Altering adipose tissue responses to glucocorticoids through genetic manipulation of the 11β-HSD1 gene

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Summary

Glucocorticoids (GC) are important regulators of permissive and adaptive physiology. Exogenous and endogenous GC excess (Cushing's syndrome) can lead to a range of metabolic complications including increased adiposity, fatty liver, high blood pressure and muscle wasting. This increases the risk of developing type 2 diabetes, metabolic syndrome and cardiovascular disease. GC levels are regulated by a number of homeostatic mechanisms. Importantly, intracellular GC sensitivity is controlled by the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which reactivates GC to increase their local availability. 11β-HSD1 activity in adipose tissue has been shown to be deregulated in a range of metabolic disorders in which GC levels are generally normal. I now hypothesise that 11β-HSD1 is a critical regulator of adipose tissue sensitivity to circulating GC excess, driving a fast forward mechanism of intracellular GC excess, and that through 11β-HSD1 depletion adipose tissue will be desensitised to GCs and resist metabolic deregulation.

Using 11β-HSD1 global and adipose tissue-specific KO mice in a model of GC excess I demonstrated that 11β-HSD1 indeed mediates the adverse metabolic effects of GC excess on a global scale. Specifically, adipose tissue 11β-HSD1 activity plays a major role in determining the hepatic manifestation of GC excess.

I further investigated brown adipose tissue (BAT) in the context of GC excess. I demonstrate that 11β-HSD1 regulates BAT activity and mitochondrial function, possibly suppressing BATs thermogenic potential. I extended our studies to examine the potential for white adipose tissue (WAT) to assume markers of thermogenic and mitochondrial function in the context of 11βHSD1 and GC excess. The data suggest that 11β-HSD1 may act to suppress the potential of WAT to assume a 'BAT-like' profile, particularly in the face of GC excess.

These data show that 11β-HSD1 loss of function in mice confers a protective phenotype in the face of GC excess and highlights the important role of this enzyme in mediating the metabolic phenotype associated with GCs. The effects of GC excess on adipose tissue are profound and extend beyond the usual description of lipid and glucose regulation. Indeed these data support the idea that GCs can influence BAT and WAT thermogenic potential and may help to extend what I understand about the metabolic dysregulation in humans suffering from GC excess syndromes. This therefore highlights 11β-HSD1 as an exciting potential target for the treatment for the metabolic disease associated with GC excess.

Abbreviations

ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATGL	Adipose triglyceride lipase
AVP	Arginine vasopressin
11β-HSD1	11beta-hydroxysteroid dehydrogenase type 1
11β-HSD2	11beta-hydroxysteroid dehydrogenase type 2
BAT	Brown adipose tissue
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CBP	CREB- binding protein
CBG	Corticosteroid-binding globulin
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
Cidea	Cell death-inducing DFFA-like effector a
CHOP	CCAAT/Enhancer-Binding Protein Homologous Protein
CIV-MTCO1	Cytochrome c oxidase
CNS	Central nervous system
CO_2	Carbon Dioxide
CORT	Corticosterone
CoxIV	Cytochrome c oxidase iv
Cox7a1	Cytochrome c oxidase 7a1
Cox8b	Cytochrome c oxidase 8b
CRF	Corticotrophin-releasing factor
CS	Cushing's syndrome
CII-SDHB	Succinate dehydrogenase B
Dex	Dexamethasone
20-DHB	20-dihydrocorticosterone
11-DHC	11-dehydrocorticosterone
DiOH-HHA	Dihydroxy-hexahydro 11-dehydro-corticosterone
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
Elovl3	Elongation of very long chain fatty acids
ER	Endoplasmic reticulum
^{18}FDG-PET	^{18}fluoro-labeled 2-deoxy-glucose positron emission tomography
FFA	Free fatty acids
FKO	Adipose specific knock-out
GC	Glucocorticoid
GC/MS	Gas chromatography/Mass spectrometry
GKO	Global knock-out
G6P	Glucose-6-phosphate
GR	Glucocorticoid receptor
GRa	Glucocorticoid receptor alpha
GRb	Glucocorticoid receptor beta
GRE	Glucocorticoid response element

