2 from human placenta utilizing a novel affinity labelling technique." Biochem J **313 (Pt 3)**: 997-1005.

Brown, R. W., R. Diaz, et al. (1996). "The ontogeny of 11 beta-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development." Endocrinology **137**(2): 794-7.

Buemann, B., M. C. Vohl, et al. (1997). "Abdominal visceral fat is associated with a BclI restriction fragment length polymorphism at the glucocorticoid receptor gene locus." Obes Res **5**(3): 186-192.

Bujalska, I. J., L. L. Gathercole, et al. (2008). "A novel selective 11beta-hydroxysteroid dehydrogenase type 1 inhibitor prevents human adipogenesis." J Endocrinol **197**(2): 297-307.

Burant, C. F., S. Sreenan, et al. (1997). "Troglitazone action is independent of adipose tissue." J Clin Invest **100**(11): 2900-8.

Buren, J., Y. C. Lai, et al. (2008). "Insulin action and signalling in fat and muscle from dexamethasone-treated rats." Arch Biochem Biophys **474**(1): 91-101.

Buren, J., H. X. Liu, et al. (2002). "Dexamethasone impairs insulin signalling and glucose transport by depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase and protein kinase B in primary cultured rat adipocytes." Eur J Endocrinol **146**(3): 419-29.

Carter, R., J. M. Paterson, et al. (2009). "Hypothalamic-pituitary-adrenal axis abnormalities in response to deletion of 11ss-HSD1 is strain dependent." J Neuroendocrinol.

Chao, H. M., P. H. Choo, et al. (1989). "Glucocorticoid and mineralocorticoid receptor mRNA expression in rat brain." Neuroendocrinology **50**(4): 365-71.

Chao, H. M., L. Y. Ma, et al. (1998). "Regulation of glucocorticoid receptor and mineralocorticoid receptor messenger ribonucleic acids by selective agonists in the rat hippocampus." Endocrinology **139**(4): 1810-4.

Chao, H. M. and B. S. McEwen (1994). "Glucocorticoids and the expression of mRNAs for neurotrophins, their receptors and GAP-43 in the rat hippocampus." Brain Res Mol Brain Res **26**(1-2): 271-6.

Chao, H. M., R. R. Sakai, et al. (1998). "Adrenal steroid regulation of neurotrophic factor expression in the rat hippocampus." Endocrinology **139**(7): 3112-8.

Chapman, K. E., A. E. Coutinho, et al. (2009). "The role and regulation of 11beta-hydroxysteroid dehydrogenase type 1 in the inflammatory response." Mol Cell Endocrinol **301**(1-2): 123-31.

Charmandari, E., T. Kino, et al. (2006). "Functional characterization of the natural human glucocorticoid receptor (hGR) mutants hGRalphaR477H and hGRalphaG679S associated with generalized glucocorticoid resistance." J Clin Endocrinol Metab **91**(4): 1535-43.

Charmandari, E., A. Raji, et al. (2005). "A novel point mutation in the ligand-binding domain (LBD) of the human glucocorticoid receptor (hGR) causing generalized glucocorticoid resistance: the importance of the C terminus of hGR LBD in conferring transactivational activity." J Clin Endocrinol Metab **90**(6): 3696-705.

Chebotaev, D., A. Yemelyanov, et al. (2006). "The tumor suppressor effect of the glucocorticoid receptor in skin is mediated via its effect on follicular epithelial stem cells." Oncogene.

Christopher, M. M., J. G. White, et al. (1990). "Erythrocyte pathology and mechanisms of Heinz body-mediated hemolysis in cats." Vet Pathol **27**(5): 299-310.

Coirini, H., A. M. Magarinos, et al. (1985). "Further studies of brain aldosterone binding sites employing new mineralocorticoid and glucocorticoid receptor markers in vitro." Brain Res **361**(1-2): 212-6.

Cole, T. G., H. G. Wilcox, et al. (1982). "Effects of adrenalectomy and dexamethasone on hepatic lipid metabolism." J Lipid Res **23**(1): 81-91.

Cole, T. J., J. A. Blendy, et al. (1995). "Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation." Genes Dev **9**(13): 1608-21.

Cole, T. J., K. Myles, et al. (2001). "GRKO mice express an aberrant dexamethasone-binding glucocorticoid receptor, but are profoundly glucocorticoid resistant." Mol Cell Endocrinol **173**(1-2): 193-202.

Cole, T. J., K. Myles, et al. (2001). "GRKO mice express an aberrant dexamethasone-binding glucocorticoid receptor, but are profoundly glucocorticoid resistant." Mol Cell Endocrinol **173**(1-2): 193-202.

Cole, T. J., N. M. Solomon, et al. (2004). "Altered epithelial cell proportions in the fetal lung of glucocorticoid receptor null mice." Am J Respir Cell Mol Biol **30**(5): 613-619.

Conrad, C. D., S. J. Lupien, et al. (1999). "Support for a bimodal role for type II adrenal steroid receptors in spatial memory." Neurobiol Learn Mem **72**(1): 39-46.

Corvol, H., N. Nathan, et al. (2007). "Glucocorticoid receptor gene polymorphisms associated with progression of lung disease in young patients with cystic fibrosis." Respir Res **8**(1): 88.

Cottrell, E. C. and J. R. Seckl (2009). "Prenatal stress, glucocorticoids and the programming of adult disease." Front Behav Neurosci **3**: 19.

Dallman, M. F. (2009). "Stress-induced obesity and the emotional nervous system." Trends Endocrinol Metab.

Dallman, M. F., S. F. Akana, et al. (1995). "The neural network that regulates energy balance is responsive to glucocorticoids and insulin and also regulates HPA axis responsivity at a site proximal to CRF neurons." Ann N Y Acad Sci **771**: 730-42.

Dallman, M. F., S. E. la Fleur, et al. (2004). "Minireview: glucocorticoids--food intake, abdominal obesity, and wealthy nations in 2004." Endocrinology **145**(6): 2633-8.

Dallman, M. F., N. C. Pecoraro, et al. (2005). "Chronic stress and comfort foods: self-medication and abdominal obesity." Brain Behav Immun **19**(4): 275-80.

Dalman, F. C., E. R. Sanchez, et al. (1988). "Localization of phosphorylation sites with respect to the functional domains of the mouse L cell glucocorticoid receptor." J Biol Chem **263**(25): 12259-67.

Datson, N. A., M. C. Morsink, et al. (2008). "Central corticosteroid actions: Search for gene targets." Eur J Pharmacol **583**(2-3): 272-89.

De Sousa Peixoto, R. A., S. Turban, et al. (2008). "Preadipocyte 11beta-hydroxysteroid dehydrogenase type 1 is a keto-reductase and contributes to diet-induced visceral obesity in vivo." Endocrinology **149**(4): 1861-8.

De Kloet, E. R. and R. Derijk (2004). "Signaling pathways in brain involved in predisposition and pathogenesis of stress-related disease: genetic and kinetic factors affecting the MR/GR balance." Ann N Y Acad Sci **1032**: 14-34.

De Kloet, E. R. and B. S. McEwen (1976). "A putative glucocorticoid receptor and a transcortin-like macromolecule in pituitary cytosol." Biochim Biophys Acta **421**(1): 115-23.

De Kloet, E. R. and B. S. McEwen (1976). "Differences between cytosol receptor complexes with corticosterone and dexamethasone in hippocampal tissue from rat brain." Biochim Biophys Acta **421**(1): 124-32.

Delaunay, F., A. Khan, et al. (1997). "Pancreatic ß cells are important targets for the diabetogenic effects of glucocorticoids." J Clin Invest **100**: 2094-2098.

Delaunay, F., A. Khan, et al. (1997). "Pancreatic beta cells are important targets for the diabetogenic effects of glucocorticoids." J Clin Invest **100**(8): 2094-8.

Derijk, R. and E. R. de Kloet (2005). "Corticosteroid receptor genetic polymorphisms and stress responsivity." Endocrine **28**(3): 263-270.

Derijk, R. H. and E. R. de Kloet (2008). "Corticosteroid receptor polymorphisms: determinants of vulnerability and resilience." Eur J Pharmacol **583**(2-3): 303-11.

DeRijk, R. H., M. Schaaf, et al. (2002). "Glucocorticoid receptor variants: clinical implications." J Steroid Biochem Mol Biol **81**(2): 103-22.

Derijk, R. H., M. J. Schaaf, et al. (2001). "A human glucocorticoid receptor gene variant that increases the stability of the glucocorticoid receptor beta-isoform mRNA is associated with rheumatoid arthritis." J Rheumatol **28**(11): 2383-8.

Dhabhar, F. S., B. S. McEwen, et al. (1993). "Stress response, adrenal steroid receptor levels and corticosteroid-binding globulin levels--a comparison between Sprague-Dawley, Fischer 344 and Lewis rats." Brain Res **616**(1-2): 89-98.

Dhabhar, F. S., A. H. Miller, et al. (1995). "Differential activation of adrenal steroid receptors in neural and immune tissues of Sprague Dawley, Fischer 344, and Lewis rats." J Neuroimmunol **56**(1): 77-90.

Dhabhar, F. S., A. H. Miller, et al. (1996). "Stress-induced changes in blood leukocyte distribution. Role of adrenal steroid hormones." J Immunol **157**(4): 1638-44.

Diaz, R., R. W. Brown, et al. (1998). "Distinct ontogeny of glucocorticoid and mineralocorticoid receptor and 11beta-hydroxysteroid dehydrogenase types I and II mRNAs in the fetal rat brain suggest a complex control of glucocorticoid actions." J Neurosci **18**(7): 2570-80.