GTT	Glucose tolerance test
GH	Growth hormone
^{3}H-GC	^{3}H-11-dehydrocorticosteone
H&E	Haematoxylin and eosin
HFD	High fat diet
HPA	Hypothalamic-pituitary-adrenal
H6PDH	Hexose-6-phosphate dehydrogenase
h	Hour
HSL	Hormone sensitive lipase
Hox7	Homeobox 7
HoxC8	Homeobox protein 8
H11β-HSD1	Humanised 11β-hydroxysteroid dehydrogenase type 1
hHSD	Humanised 11β-hydroxysteroid dehydrogenase type 1
IMS	Industrial methylated spirits
IP	Intraperitoneal
ITT	Insulin tolerance test
KI	Knock-in
KO	Knock-out
LAP	Liver-enriched activator protein
LB	Lysogeny broth
LIP	Liver-enriched inhibitory protein
LKO	Liver specific knock-out
LXRa	Liver x receptor alpha
LXRb	Liver x receptor beta
min	Minute
MR	Mineralocorticoid receptor
mRNA	Messenger deoxyribonucleic acid
MS	Metabolic syndrome
Myf5	Myogenic factor 5
MyoD	Myogenic differentiation
N_2	Nitrogen
NaCl	Sodium Chloride
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Dihydronicotinamide adenine dinucleotide phosphate
NRF1	Nuclear respiratory factor 1
Nr3c1	Nuclear receptor subfamily 3, group c, member 1
OD	Optical density
OH-HHA	hydroxy-hexahydro 11-dehydro-corticosterone
OH-HHB	hydroxy-hexahydro corticosterone
OH-THA	hydroxyl-tetrahydro 11-dehydro-corticosterone
OH-THB	hydroxy-tetrahydrocorticosterone
p	P value
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
6PG	6-phosphoglutanate
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PKA	Protein kinase A
PPARγ	Peroxisome proliferator-activated receptor gamma
PRDM16	Pr domain containing 16
PVN	Periventricular nucleus of the hypothalamus
RIPA	Radioimmunoprecipitation assay
RT-PCR	Real time-polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Real-time polymerase chain reaction
s	second
18S	18S ribosomal subunit
SDR	Short chain dehydrogenase/reductase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SIRT1	Sirtuin 1
SNS	Sympathetic nervous system
SRC-1	Steroid receptor coactivator 1
Subcut	Subcutaneous
TAG	Triacylglycerol's
Tbx15	T-box 15
Tcf21	Transcription factor 21
TFAM	Mitochondrial transcription factor A
TLC	Thin layer chromatography
TNFa	Tumour necrosis factor alpha
UCP1	Uncoupling protein 1
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
WAT	White adipose tissue
WHO	World Health Organisation
WT	Wild-type
zF	Zona fasciculate
zG	Zona glomerulosa
zR	Zona reticularis

Contents

Published work

Morgan. S. A, McCabe. E. L, Gathercole. L. L, Hassan-Smith. Z. K, Larner. D. P, Bujalska. I. J, Stewart. P. M, Tomlinson. J. W, Lavery. G. G. 2014. 11β-HSD1 is the major regulator of the tissue-specific effects of circulating glucocorticoid excess. Proceedings of the National Academy of Sciences. 111(24). E2482-91

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1. Chapter 1 – General introduction

1.1 The clinical problem

The global prevalence of obesity is on the increase, leading to ever escalating costs and burdens on health care systems to cope with this modern day epidemic. On its own obesity poses a striking medical problem, however it is additionally linked to a myriad of other diseases. These include metabolic syndrome and type 2 diabetes; the incidences of which are also on the increase. It is therefore highly desirable to define novel approaches to decrease the incidence of obesity and its related metabolic disorders.

1.1.1 Obesity

Obesity is as an accumulation of adipose tissue which is of sufficient levels to impair health (WHO, 2014). The incidence of obesity has escalated to a global epidemic with as many as 30% of adults in the US being obese according to the US Department for Health, with complications substantially contributing to health care costs and mortality, placing a huge financial burden on the economy (Stryjecki et al 2011). Current figures estimated by the World Health Organisation (WHO) place 1.6 billion adults as overweight and at least 400 million of them clinically obese. The increasing trend in obesity is shown in figure 1-1. Within the US alone the cost associated with obesity is around $100 billion per year.

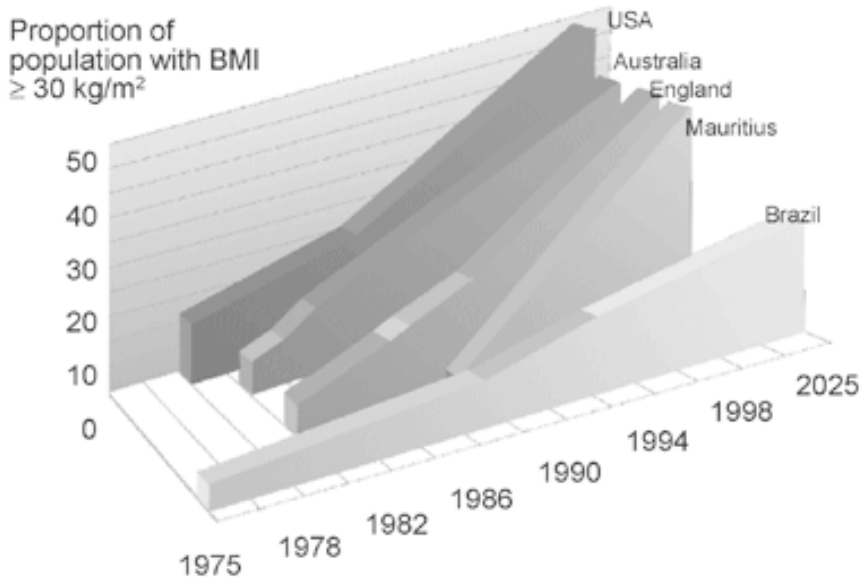

Figure 1-1 Rising incidence of obesity
The level of obesity worldwide has been increasing over the last 50 years and this trend is expected to continue. Further to this the increasing incidence of obesity is not limited to one country or area; it is a global issue with global consequences. Cited from Drugs Today 2006, 42(Suppl. C): 5.

Obesity is the result of a greater degree of energy intake as opposed to energy expenditure (Rohner-Jeanrenaud et al 2015). It is a consequence of the ingestion of energy-dense foods, a decrease in physical expenditure, and an inability of the central nervous system (CNS) to correctly suppress appetite, this leads to an increase in the concentration of white adipose tissue (Cypress et al 2010; Wickelgrem et al 1998).

Despite obesity being driven by environmental factors there is evidence to suggest that inherited factors also affect human fat mass. It has been demonstrated that genetic factors play a role in the development of obesity highlighting that the burden of obesity is not limited solely to the effects of the environment (Faroogi et al 2008).

In addition to the direct complications of obesity, it represents a major risk factor for the development of insulin resistance, and has been implicated in a wide range of pathological

states such as hyperlipidaemia, type 2 diabetes, and cardiovascular disorders (Wickelgren et al 1998). These complications are more frequently attributed to central obesity, in particular at the visceral level, as compared with generalised obesity (Kissebah et al 1994). It has been demonstrated that accumulation of visceral fat strongly increases the risk of cardio metabolic disease, whereas peripheral fat accumulation is relatively protective (Gabriely et al 2002; Jensen et al 2008). Studies have shown that for a given body mass index, there is an increased mortality rate if adipose tissue is located centrally (visceral or omental) as opposed to general obesity (Bjorntrop et al 1991).

1.1.2 Type 2 diabetes

Type 2 diabetes is a metabolic disorder characterised by hyperglycaemia. The prevalence of type 2 diabetes is higher within women than men, and represents a growing disease burden with estimates of approximately 171 million cases worldwide in 2000, and 366 million cases predicted by 2030 (Wild et al 2004). Type 2 diabetes consequently has become a worldwide epidemic and is closely associated with the metabolic syndrome (MS).

Initial treatment for type 2 diabetes is an increase in exercise and dietary modifications; however, if this does not produce a clinical improvement then medications may be required. However the current medications available come with selection of associated negative side effects. Metformin is the first-line treatment choice for the majority of patients with type 2 diabetes, however it does not improve glycaemic control in all patients and can cause gastrointestinal upset (Bolen et al 2007). Thiazolidinedione's such as rosiglitazone are good insulin sensitises but can lead to an increase in body weight, cause fluid retention and induce congestive heart failure (Bolen et al 2007). In addition sulphonylureas such as gliclazide can lead to weight gain and are prone to causing hypoglycaemia (Bolen et al 2007). Due to this improved medications with decreased levels of side effects are highly desirable.