Dong, Q., A. Salva, et al. (2004). "Rapid glucocorticoid mediation of suppressed testosterone biosynthesis in male mice subjected to immobilization stress." J Androl **25**(6): 973-81.

Donn, R., D. Payne, et al. (2007). "Glucocorticoid receptor gene polymorphisms and susceptibility to rheumatoid arthritis." Clin Endocrinol (Oxf).

Drake, A. J., D. E. Livingstone, et al. (2005). "Reduced adipose glucocorticoid reactivation and increased hepatic glucocorticoid clearance as an early adaptation to high-fat feeding in Wistar rats." Endocrinology **146**(2): 913-9.

Duplus, E., M. Glorian, et al. (2000). "Fatty acid regulation of gene transcription." J Biol Chem **275**(40): 30749-52.
Dussault, I. and B. M. Forman (2000). "Prostaglandins and fatty acids regulate transcriptional signaling via the peroxisome proliferator activated receptor nuclear receptors." Prostaglandins Other Lipid Mediat **62**(1): 1-13.
Ehrchen, J., L. Steinmuller, et al. (2007). "Glucocorticoids induce differentiation of a specifically activated, anti-inflammatory subtype of human monocytes." Blood **109**(3): 1265-1274.
Erdmann, G., S. Berger, et al. (2008). "Genetic dissection of glucocorticoid receptor function in the mouse brain." J Neuroendocrinol **20**(6): 655-9.
Felt, B. T., R. M. Sapolsky, et al. (1984). "Regulation of hippocampal corticosterone receptors by a vasopressin analogue." Peptides **5**(6): 1225-7.
Fievet, C. and B. Staels (2009). "Liver X receptor modulators: effects on lipid metabolism and potential use in the treatment of atherosclerosis." Biochem Pharmacol **77**(8): 1316-27.
Finken, M. J. J., I. Meulenbelt, et al. (2007). "The 23K Variant of the R23K Polymorphism in the Glucocorticoid Receptor Gene Protects Against Postnatal Growth Failure and Insulin Resistance After Preterm Birth." J Clin Endocrinol Metab %R 10.1210/jc.2007-1290: jc.2007-1290.
Finotto, S., K. Krieglstein, et al. (1999). "Analysis of mice carrying targeted mutations of the glucocorticoid receptor gene argues against an essential role of glucocorticoid signalling for generating adrenal chromaffin cells." Development **126**(13): 2935-44.
Fleury, I., P. Beaulieu, et al. (2003). "Characterization of the BclI polymorphism in the glucocorticoid receptor gene." Clin Chem **49**(9): 1528-31.
Franchimont, D. (2004). "Overview of the actions of glucocorticoids on the immune response: a good model to characterize new pathways of immunosuppression for new treatment strategies." Ann N Y Acad Sci **1024**: 124-37.
Franchimont, D., J. Galon, et al. (2000). "Inhibition of Th1 immune response by glucocorticoids: dexamethasone selectively inhibits IL-12-induced Stat4 phosphorylation in T lymphocytes." J Immunol **164**(4): 1768-74.
Freeman, A. I., H. L. Munn, et al. (2004). "Glucocorticoid down-regulation of rat glucocorticoid receptor does not involve differential promoter regulation." J Endocrinol **183**(2): 365-74.
Friedman, J. M. (2000). "Obesity in the new millennium." Nature **404**(6778): 632-4.
Fujikawa, T., H. Soya, et al. (2000). "A biphasic regulation of receptor mRNA expressions for growth hormone, glucocorticoid and mineralocorticoid in the rat dentate gyrus during acute stress." Brain Res **874**(2): 186-93.
Fujioka, S., Y. Matsuzawa, et al. (1987). "Contribution of intra-abdominal fat accumulation to the impairment of glucose and lipid metabolism in human obesity." Metabolism **36**(1): 54-9.
Funder, J. W., P. T. Pearce, et al. (1990). "Apparent mineralocorticoid excess, pseudohypoaldosteronism, and urinary electrolyte excretion: toward a redefinition of mineralocorticoid action." FASEB J **4**(14): 3234-8.
Gagnon, S., W. Atmodjo, et al. (2006). "Transgenic glucocorticoid receptor expression driven by the SP-C promoter reduces neonatal lung cellularity and midkine expression in GRhypo mice." Biol Neonate **90**(1): 46-57.

Galon, J., D. Franchimont, et al. (2002). "Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells." FASEB J **16**(1): 61-71.

Gannon, M. N., R. L. Spencer, et al. (1990). "Pharmacological characterization of type II glucocorticoid binding sites in AtT20 pituitary cell culture." J Steroid Biochem **36**(1-2): 83-8.

Garbrecht, M. R., J. M. Klein, et al. (2006). "Glucocorticoid metabolism in the human fetal lung: implications for lung development and the pulmonary surfactant system." Biol Neonate **89**(2): 109-19.

Gathercole, L. L., I. J. Bujalska, et al. (2007). "Glucocorticoid modulation of insulin signaling in human subcutaneous adipose tissue." J Clin Endocrinol Metab **92**(11): 4332-9.

Giguere, V., S. M. Hollenberg, et al. (1986). "Functional domains of the human glucocorticoid receptor." Cell **46**(5): 645-52.

Gilmour, J. S., A. E. Coutinho, et al. (2006). "Local amplification of glucocorticoids by 11 beta-hydroxysteroid dehydrogenase type 1 promotes macrophage phagocytosis of apoptotic leukocytes." J Immunol **176**(12): 7605-11.

Goedecke, J. H., D. J. Wake, et al. (2006). "Glucocorticoid metabolism within superficial subcutaneous rather than visceral adipose tissue is associated with features of the metabolic syndrome in South African women." Clin Endocrinol (Oxf) **65**(1): 81-7.

Gorospe, J. R., S. Naidu, et al. (2002). "Molecular findings in symptomatic and pre-symptomatic Alexander disease patients." Neurology **58**(10): 1494-500.

Graves, C. W., S. T. Philips, et al. (2008). "Use of a cryptic splice site for the expression of huntingtin interacting protein 1 in select normal and neoplastic tissues." Cancer Res **68**(4): 1064-73.

Graves, R. A., P. Tontonoz, et al. (1992). "Identification of a fat cell enhancer: analysis of requirements for adipose tissue-specific gene expression." J Cell Biochem **49**(3): 219-24.

Graves, R. A., P. Tontonoz, et al. (1991). "Identification of a potent adipocyte-specific enhancer: involvement of an NF-1-like factor." Genes Dev **5**(3): 428-37.

Grillo, C., S. Vallee, et al. (1990). "Properties and distribution of binding sites for the mineralocorticoid receptor antagonist [3H]ZK 91587 in brain." J Steroid Biochem **35**(1): 11-5.

Grillo, C., S. M. Vallee, et al. (1992). "Transformation and nuclear translocation of brain type L corticosteroid receptors complexed with the mineralocorticoid antagonist ZK 91587, aldosterone or dexamethasone." J Steroid Biochem Mol Biol **42**(5): 515-20.

Grose, R., S. Werner, et al. (2002). "A role for endogenous glucocorticoids in wound repair." EMBO Rep **3**(6): 575-82.

Grossman, R., R. Yehuda, et al. (2006). "Cognitive effects of intravenous hydrocortisone in subjects with PTSD and healthy control subjects." Ann N Y Acad Sci **1071**: 410-21.

Grundy, S. M. (1998). "Cholesterol management in high-risk patients without heart disease. When is lipid-lowering medication warranted for primary prevention?" Postgrad Med **104**(5): 117-20, 123-4, 129.

Gur, S., P. J. Kadowitz, et al. (2007). "Purinergic (P2) receptor control of lower genitourinary tract function and new avenues for drug action: an overview." Curr Pharm Des **13**(31): 3236-44.

Hache, R. J., R. Tse, et al. (1999). "Nucleocytoplasmic trafficking of steroid-free glucocorticoid receptor." J Biol Chem **274**(3): 1432-9.

Hahn, T., S. Barth, et al. (1999). "Placental glucose transporter expression is regulated by glucocorticoids." J Clin Endocrinol Metab **84**(4): 1445-1452.

Hanson, R. W. and L. Reshef (1997). "Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression." Annu Rev Biochem **66**: 581-611.

Hansson, G. K. and P. Libby (2006). "The immune response in atherosclerosis: a double-edged sword." Nat Rev Immunol **6**(7): 508-19.

Hardy, R., E. H. Rabbitt, et al. (2008). "Local and systemic glucocorticoid metabolism in inflammatory arthritis." Ann Rheum Dis **67**(9): 1204-10.

Hardy, R. S., A. Filer, et al. (2006). "Differential expression, function and response to inflammatory stimuli of 11beta-hydroxysteroid dehydrogenase type 1 in human fibroblasts: a mechanism for tissue-specific regulation of inflammation." Arthritis Res Ther **8**(4): R108.

Harris, H., R. Henderson, et al. (2001). "Regulation of metallothionein II messenger ribonucleic acid measures exogenous estrogen receptor-beta activity in SAOS-2 and LNCaPLN3 cells." Endocrinology **142**(2): 645-52.

Harris, H. J., Y. Kotelevtsev, et al. (2001). "Intracellular regeneration of glucocorticoids by 11beta-hydroxysteroid dehydrogenase (11beta-HSD)-1 plays a key role in regulation of the hypothalamic-pituitary-adrenal axis: analysis of 11beta-HSD-1-deficient mice." Endocrinology **142**(1): 114-20.

Harvey, B. H., B. S. McEwen, et al. (2003). "Neurobiology of antidepressant withdrawal: implications for the longitudinal outcome of depression." Biol Psychiatry **54**(10): 1105-17.