1.2 Adipose tissue

Adipose tissue represents an active endocrine organ (Rosen et al 2006). There are three separate types of adipose tissue within the body: white, brown and brite. Within the body adipocytes maintain lipid homeostasis and energy balance, this is achieved by storing triglycerides and releasing free fatty acids as a response to fluctuating energy needs. In addition, adipocytes produce and secrete signalling molecules, such as leptin and TNFα. (Halaas et al 1995; Hotamisligil et al 1994).

White adipose tissue (WAT) is located primarily within three anatomically distinct areas; subcutaneous, dermal and intraperitoneal. WAT stores energy in the form of triglycerides during times of energy excess and releases fatty acids in times of need, thereby regulating whole body energy homeostasis. Brown adipose tissue (BAT) is located primarily perinatally within humans but has also been found in the interscapular, subclavicular, cervical, auxiliary and paravertebral regions (Sacks et al 2013). BAT releases energy in the form of heat in response to a given stimulus. Brite adipocytes are located within WAT and are induced by a variety of environmental stimuli such as cold exposure, or β-adrenergic receptor agonists. Brite adipocytes exhibit similar molecular and functional features to brown adipocytes, are multi-ocular and express uncoupling protein 1 (UCP1).

1.2.1 Adipose cell lineage

It has recently been demonstrated that brown and white adipocytes originate from separate cell lineages, where primary brown cell cultures are phenotypically distinct from white preadipocytes, and a number of developmental genes are differentially expressed between the two adipocyte depots (Timmons et al 2007).

A selection of experiments have shown that brown and white adipose cells are not sister cells, and that brown adipocytes are in fact more closely related to myocytes where both

originate from a common precursor (Seale et al 2008; Atit et al 2006). This relationship includes a common early transcriptional programme, which is suppressed early in differentiation (Timmons et al. 2007).

Myogenic genes including Myf5 have been shown to be expressed within brown preadipocytes and skeletal muscle, but are absent from white adipose cells (Seale et al 2008). In addition distinct expression patterns have been shown for several developmental genes, including tbx15, hoxc8, and hox7 between brown and white preadipocytes. Further, white adipocytes and not brown adipocytes or skeletal muscle express Tcf21, which suppresses myogenesis. (Timmons et al 2007).

The link between brown adipocytes and myocytes occurs early in differentiation where the expression of MyoD is lost one day into differentiation in brown adipocytes. This could provide an explanation for the decreased expression of myogenin leading to the down-regulation of the skeletal muscle gene expression pattern within brown adipocytes. This decrease in MyoD has been shown to be a consequence of the action of SIRT1 which deacetylates MyoD (Timmons et al 2007).

The similarities between the myocyte and the brown preadipocyte demonstrate the distinct origin for BAT compared to WAT and provides an explanation as to why brown adipocytes ultimately specialise in lipid catabolism rather than storage, similar to oxidative skeletal muscle tissue (Timmons et al 2007).

Further studies have shown that classical white adipocytes contain two types of cells, pure white adipocytes and a more brown-like cell type, named brite cells which have the ability to express UCP1 which until recently was believed to be a unique marker for brown adipocytes. However the full the molecular characteristics of brown adipocytes are not shared with brite cells (Petrovic et al 2009; Guerra et al 1998; Seale et al 2008). Brite cells are not

derived from the Myf-5 lineage like traditional brown adipocytes. They have been shown to have a related but distinct gene expression profile from brown adipose cells (Long et al 2014; Petrovic et al 2009).

Brite cells are most commonly seen following cold acclimatisation, which induces neither an increase in DNA content nor an increase in adipocyte number (Petrovic et al 2009). This supports the notion that a distinct pool of progenitors in the white adipose tissue depot can give rise to beige cells that are similar but not identical to brown fat cells (Wu et al 2012).