Hastings, N. B., M. Orchinik, et al. (1999). "Pharmacological characterization of central and peripheral type I and type II adrenal steroid receptors in the prairie vole, a glucocorticoid-resistant rodent." Endocrinology **140**(10): 4459-69.

Hermanowski-Vosatka, A., J. M. Balkovec, et al. (2005). "11beta-HSD1 inhibition ameliorates metabolic syndrome and prevents progression of atherosclerosis in mice." J Exp Med **202**(4): 517-27.

Hill, M. N. and B. S. McEwen (2009). "Endocannabinoids: The silent partner of glucocorticoids in the synapse." Proc Natl Acad Sci U S A **106**(12): 4579-80.

Hoeck, W. and B. Groner (1990). "Hormone-dependent phosphorylation of the glucocorticoid receptor occurs mainly in the amino-terminal transactivation domain." J Biol Chem **265**(10): 5403-8.

Holmes, M. C., K. L. French, et al. (1995). "Modulation of serotonin and corticosteroid receptor gene expression in the rat hippocampus with circadian rhythm and stress." Brain Res Mol Brain Res **28**(2): 186-92.

Holmes, M. C., K. L. French, et al. (1997). "Dysregulation of diurnal rhythms of serotonin 5-HT2C and corticosteroid receptor gene expression in the hippocampus with food restriction and glucocorticoids." J Neurosci **17**(11): 4056-65.

Holmes, M. C., Y. Kotelevtsev, et al. (2001). "Phenotypic analysis of mice bearing targeted deletions of 11beta-hydroxysteroid dehydrogenases 1 and 2 genes." Mol Cell Endocrinol **171**(1-2): 15-20.

Holmes, M. C., M. Sangra, et al. (2006). "11beta-Hydroxysteroid dehydrogenase type 2 protects the neonatal cerebellum from deleterious effects of glucocorticoids." Neuroscience **137**(3): 865-73.

Holmes, M. C. and J. R. Seckl (2006). "The role of 11beta-hydroxysteroid dehydrogenases in the brain." Mol Cell Endocrinol **248**(1-2): 9-14.

Horner, H. C., A. Munck, et al. (1987). "Dexamethasone causes translocation of glucose transporters from the plasma membrane to an intracellular site in human fibroblasts." J Biol Chem **262**(36): 17696-702.

Hotamisligil, G. S. (2006). "Inflammation and metabolic disorders." Nature **444**(7121): 860-7.

Hotamisligil, G. S. and E. Erbay (2008). "Nutrient sensing and inflammation in metabolic diseases." Nat Rev Immunol **8**(12): 923-34.

Howell, M. P. and L. J. Muglia (2006). "Effects of genetically altered brain glucocorticoid receptor action on behavior and adrenal axis regulation in mice." Front Neuroendocrinol **27**(3): 275-84.

Huizenga, N. A., J. W. Koper, et al. (1998). "A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids in vivo." J Clin Endocrinol Metab **83**(1): 144-51.

Hundertmark, S., V. Ragosch, et al. (1993). "11-beta-Hydroxysteroid dehydrogenase of rat lung: enzyme kinetic, oxidase-reductase ratio, electrolyte and trace element dependence." Enzyme Protein **47**(2): 83-91.

Hutchison, K. A., F. C. Dalman, et al. (1993). "Localization of the ~12 kDa Mr discrepancy in gel migration of the mouse glucocorticoid receptor to the major phosphorylated cyanogen bromide fragment in the transactivating domain." The Journal of Steroid Biochemistry and Molecular Biology **46**(6): 681-686.

Ikeda, Y., T. Suehiro, et al. (2001). "A polymorphism in the promoter region of the glucocorticoid receptor gene is associated with its transcriptional activity." Endocr J **48**(6): 723-6.

Irving, J. A., L. Bloodworth, et al. (2005). "Loss of heterozygosity in childhood acute lymphoblastic leukemia detected by genome-wide microarray single nucleotide polymorphism analysis." Cancer Res **65**(8): 3053-8.

Ishii, T., H. Masuzaki, et al. (2007). "Augmentation of 11β-hydroxysteroid dehydrogenase type 1 in LPS-activated J774.1 macrophages - role of 11β-HSD1 in pro-inflammatory properties in macrophages." FEBS Lett **581**(3): 349-54.

James, W. P., N. Rigby, et al. (2006). "Obesity and the metabolic syndrome: the stress on society." Ann N Y Acad Sci **1083**: 1-10.

Jang, C., V. R. Obeyesekere, et al. (2009). "Skeletal muscle 11beta hydroxysteroid dehydrogenase type 1 activity is upregulated following elective abdominal surgery." Eur J Endocrinol **160**(2): 249-55.

Jewell, C. M. and J. A. Cidlowski (2007). "Molecular Evidence for a Link between the N363S Glucocorticoid Receptor Polymorphism and Altered Gene Expression." J Clin Endocrinol Metab %R 10.1210/jc.2007-0642: jc.2007-0642.

Johnson, L. R., C. Farb, et al. (2005). "Localization of glucocorticoid receptors at postsynaptic membranes in the lateral amygdala." Neuroscience **136**(1): 289-99.

Jump, D. B. and S. D. Clarke (1999). "Regulation of gene expression by dietary fat." Annu Rev Nutr **19**: 63-90.

Justesen, J., L. Mosekilde, et al. (2004). "Mice deficient in 11beta-hydroxysteroid dehydrogenase type 1 lack bone marrow adipocytes, but maintain normal bone formation." Endocrinology **145**(4): 1916-25.

Juvet, L. K., S. M. Andresen, et al. (2003). "On the role of liver X receptors in lipid accumulation in adipocytes." Mol Endocrinol **17**(2): 172-82.

Karanth, S., A. C. Linthorst, et al. (1997). "Hypothalamic-pituitary-adrenocortical axis changes in a transgenic mouse with impaired glucocorticoid receptor function." Endocrinology **138**(8): 3476-85.

Kasai, Y. (1990). "Two naturally-occurring isoforms and their expression of a glucocorticoid receptor gene from an androgen-dependent mouse tumor." FEBS Lett **274**(1-2): 99-102.

Kellendonk, C., S. Eiden, et al. (2002). "Inactivation of the GR in the nervous system affects energy accumulation." Endocrinology **143**(6): 2333-40.

Kershaw, E. E., N. M. Morton, et al. (2005). "Adipocyte-specific glucocorticoid inactivation protects against diet-induced obesity." Diabetes **54**(4): 1023-31.

Kim, D. H., G. S. Kim, et al. (2008). "Functional conservation of the glutamine-rich domains of yeast Gal11 and human SRC-1 in the transactivation of glucocorticoid receptor Tau 1 in Saccharomyces cerevisiae." Mol Cell Biol **28**(3): 913-25.

Kim, J. J. and J. Haller (2007). "Glucocorticoid hyper- and hypofunction: stress effects on cognition and aggression." Ann N Y Acad Sci **1113**: 291-303.

King, L. B., M. S. Vacchio, et al. (1995). "A targeted glucocorticoid receptor antisense transgene increases thymocyte apoptosis and alters thymocyte development." Immunity **3**(5): 647-56.

Kino, T. and G. P. Chrousos (2004). "Glucocorticoid and mineralocorticoid receptors and associated diseases." Essays Biochem **40**: 137-55.

Kino, T., A. Vottero, et al. (2002). "Familial/sporadic glucocorticoid resistance syndrome and hypertension." Ann N Y Acad Sci **970**: 101-11.

Kitay, J. I. (1961). "Sex differences in adrenal cortical secretion in the rat." Endocrinology **68**: 818-24.

Koeijvoets, K. C., J. B. van der Net, et al. (2008). "Two common haplotypes of the glucocorticoid receptor gene are associated with increased susceptibility to cardiovascular disease in men with familial hypercholesterolemia." J Clin Endocrinol Metab **93**(12): 4902-8.

Koeijvoets, K. C., J. B. van der Net, et al. (2008). "Two common haplotypes of the glucocorticoid receptor gene are associated with increased susceptibility to cardiovascular disease in men with familial hypercholesterolemia." J Clin Endocrinol Metab.

Kolber, B. J. and L. J. Muglia (2009). "Defining brain region-specific glucocorticoid action during stress by conditional gene disruption in mice." Brain Res.

Kolber, B. J., L. Wieczorek, et al. (2008). "Hypothalamic-pituitary-adrenal axis dysregulation and behavioral analysis of mouse mutants with altered glucocorticoid or mineralocorticoid receptor function." Stress **11**(5): 321-38.

Kopecky, J., Z. Hodny, et al. (1996). "Reduction of dietary obesity in aP2-Ucp transgenic mice: physiology and adipose tissue distribution." Am J Physiol **270**(5 Pt 1): E768-75.

Kopecky, J., M. Rossmeisl, et al. (2001). "Mitochondrial uncoupling and lipid metabolism in adipocytes." Biochem Soc Trans **29**(Pt 6): 791-7.

Kopecky, J., M. Rossmeisl, et al. (2004). "Energy metabolism of adipose tissue--physiological aspects and target in obesity treatment." Physiol Res **53 Suppl 1**: S225-32.

Kopecky, J., M. Rossmeisl, et al. (1996). "Reduction of dietary obesity in aP2-Ucp transgenic mice: mechanism and adipose tissue morphology." Am J Physiol **270**(5 Pt 1): E776-86.

Koper, J. W., R. P. Stolk, et al. (1997). "Lack of association between five polymorphisms in the human glucocorticoid receptor gene and glucocorticoid resistance." Hum Genet **99**(5): 663-8.

Kotelevtsev, Y., M. C. Holmes, et al. (1997). "11beta-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress." Proc Natl Acad Sci U S A **94**(26): 14924-9.

Koyano, S., Y. Saito, et al. (2005). "Novel genetic polymorphisms in the NR3C1 (glucocorticoid receptor) gene in a Japanese population." Drug Metab Pharmacokinet **20**(1): 79-84.

Kronenberg, G., I. Kirste, et al. (2009). "Reduced hippocampal neurogenesis in the GR(+/-) genetic mouse model of depression." Eur Arch Psychiatry Clin Neurosci **259**(8): 499-504.

Kronenberg, G., I. Kirste, et al. (2009). "Reduced hippocampal neurogenesis in the GR(+/-) genetic mouse model of depression." Eur Arch Psychiatry Clin Neurosci.

Kumsta, R., S. Entringer, et al. (2009). "Working Memory Performance Is Associated with Common Glucocorticoid Receptor Gene Polymorphisms." Neuropsychobiology **61**(1): 49-56.

Kuningas, M., R. H. de Rijk, et al. (2006). "Mental Performance in Old Age Dependent on Cortisol and Genetic Variance in the Mineralocorticoid and Glucocorticoid Receptors." Neuropsychopharmacology.

Kuroda, Y., Y. Watanabe, et al. (1994). "Effects of adrenalectomy and type I or type II glucocorticoid receptor activation on 5-HT1A and 5-HT2 receptor binding and 5-HT transporter mRNA expression in rat brain." Brain Res **648**(1): 157-61.

Labreuche, J., P. J. Touboul, et al. (2009). "Plasma triglyceride levels and risk of stroke and carotid atherosclerosis: a systematic review of the epidemiological studies." Atherosclerosis **203**(2): 331-45.

Lai, M., K. Horsburgh, et al. (2007). "Forebrain mineralocorticoid receptor overexpression enhances memory, reduces anxiety and attenuates neuronal loss in cerebral ischaemia." Eur J Neurosci **25**(6): 1832-42.

Le Mevel, J. C., S. Abitbol, et al. (1979). "Temporal changes in plasma adrenocorticotropin concentration after repeated neurotropic stress in male and female rats." Endocrinology **105**(3): 812-7.

Lee, E. B., J. Y. Kim, et al. (2005). "Glucocorticoid receptor polymorphisms in Korean patients with rheumatoid arthritis." Ann Rheum Dis **64**(3): 503-4.

Lee, J. Y., K. H. Sohn, et al. (2001). "Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4." J Biol Chem **276**(20): 16683-9.

Leite, J. O., R. DeOgburn, et al. (2009). "Low-carbohydrate diet disrupts the association between insulin resistance and weight gain." Metabolism **58**(8): 1116-22.

Levitt, N. S., R. S. Lindsay, et al. (1996). "Dexamethasone in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in the adult offspring in the rat." Neuroendocrinology **64**(6): 412-8.

Lindsay, R. S., R. M. Lindsay, et al. (1996). "Inhibition of 11-beta-hydroxysteroid dehydrogenase in pregnant rats and the programming of blood pressure in the offspring." Hypertension **27**(6): 1200-4.

Linthorst, A. C., S. Karanth, et al. (1999). "Impaired glucocorticoid receptor function evolves in aberrant physiological responses to bacterial endotoxin." Eur J Neurosci **11**(1): 178-86.

Linthorst, A. C. E., S. Karanth, et al. (1999). "Impaired glucocorticoid receptor function evolves in aberrant physiological responses to bacterial endotoxin." European Journal of Neuroscience **11**(1): 178-186.

Liu, Q. Y., E. Quinet, et al. (2007). "Adipocyte fatty acid-binding protein (aP2), a newly identified LXR target gene, is induced by LXR agonists in human THP-1 cells." Mol Cell Biochem **302**(1-2): 203-13.

Lloyd, D. J., J. Helmering, et al. (2009). "Antidiabetic effects of 11beta-HSD1 inhibition in a mouse model of combined diabetes, dyslipidaemia and atherosclerosis." Diabetes Obes Metab **11**(7): 688-99.

Lovejoy, J. C. and A. Sainsbury (2009). "Sex differences in obesity and the regulation of energy homeostasis." Obes Rev **10**(2): 154-67.

Lu, F. W., K. Yasutomo, et al. (2000). "Thymocyte resistance to glucocorticoids leads to antigen-specific unresponsiveness due to "holes" in the T cell repertoire." Immunity **12**(2): 183-92.

Lu, N. Z. and J. A. Cidlowski (2004). "The origin and functions of multiple human glucocorticoid receptor isoforms." Ann N Y Acad Sci **1024**: 102-23.

Lu, N. Z. and J. A. Cidlowski (2005). "Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes." Mol Cell **18**(3): 331-42.

Lu, N. Z. and J. A. Cidlowski (2006). "Glucocorticoid receptor isoforms generate transcription specificity." Trends Cell Biol **16**(6): 301-7.

Luca, F., S. Kashyap, et al. (2009). "Adaptive Variation Regulates the Expression of the Human SGK1 Gene in Response to Stress." PLoS Genet **5**(5): e1000489.

Lucas, L. R., P. Pompei, et al. (1998). "Effects of adrenal steroids on basal ganglia neuropeptide mRNA and tyrosine hydroxylase radioimmunoreactive levels in the adrenalectomized rat." J Neurochem **71**(2): 833-43.

Lumeng, C. N., I. Maillard, et al. (2009). "T-ing up inflammation in fat." Nat Med **15**(8): 846-7.

Lundgren, M., J. Buren, et al. (2004). "Glucocorticoids down-regulate glucose uptake capacity and insulin-signaling proteins in omental but not subcutaneous human adipocytes." J Clin Endocrinol Metab **89**(6): 2989-97.

Lupien, S. J. and B. S. McEwen (1997). "The acute effects of corticosteroids on cognition: Integration of animal and human model studies." Brain Research Reviews **24**(1): 1-27.

Ma, L. Y., B. S. McEwen, et al. (1993). "Glucocorticoids facilitate mineralocorticoid-induced sodium intake in the rat." Horm Behav **27**(2): 240-50.

MacLusky, N. J. and B. S. McEwen (1980). "Progestin receptors in rat brain: distribution and properties of cytoplasmic progestin-binding sites." Endocrinology **106**(1): 192-202.

Madsen, L., R. K. Petersen, et al. (2005). "Regulation of adipocyte differentiation and function by polyunsaturated fatty acids." Biochim Biophys Acta **1740**(2): 266-86.

Magarinos, A. M. and B. S. McEwen (1995). "Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: involvement of glucocorticoid secretion and excitatory amino acid receptors." Neuroscience **69**(1): 89-98.

Marchetti, B., M. C. Morale, et al. (2002). "Exposure to a Dysfunctional Glucocorticoid Receptor from Early Embryonic Life Programs the Resistance to Experimental Autoimmune Encephalomyelitis Via Nitric Oxide-Induced Immunosuppression." J Immunol **168**(11): 5848-5859.

Marchetti, B., M. C. Morale, et al. (2002). "Exposure to a dysfunctional glucocorticoid receptor from early embryonic life programs the resistance to experimental autoimmune encephalomyelitis via nitric oxide-induced immunosuppression." J Immunol **168**(11): 5848-59.

Marti, A., M. C. Ochoa, et al. (2006). "Meta-analysis on the effect of the N363S polymorphism of the glucocorticoid receptor gene (GRL) on human obesity." BMC Med Genet **7**(1): 50.

Massiera, F., M. Bloch-Faure, et al. (2001). "Adipose angiotensinogen is involved in adipose tissue growth and blood pressure regulation." FASEB J **15**(14): 2727-9.

Masuzaki, H., J. Paterson, et al. (2001). "A transgenic model of visceral obesity and the metabolic syndrome." Science **294**(5549): 2166-70.

Mattsson, C. and T. Olsson (2007). "Estrogens and glucocorticoid hormones in adipose tissue metabolism." Curr Med Chem **14**(27): 2918-24.

McEwen, B. S. (1979). "Influences of adrenocortical hormones on pituitary and brain function." Monogr Endocrinol **12**: 467-92.

McEwen, B. S. (1987). "Steroid hormones and brain development: some guidelines for understanding actions of pseudohormones and other toxic agents." Environ Health Perspect **74**: 177-84.

McEwen, B. S. (1992). "Steroid hormones: effect on brain development and function." Horm Res **37**(Suppl 3): 1-10.

McEwen, B. S. (2003). "Interacting mediators of allostasis and allostatic load: towards an understanding of resilience in aging." Metabolism **52**(10 Suppl 2): 10-6.

McEwen, B. S. (2005). "Glucocorticoids, depression, and mood disorders: structural remodeling in the brain." Metabolism **54**(5 Suppl 1): 20-3.

McEwen, B. S., C. A. Biron, et al. (1997). "The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions." Brain Res Rev **23**(1-2): 79-133.

McEwen, B. S., H. Coirini, et al. (1990). "Steroid effects on neuronal activity: when is the genome involved?" Ciba Found Symp **153**: 3-12; discussion 12-21.
McEwen, B. S., C. D. Conrad, et al. (1997). "Prevention of stress-induced morphological and cognitive consequences." Eur Neuropsychopharmacol **7 Suppl 3**: S323-8.
McEwen, B. S., R. de Kloet, et al. (1976). "Interactions in vivo and in vitro of corticoids and progesterone with cell nuclei and soluble macromolecules from rat brain regions and pituitary." Brain Res **105**(1): 129-36.
McEwen, B. S., E. A. Gould, et al. (1992). "The vulnerability of the hippocampus to protective and destructive effects of glucocorticoids in relation to stress." Br J Psychiatry Suppl(15): 18-23.
McEwen, B. S. and A. M. Magarinos (1997). "Stress effects on morphology and function of the hippocampus." Ann N Y Acad Sci **821**: 271-84.
McKinnell, J., D. Roscoe, et al. (2000). "Regulation of 11beta-hydroxysteroid dehydrogenase enzymes by dietary sodium in the rat." Endocr Res **26**(1): 81-95.
McKittrick, C. R., D. C. Blanchard, et al. (1995). "Serotonin receptor binding in a colony model of chronic social stress." Biol Psychiatry **37**(6): 383-93.
Meaney, M. J., R. M. Sapolsky, et al. (1985). "[3H]dexamethasone binding in the limbic brain of the fetal rat." Brain Res **355**(2): 297-300.
Meaney, M. J., R. M. Sapolsky, et al. (1985). "The development of the glucocorticoid receptor system in the rat limbic brain. I. Ontogeny and autoregulation." Brain Res **350**(1-2): 159-64.
Meaney, M. J., R. M. Sapolsky, et al. (1985). "The development of the glucocorticoid receptor system in the rat limbic brain. II. An autoradiographic study." Brain Res **350**(1-2): 165-8.
Meyer, J. S., P. J. Leveille, et al. (1982). "Evidence for glucocorticoid target cells in the rat optic nerve. Hormone binding and glycerolphosphate dehydrogenase induction." J Neurochem **39**(2): 423-34.
Michailidou, Z., R. N. Carter, et al. (2008). "Glucocorticoid receptor haploinsufficiency causes hypertension and attenuates hypothalamic-pituitary-adrenal axis and blood pressure adaptions to high-fat diet." Faseb J **22**: 3896-3907.
Michailidou, Z., M. D. Jensen, et al. (2007). "Omental 11beta-hydroxysteroid dehydrogenase 1 correlates with fat cell size independently of obesity." Obesity (Silver Spring) **15**(5): 1155-63.
Michelsohn, A. M. and D. J. Anderson (1992). "Changes in competence determine the timing of two sequential glucocorticoid effects on sympathoadrenal progenitors." Neuron **8**(3): 589-604.
Miller, A. H., R. L. Spencer, et al. (1994). "Effects of selective type I and II adrenal steroid agonists on immune cell distribution." Endocrinology **135**(5): 1934-44.
Miller, A. H., R. L. Spencer, et al. (1998). "Glucocorticoid receptors are differentially expressed in the cells and tissues of the immune system." Cell Immunol **186**(1): 45-54.
Miller, A. H., R. L. Spencer, et al. (1997). "1996 Curt P. Richter Award. Effects of viral infection on corticosterone secretion and glucocorticoid receptor binding in immune tissues." Psychoneuroendocrinology **22**(6): 455-74.

Miller, A. H., R. L. Spencer, et al. (1992). "Adrenal steroid receptor activation in rat brain and pituitary following dexamethasone: implications for the dexamethasone suppression test." Biol Psychiatry **32**(10): 850-69.

Miller, A. H., R. L. Spencer, et al. (1990). "Adrenal steroid receptor binding in spleen and thymus after stress or dexamethasone." Am J Physiol **259**(3 Pt 1): E405-12.

Miller, A. H., R. L. Spencer, et al. (1991). "Adrenal steroid receptor activation in vivo and immune function." Am J Physiol **261**(1 Pt 1): E126-31.

Mittelstadt, P. R., J. Galon, et al. (2002). "Glucocorticoid-inducible genes that regulate T-cell function." Ernst Schering Res Found Workshop(40): 319-39.

Moisan, M. P., J. R. Seckl, et al. (1990). "11 beta-hydroxysteroid dehydrogenase bioactivity and messenger RNA expression in rat forebrain: localization in hypothalamus, hippocampus, and cortex." Endocrinology **127**(3): 1450-5.

Moitra, J., M. M. Mason, et al. (1998). "Life without white fat: a transgenic mouse." Genes Dev **12**(20): 3168-81.

Montkowski, A., N. Barden, et al. (1995). "Long-term antidepressant treatment reduces behavioral deficits in transgenic mice with impaired glucocorticoid receptor function." Journal of Neuroendocrinology **7**: 841-845.

Morale, M. C., N. Batticane, et al. (1995). "Disruption of hypothalamic-pituitary-adrenocortical system in transgenic mice expressing type II glucocorticoid receptor antisense ribonucleic acid permanently impairs T cell function: effects on T cell trafficking and T cell responsiveness during postnatal development." Endocrinology **136**(9): 3949-60.

Morgan, S. A., M. Sherlock, et al. (2009). "11beta-hydroxysteroid dehydrogenase type 1 regulates glucocorticoid-induced insulin resistance in skeletal muscle." Diabetes **58**(11): 2506-15.

Morton, G. J., D. E. Cummings, et al. (2006). "Central nervous system control of food intake and body weight." Nature **443**(7109): 289-95.

Morton, N. M., M. C. Holmes, et al. (2001). "Improved lipid and lipoprotein profile, hepatic insulin sensitivity, and glucose tolerance in 11beta-hydroxysteroid dehydrogenase type 1 null mice." J Biol Chem **276**(44): 41293-300.

Morton, N. M., J. M. Paterson, et al. (2004). "Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11 beta-hydroxysteroid dehydrogenase type 1-deficient mice." Diabetes **53**(4): 931-8.

Morton, N. M., L. Ramage, et al. (2004). "Down-regulation of adipose 11beta-hydroxysteroid dehydrogenase type 1 by high-fat feeding in mice: a potential adaptive mechanism counteracting metabolic disease." Endocrinology **145**(6): 2707-12.

Najafipour, H. and W. R. Ferrell (1993). "Sympathetic innervation and beta-adrenoceptor profile of blood vessels in the posterior region of the rabbit knee joint." Exp Physiol **78**(5): 625-37.

Napolitano, A., M. W. Voice, et al. (1998). "11Beta-hydroxysteroid dehydrogenase 1 in adipocytes: expression is differentiation-dependent and hormonally regulated." J Steroid Biochem Mol Biol **64**(5-6): 251-60.

Nicholson, S., M. Holmes, et al. (1986). "Effects of 6-hydroxydopamine on the hypothalamo-pituitary-adrenocortical axis." Neuroendocrinology **43**(4): 483-90.

Nishi, M., T. Usuku, et al. (2007). "Direct visualization of glucocorticoid receptor positive cells in the hippocampal regions using green fluorescent protein transgenic mice." Neuroscience.

Niu, N., V. Manickam, et al. (2009). "Human Glucocorticoid Receptor Alpha Gene (NR3C1) Pharmacogenomics: Gene Resequencing and Functional Genomics." J Clin Endocrinol Metab %R 10.1210/jc.2008-2109: jc.2008-2109.

Nohno, T., Y. Kasai, et al. (1989). "Novel cDNA sequence possibly generated by alternative splicing of a mouse glucocorticoid receptor gene transcript from Shionogi carcinoma 115." Nucleic Acids Res **17**(1): 445.

Nuber, U. A., S. Kriaucionis, et al. (2005). "Up-regulation of glucocorticoid-regulated genes in a mouse model of Rett syndrome." Hum Mol Genet **14**(15): 2247-56.

Nyirenda, M. J., R. S. Lindsay, et al. (1998). "Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring." J Clin Invest **101**: 2174-2181.

O'Regan, D., C. J. Kenyon, et al. (2004). "Glucocorticoid exposure in late gestation in the rat permanently programs gender-specific differences in adult cardiovascular and metabolic physiology." Am J Physiol Endocrinol Metab **287**(5): E863-70.

Oitzl, M. S., E. R. deKloet, et al. (1997). "Spatial learning deficits in mice with a targeted glucocorticoid receptor gene disruption." European Journal of Neuroscience **9**(11): 2284-2296.

Oitzl, M. S., H. M. Reichardt, et al. (2001). "Point mutation in the mouse glucocorticoid receptor preventing DNA binding impairs spatial memory." Proc Natl Acad Sci U S A **98**(22): 12790-5.

Oller do Nascimento, C. M., E. B. Ribeiro, et al. (2009). "Metabolism and secretory function of white adipose tissue: effect of dietary fat." An Acad Bras Cienc **81**(3): 453-66.

Olpe, H. R. and B. S. McEwen (1976). "Glucocorticoid binding to receptor-like proteins in rat brain and pituitary: ontogenetic and experimentally induced changes." Brain Res **105**(1): 121-8.

Opherk, C., F. Tronche, et al. (2004). "Inactivation of the glucocorticoid receptor in hepatocytes leads to fasting hypoglycemia and ameliorates hyperglycemia in streptozotocin-induced diabetes mellitus." Mol Endocrinol **18**(6): 1346-53.

Orange, J. S., T. P. Salazar-Mather, et al. (1995). "Mechanism of interleukin 12-mediated toxicities during experimental viral infections: role of tumor necrosis factor and glucocorticoids." J Exp Med **181**(3): 901-14.

Osborne, T. F. (2000). "Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action." J Biol Chem **275**(42): 32379-82.

Ozols, J. (1995). "Lumenal orientation and posttranslational modifications of the liver microsomal 11β-hydroxysteroid dehydrogenase." J Biol Chem **270**: 2305-2312.

Panarelli, M., C. D. Holloway, et al. (1998). "Glucocorticoid receptor polymorphism, skin vasoconstriction, and other metabolic intermediate

phenotypes in normal human subjects." J Clin Endocrinol Metab **83**(6): 1846-1852.
Paterson, J. M., M. C. Holmes, et al. (2007). "Liver-selective transgene rescue of hypothalamic-pituitary-adrenal axis dysfunction in 11beta-hydroxysteroid dehydrogenase type 1-deficient mice." Endocrinology **148**(3): 961-6.
Paterson, J. M., N. M. Morton, et al. (2004). "Metabolic syndrome without obesity: Hepatic overexpression of 11beta-hydroxysteroid dehydrogenase type 1 in transgenic mice." Proc Natl Acad Sci U S A **101**(18): 7088-93.
Pavlides, C. and B. S. McEwen (1999). "Effects of mineralocorticoid and glucocorticoid receptors on long-term potentiation in the CA3 hippocampal field." Brain Res **851**(1-2): 204-14.
Pavlides, C., S. Ogawa, et al. (1996). "Role of adrenal steroid mineralocorticoid and glucocorticoid receptors in long-term potentiation in the CA1 field of hippocampal slices." Brain Res **738**(2): 229-35.
Pazirandeh, A., M. Jondal, et al. (2005). "Conditional expression of a glucocorticoid receptor transgene in thymocytes reveals a role for thymic-derived glucocorticoids in thymopoiesis *in vivo*." Endocrinology **146**(6): 2501-2507.
Pazirandeh, A., Y. Xue, et al. (2002). "Effects of altered glucocorticoid sensitivity in the T-cell lineage on thymocyte and T-cell homeostasis." Faseb J **16**: 727-729.
Pepin, M. C., F. Pothier, et al. (1992). "Impaired type II glucocorticoid-receptor function in mice bearing antisense RNA transgene." Nature **355**(6362): 725-8.
Piroli, G. G., C. A. Grillo, et al. (2007). "Corticosterone impairs insulin-stimulated translocation of GLUT4 in the rat hippocampus." Neuroendocrinology **85**(2): 71-80.
Prasad, R., R. S. Dayal, et al. (1976). "Beta1 C (C'3) complement estimation in various types on nephritis in children." Indian Pediatr **13**(2): 97-102.
Priebe, K., R. D. Romeo, et al. (2005). "Maternal influences on adult stress and anxiety-like behavior in C57BL/6J and BALB/cJ mice: a cross-fostering study." Dev Psychobiol **47**(4): 398-407.
Purton, J. F., R. L. Boyd, et al. (2000). "Intrathymic T cell development and selection proceeds normally in the absence of glucocorticoid receptor signaling." Immunity **13**(2): 179-186.
Purton, J. F., Y. Zhan, et al. (2002). "Glucocorticoid receptor deficient thymic and peripheral T cells develop normally in adult mice." Eur J Immunol **32**(12): 3546-3555.
Purton, J. F., Y. Zhan, et al. (2002). "Glucocorticoid receptor deficient thymic and peripheral T cells develop normally in adult mice." Eur J Immunol **32**(12): 3546-55.
Rajeevan, M. S., A. K. Smith, et al. (2006). "Glucocorticoid receptor polymorphisms and haplotypes associated with chronic fatigue syndrome." Genes Brain Behav.
Ramirez, G., A. Ganguly, et al. (1988). "Acute effect of captopril on aldosterone secretory responses to endogenous or exogenous adrenocorticotropin." J Clin Endocrinol Metab **66**(1): 46-50.
Reagan, L. P., A. M. Magarinos, et al. (1999). "Neurological changes induced by stress in streptozotocin diabetic rats." Ann N Y Acad Sci **893**: 126-37.

Reagan, L. P. and B. S. McEwen (1997). "Controversies surrounding glucocorticoid-mediated cell death in the hippocampus." J Chem Neuroanat **13**(3): 149-67.
Reagan, L. P., C. R. McKittrick, et al. (1999). "Corticosterone and phenytoin reduce neuronal nitric oxide synthase messenger RNA expression in rat hippocampus." Neuroscience **91**(1): 211-9.
Reaven, G. M. (2006). "The metabolic syndrome: is this diagnosis necessary?" Am J Clin Nutr **83**(6): 1237-47.
Reichardt, H. M., K. H. Kaestner, et al. (1998). "DNA binding of the glucocorticoid receptor is not essential for survival." Cell **93**(4): 531-41.
Reichardt, H. M., J. P. Tuckermann, et al. (2000). "Molecular genetic dissection of glucocorticoid receptor function in vivo." Z Rheumatol **59 Suppl 2**: II/1-5.
Reshef, L., Y. Olswang, et al. (2003). "Glyceroneogenesis and the triglyceride/fatty acid cycle." J Biol Chem **278**(33): 30413-6.
Reul, J. M. and E. R. de Kloet (1985). "Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation." Endocrinology **117**(6): 2505-11.
Richard, D., S. Chapdelaine, et al. (1993). "Energy balance and lipid metabolism in transgenic mice bearing an antisense GCR gene construct." Am J Physiol **265**(1 Pt 2): R146-50.
Ricketts, M. L., K. J. Shoesmith, et al. (1998). "Regulation of 11 beta-hydroxysteroid dehydrogenase type 1 in primary cultures of rat and human hepatocytes." J Endocrinol **156**(1): 159-68.
Ridder, S., S. Chourbaji, et al. (2005). "Mice with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive reactions." J Neurosci **25**(26): 6243-50.
Riftina, F., J. Angulo, et al. (1995). "Regulation of angiotensinogen gene expression in the rat forebrain by adrenal steroids and relation to salt appetite." Brain Res Mol Brain Res **33**(2): 201-8.
Rivers, C., A. Levy, et al. (1999). "Insertion of an amino acid in the DNA-binding domain of the glucocorticoid receptor as a result of alternative splicing." J Clin Endocrinol Metab **84**(11): 4283-6.
Robson, A. C., C. M. Leckie, et al. (1998). "11 Beta-hydroxysteroid dehydrogenase type 2 in the postnatal and adult rat brain." Brain Res Mol Brain Res **61**(1-2): 1-10.
Romeo, R. D., F. S. Ali, et al. (2008). "Glucocorticoid receptor mRNA expression in the hippocampal formation of male rats before and after pubertal development in response to acute or repeated stress." Neuroendocrinology **87**(3): 160-7.
Ronchi, E., R. L. Spencer, et al. (1998). "Effects of photoperiod on brain corticosteroid receptors and the stress response in the golden hamster (Mesocricetus auratus)." Brain Res **780**(2): 348-51.
Rosmond, R. (2002). "The glucocorticoid receptor gene and its association to metabolic syndrome." Obes Res **10**(10): 1078-86.
Rosmond, R., C. Bouchard, et al. (2001). "Tsp509I polymorphism in exon 2 of the glucocorticoid receptor gene in relation to obesity and cortisol secretion: cohort study." Bmj **322**(7287): 652-3.

Rosmond, R., Y. C. Chagnon, et al. (2000). "A polymorphism of the 5'-flanking region of the glucocorticoid receptor gene locus is associated with basal cortisol secretion in men." Metabolism **49**: 1197-1199.

Rosmond, R., Y. C. Chagnon, et al. (2000). "A glucocorticoid receptor gene marker is associated with abdominal obesity, leptin, and dysregulation of the hypothalamic-pituitary-adrenal axis." Obes Res **8**(3): 211-8.

Rosmond, R., V. Radulovic, et al. (2006). "A brief update of glucocorticoid receptor variants and obesity risk." Ann N Y Acad Sci **1083**: 153-64.

Ross, S. R., L. Choy, et al. (1992). "Hibernoma formation in transgenic mice and isolation of a brown adipocyte cell line expressing the uncoupling protein gene." Proc Natl Acad Sci U S A **89**(16): 7561-5.

Ross, S. R., R. A. Graves, et al. (1993). "Targeted expression of a toxin gene to adipose tissue: transgenic mice resistant to obesity." Genes Dev **7**(7B): 1318-24.

Rossmeisl, M., G. Barbatelli, et al. (2002). "Expression of the uncoupling protein 1 from the aP2 gene promoter stimulates mitochondrial biogenesis in unilocular adipocytes in vivo." Eur J Biochem **269**(1): 19-28.

Rossmeisl, M., P. Flachs, et al. (2004). "Role of energy charge and AMP-activated protein kinase in adipocytes in the control of body fat stores." Int J Obes Relat Metab Disord **28 Suppl 4**: S38-44.

Rossmeisl, M., J. Kovar, et al. (2005). "Triglyceride-lowering effect of respiratory uncoupling in white adipose tissue." Obes Res **13**(5): 835-44.

Rossmeisl, M., I. Syrovy, et al. (2000). "Decreased fatty acid synthesis due to mitochondrial uncoupling in adipose tissue." FASEB J **14**(12): 1793-800.

Russcher, H., E. F. van Rossum, et al. (2005). "Increased Expression of the Glucocorticoid Receptor-A Translational Isoform as a Result of the ER22/23EK Polymorphism." Mol Endocrinol.

Sacedon, R., A. Vicente, et al. (1999). "Partial blockade of T-cell differentiation during ontogeny and marked alterations of the thymic microenvironment in transgenic mice with impaired glucocorticoid receptor function." J Neuroimmunol **98**(2): 157-67.

Sainte-Marie, Y., A. Nguyen Dinh Cat, et al. (2007). "Conditional glucocorticoid receptor expression in the heart induces atrio-ventricular block." FASEB J **21**(12): 3133-41.

Sakai, R. R., L. Y. Ma, et al. (1996). "Intracerebral administration of mineralocorticoid receptor antisense oligonucleotides attenuate adrenal steroid-induced salt appetite in rats." Neuroendocrinology **64**(6): 425-9.

Sakai, R. R., B. S. McEwen, et al. (2000). "The amygdala: site of genomic and nongenomic arousal of aldosterone-induced sodium intake." Kidney Int **57**(4): 1337-45.

Sakoda, H., T. Ogihara, et al. (2000). "Dexamethasone-induced insulin resistance in 3T3-L1 adipocytes is due to inhibition of glucose transport rather than insulin signal transduction." Diabetes **49**(10): 1700-8.

Sampath, H. and J. M. Ntambi (2005). "The fate and intermediary metabolism of stearic acid." Lipids **40**(12): 1187-91.

Samra, J. S., M. L. Clark, et al. (1998). "Effects of physiological hypercortisolemia on the regulation of lipolysis in subcutaneous adipose tissue." J Clin Endocrinol Metab **83**(2): 626-31.

Sapolsky, R. M., L. C. Krey, et al. (1983). "Corticosterone receptors decline in a site-specific manner in the aged rat brain." Brain Res **289**(1-2): 235-40.
Sapolsky, R. M., L. C. Krey, et al. (1984). "Glucocorticoid-sensitive hippocampal neurons are involved in terminating the adrenocortical stress response." Proc Natl Acad Sci U S A **81**(19): 6174-7.
Sapolsky, R. M., L. C. Krey, et al. (1984). "Stress down-regulates corticosterone receptors in a site-specific manner in the brain." Endocrinology **114**(1): 287-92.
Sapolsky, R. M., L. C. Krey, et al. (1985). "Prolonged glucocorticoid exposure reduces hippocampal neuron number: implications for aging." J Neurosci **5**(5): 1222-7.
Sapolsky, R. M., L. C. Krey, et al. (1984). "Do vasopressin-related peptides induce hippocampal corticosterone receptors? Implications for aging." J Neurosci **4**(6): 1479-85.
Sapolsky, R. M. and B. S. McEwen (1985). "Down-regulation of neural corticosterone receptors by corticosterone and dexamethasone." Brain Res **339**(1): 161-5.
Sapolsky, R. M., B. S. McEwen, et al. (1983). "Quantitative autoradiography of [3H]corticosterone receptors in rat brain." Brain Res **271**(2): 331-4.
Sapolsky, R. M., M. J. Meaney, et al. (1985). "The development of the glucocorticoid receptor system in the rat limbic brain. III. Negative-feedback regulation." Brain Res **350**(1-2): 169-73.
Sarrieau, A., M. Vial, et al. (1986). "Corticosteroid receptors in rat hippocampal sections: effect of adrenalectomy and corticosterone replacement." J Steroid Biochem **24**(3): 721-4.
Schaaf, M. J. and J. A. Cidlowski (2002). "Molecular mechanisms of glucocorticoid action and resistance." J Steroid Biochem Mol Biol **83**(1-5): 37-48.
Schaaf, M. J. and J. A. Cidlowski (2003). "Molecular determinants of glucocorticoid receptor mobility in living cells: the importance of ligand affinity." Mol Cell Biol **23**(6): 1922-34.
Schakman, O., H. Gilson, et al. (2008). "Mechanisms of glucocorticoid-induced myopathy." J Endocrinol **197**(1): 1-10.
Schmidt, M., C. Weidler, et al. (2005). "Reduced capacity for the reactivation of glucocorticoids in rheumatoid arthritis synovial cells: possible role of the sympathetic nervous system?" Arthritis Rheum **52**(6): 1711-20.
Seckl, J. R., N. M. Morton, et al. (2004). "Glucocorticoids and 11beta-hydroxysteroid dehydrogenase in adipose tissue." Recent Prog Horm Res **59**: 359-93.
Sell, H., Y. Deshaies, et al. (2004). "The brown adipocyte: update on its metabolic role." Int J Biochem Cell Biol **36**(11): 2098-104.
Sharma, A. M. and B. Staels (2007). "Review: Peroxisome proliferator-activated receptor gamma and adipose tissue--understanding obesity-related changes in regulation of lipid and glucose metabolism." J Clin Endocrinol Metab **92**(2): 386-95.
Sheward, W. J., E. S. Maywood, et al. (2007). "Entrainment to feeding but not to light: circadian phenotype of VPAC2 receptor-null mice." J Neurosci **27**(16): 4351-8.

Shi, H., M. V. Kokoeva, et al. (2006). "TLR4 links innate immunity and fatty acid-induced insulin resistance." J Clin Invest **116**(11): 3015-25.
Shi, H., R. J. Seeley, et al. (2009). "Sexual differences in the control of energy homeostasis." Front Neuroendocrinol **30**(3): 396-404.
Shimomura, I., R. E. Hammer, et al. (1998). "Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy." Genes Dev **12**(20): 3182-94.
Sierra, A., A. Gottfried-Blackmore, et al. (2008). "Steroid hormone receptor expression and function in microglia." Glia **56**(6): 659-74.
Slavin, B. G., J. M. Ong, et al. (1994). "Hormonal regulation of hormone-sensitive lipase activity and mRNA levels in isolated rat adipocytes." J Lipid Res **35**(9): 1535-41.
Soloveva, V., R. A. Graves, et al. (1997). "Transgenic mice overexpressing the beta 1-adrenergic receptor in adipose tissue are resistant to obesity." Mol Endocrinol **11**(1): 27-38.
Somponpun, S. J., M. C. Holmes, et al. (2004). "Modulation of oestrogen receptor-beta mRNA expression in rat paraventricular and supraoptic nucleus neurones following adrenal steroid manipulation and hyperosmotic stimulation." J Neuroendocrinol **16**(5): 472-82.
Sonino, N., G. A. Fava, et al. (1993). "Course of depression in Cushing's syndrome: response to treatment and comparison with Graves' disease." Horm Res **39**(5-6): 202-6.
Spencer, R. L., A. H. Miller, et al. (1993). "Diurnal differences in basal and acute stress levels of type I and type II adrenal steroid receptor activation in neural and immune tissues." Endocrinology **133**(5): 1941-50.
Spencer, R. L., E. A. Young, et al. (1990). "Adrenal steroid type I and type II receptor binding: estimates of in vivo receptor number, occupancy, and activation with varying level of steroid." Brain Res **514**(1): 37-48.
Stahn, C., M. Lowenberg, et al. (2007). "Molecular mechanisms of glucocorticoid action and selective glucocorticoid receptor agonists." Mol Cell Endocrinol **275**(1-2): 71-8.
Steffensen, K. R. and J. A. Gustafsson (2004). "Putative metabolic effects of the liver X receptor (LXR)." Diabetes **53 Suppl 1**: S36-42.
Stefl, B., A. Janovska, et al. (1998). "Brown fat is essential for cold-induced thermogenesis but not for obesity resistance in aP2-Ucp mice." Am J Physiol **274**(3 Pt 1): E527-33.
Steinbach, J. H., D. Schubert, et al. (1974). "Inhibition of acetylcholine synthesis in neuroblastoma cells by a styrylpyridine analog." J Neurochem **22**(4): 611-3.
Stewart, P. M., A. Boulton, et al. (1999). "Cortisol metabolism in human obesity: impaired cortisone->cortisol conversion in subjects with central adiposity." J Clin Endocrinol Metab **84**(3): 1022-1027.
Stewart, P. M., J. E. Corrie, et al. (1988). "Syndrome of apparent mineralocorticoid excess. A defect in the cortisol-cortisone shuttle." J Clin Invest **82**(1): 340-9.
Stutzmann, G. E., B. S. McEwen, et al. (1998). "Serotonin modulation of sensory inputs to the lateral amygdala: dependency on corticosterone." J Neurosci **18**(22): 9529-38.

Summers, S. A. and D. H. Nelson (2005). "A role for sphingolipids in producing the common features of type 2 diabetes, metabolic syndrome X, and Cushing's syndrome." Diabetes **54**(3): 591-602.

Sun, L., J. S. Trausch-Azar, et al. (2008). "Glucocorticoids differentially regulate degradation of MyoD and Id1 by N-terminal ubiquitination to promote muscle protein catabolism." Proc Natl Acad Sci U S A **105**(9): 3339-44.

Tang, C. N., K. K. Tsui, et al. (2006). "A single-centre experience of 40 laparoscopic liver resections." Hong Kong Med J **12**(6): 419-25.

Tempel, D. L., B. S. McEwen, et al. (1992). "Effects of adrenal steroid agonists on food intake and macronutrient selection." Physiol Behav **52**(6): 1161-6.

Tempel, D. L., B. S. McEwen, et al. (1993). "Adrenal steroid receptors in the PVN: studies with steroid antagonists in relation to macronutrient intake." Neuroendocrinology **57**(6): 1106-13.

Thieringer, R., C. B. Le Grand, et al. (2001). "11 Beta-hydroxysteroid dehydrogenase type 1 is induced in human monocytes upon differentiation to macrophages." J Immunol **167**(1): 30-5.

Tokarz, R. R., D. Crews, et al. (1981). "Estrogen-sensitive progestin binding sites in the brain of the lizard, Anolis carolinensis." Brain Res **220**(1): 95-105.

Tolosa, E., L. B. King, et al. (1998). "Thymocyte glucocorticoid resistance alters positive selection and inhibits autoimmunity and lymphoproliferative disease in MRL-lpr/lpr mice." Immunity **8**(1): 67-76.

Tomlinson, J. W. and P. M. Stewart (2007). "Modulation of glucocorticoid action and the treatment of type-2 diabetes." Best Pract Res Clin Endocrinol Metab **21**(4): 607-19.

Tomlinson, J. W., E. A. Walker, et al. (2004). "11beta-hydroxysteroid dehydrogenase type 1: a tissue-specific regulator of glucocorticoid response." Endocr Rev **25**(5): 831-66.

Tornello, S., E. Orti, et al. (1982). "Regulation of glucocorticoid receptors in brain by corticosterone treatment of adrenalectomized rats." Neuroendocrinology **35**(6): 411-7.

Tronche, F., C. Kellendonk, et al. (1999). "Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety." Nat Genet **23**(1): 99-103.

Tronche, F., C. Opherk, et al. (2004). "Glucocorticoid receptor function in hepatocytes is essential to promote postnatal body growth." Genes Dev **18**(5): 492-497.

Tronche, F., C. Opherk, et al. (2004). "Glucocorticoid receptor function in hepatocytes is essential to promote postnatal body growth." Genes Dev **18**(5): 492-7.

Tuckermann, J. P., A. Kleiman, et al. (2007). "Macrophages and neutrophils are the targets for immune suppression by glucocorticoids in contact allergy." J Clin Invest **117**(5): 1381-90.

Tuckermann, J. P., A. Kleiman, et al. (2007). "Macrophages and neutrophils are the targets for immune suppression by glucocorticoids in contact allergy." J Clin Invest **117**(5): 1381-1390.

Tuckermann, J. P., H. M. Reichardt, et al. (1999). "The DNA binding-independent function of the glucocorticoid receptor mediates repression of AP-1-dependent genes in skin." J Cell Biol **147**(7): 1365-70.

Turner, B. B. and B. S. McEwen (1980). "Hippocampal cytosol binding capacity of corticosterone: no depletion with nuclear loading." Brain Res **189**(1): 169-82.
Uchida, S., A. Nishida, et al. (2008). "Characterization of the vulnerability to repeated stress in Fischer 344 rats: possible involvement of microRNA-mediated down-regulation of the glucocorticoid receptor." Eur J Neurosci **27**(9): 2250-61.
Valsamakis, G., A. Anwar, et al. (2004). "11beta-hydroxysteroid dehydrogenase type 1 activity in lean and obese males with type 2 diabetes mellitus." J Clin Endocrinol Metab **89**(9): 4755-61.
van den Akker, E. L., J. L. Nouwen, et al. (2006). "Staphylococcus aureus Nasal Carriage Is Associated with Glucocorticoid Receptor Gene Polymorphisms." J Infect Dis **194**(6): 814-8.
van den Akker, E. L., H. Russcher, et al. (2006). "Glucocorticoid receptor polymorphism affects transrepression but not transactivation." J Clin Endocrinol Metab **91**(7): 2800-3.
van Rossum, E. F., R. A. Feelders, et al. (2004). "Association of the ER22/23EK polymorphism in the glucocorticoid receptor gene with survival and C-reactive protein levels in elderly men." Am J Med **117**(3): 158-62.
van Rossum, E. F., J. W. Koper, et al. (2002). "A polymorphism in the glucocorticoid receptor gene, which decreases sensitivity to glucocorticoids in vivo, is associated with low insulin and cholesterol levels." Diabetes **51**(10): 3128-34.
van Rossum, E. F., J. W. Koper, et al. (2003). "Identification of the BclI polymorphism in the glucocorticoid receptor gene: association with sensitivity to glucocorticoids in vivo and body mass index." Clin Endocrinol (Oxf) **59**(5): 585-92.
van Rossum, E. F. and S. W. Lamberts (2004). "Polymorphisms in the glucocorticoid receptor gene and their associations with metabolic parameters and body composition." Recent Prog Horm Res **59**: 333-57.
van Rossum, E. F., P. H. Roks, et al. (2004). "Characterization of a promoter polymorphism in the glucocorticoid receptor gene and its relationship to three other polymorphisms." Clin Endocrinol (Oxf) **61**(5): 573-81.
van Rossum, E. F., H. Russcher, et al. (2005). "Genetic polymorphisms and multifactorial diseases: facts and fallacies revealed by the glucocorticoid receptor gene." Trends Endocrinol Metab **16**(10): 445-450.
van Winsen, L. M., T. Hooper-van Veen, et al. (2007). "Glucocorticoid receptor gene polymorphisms associated with more aggressive disease phenotype in MS." J Neuroimmunol.
van Winsen, L. M., L. Manenschijn, et al. (2009). "A glucocorticoid receptor gene haplotype (TthIII1/ER22/23EK/9beta) is associated with a more aggressive disease course in multiple sclerosis." J Clin Endocrinol Metab **94**(6): 2110-4.
van Winsen, L. M. L., L. Manenschijn, et al. (2009). "A Glucocorticoid Receptor Gene Haplotype (TthIII1/ER22/23EK/9{beta}) Is Associated with a More Aggressive Disease Course in Multiple Sclerosis." Endocr Rev **30**(3): 286-a-.
Varga, G., J. Ehrchen, et al. (2008). "Glucocorticoids induce an activated, anti-inflammatory monocyte subset in mice that resembles myeloid-derived suppressor cells." J Leukoc Biol **84**: 644-650.

Vegiopoulos, A. and S. Herzig (2007). "Glucocorticoids, metabolism and metabolic diseases." Mol Cell Endocrinol **275**(1-2): 43-61.

Viau, V. and M. J. Meaney (1991). "Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat." Endocrinology **129**(5): 2503-11.

Voorhoeve, P. G., E. L. van den Akker, et al. (2009). "Glucocorticoid receptor gene polymorphism is less frequent in children born small for gestational age without catch-up growth." Horm Res **71**(3): 162-6.

Voorhoeve, P. G., E. L. van den Akker, et al. (2009). "Glucocorticoid receptor gene variant is associated with increased body fatness in youngsters." Clin Endocrinol (Oxf).

Vottero, A., T. Kino, et al. (2002). "A novel, C-terminal dominant negative mutation of the GR causes familial glucocorticoid resistance through abnormal interactions with p160 steroid receptor coactivators." J Clin Endocrinol Metab **87**(6): 2658-67.

Wajchenberg, B. L. (2000). "Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome." Endocr Rev **21**(6): 697-738.

Walker, B. R. and R. Andrew (2006). "Tissue production of cortisol by 11beta-hydroxysteroid dehydrogenase type 1 and metabolic disease." Ann N Y Acad Sci **1083**: 165-84.

Walker, B. R., J. L. Yau, et al. (1991). "11 beta-hydroxysteroid dehydrogenase in vascular smooth muscle and heart: implications for cardiovascular responses to glucocorticoids." Endocrinology **129**(6): 3305-12.

Watt, G. C., S. B. Harrap, et al. (1992). "Abnormalities of glucocorticoid metabolism and the renin-angiotensin system: a four-corners approach to the identification of genetic determinants of blood pressure." J Hypertens **10**(5): 473-82.

Watts, L. M., V. P. Manchem, et al. (2005). "Reduction of hepatic and adipose tissue glucocorticoid receptor expression with antisense oligonucleotides improves hyperglycemia and hyperlipidemia in diabetic rodents without causing systemic glucocorticoid antagonism." Diabetes **54**(6): 1846-53.

Weaver, J. U., G. A. Hitman, et al. (1992). "An association between a Bc1I restriction fragment length polymorphism of the glucocorticoid receptor locus and hyperinsulinaemia in obese women." J Mol Endocrinol **9**(3): 295-300.

Wei, Q., X. Y. Lu, et al. (2004). "Glucocorticoid receptor overexpression in forebrain: a mouse model of increased emotional lability." Proc Natl Acad Sci U S A **101**(32): 11851-6.

Wei, Y., D. Wang, et al. (2006). "Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells." Am J Physiol Endocrinol Metab **291**(2): E275-81.

Weissman, B. A., C. M. Sottas, et al. (2009). "Normal Responses to Restraint Stress in Mice Lacking the Gene for Neuronal Nitric Oxide Synthase." J Androl.

Welberg, L. A., J. R. Seckl, et al. (2000). "Inhibition of 11beta-hydroxysteroid dehydrogenase, the foeto-placental barrier to maternal glucocorticoids, permanently programs amygdala GR mRNA expression and anxiety-like behaviour in the offspring." Eur J Neurosci **12**(3): 1047-54.

Wellen, K. E. and G. S. Hotamisligil (2005). "Inflammation, stress, and diabetes." J Clin Invest **115**(5): 1111-9.

Wust, S., J. van den Brandt, et al. (2008). "Peripheral T cells are the therapeutic targets of glucocorticoids in experimental autoimmune encephalomyelitis." J Immunol **180**(12): 8434-8443.

Wust, S., E. F. Van Rossum, et al. (2004). "Common polymorphisms in the glucocorticoid receptor gene are associated with adrenocortical responses to psychosocial stress." J Clin Endocrinol Metab **89**(2): 565-73.

Xu, D., A. Buehner, et al. (2006). "A polymorphic glucocorticoid receptor in a mouse population may explain inherited altered stress response and increased anxiety-type behaviors." Faseb J **20**(13): 2414-6.

Young, E. A., R. L. Spencer, et al. (1990). "Changes at multiple levels of the hypothalamo-pituitary adrenal axis following repeated electrically induced seizures." Psychoneuroendocrinology **15**(3): 165-72.

Yudt, M. R. and J. A. Cidlowski (2001). "Molecular identification and characterization of a and b forms of the glucocorticoid receptor." Mol Endocrinol **15**(7): 1093-103.

Zhang, J., R. Ge, et al. (2009). "Characterization of a novel gain of function glucocorticoid receptor knock-in mouse." J Biol Chem **284**(10): 6249-59.

Zhang, N., Q. A. Truong-Tran, et al. (2007). "Glucocorticoids enhance or spare innate immunity: effects in airway epithelium are mediated by CCAAT/enhancer binding proteins." J Immunol **179**(1): 578-89.