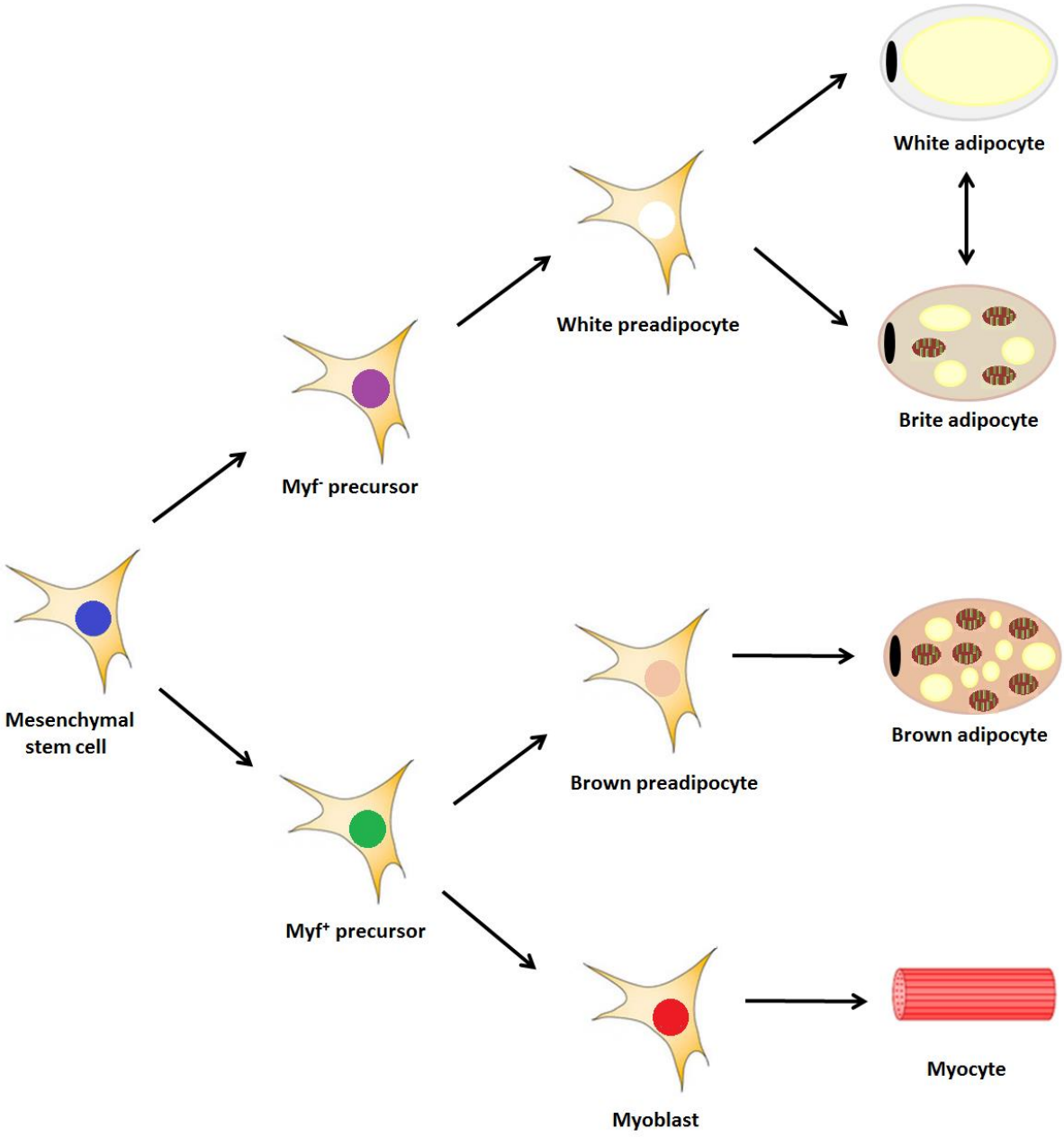

Figure 1-2 Adipose tissue cell lineage
From mesenchymal stem cells there is the production of either Myf^{+} or Myf^{-} precursor cells. Myf+ precursor cells proceed to either a brown pre adipocyte or a myoblast, which become brown adipocytes or myocytes respectively. The Myf^{-} precursor cells become white pre adipocytes which can go on to develop into white adipocytes or brite adipocytes. There is also the possibility given the correct stimulus for the inter conversion of white adipocytes and brite adipocytes.

1.2.2 Key adipocyte genes

There are a selection of key adipocyte genes which play central roles in the development and function of adipose tissue.

1.2.2.1 PRDM16

PRDM16 is cold inducible, and plays a crucial role within the biology of adipocytes (Murholm et al 2009). It primes and triggers brown adipocyte differentiation and is involved in the expression of key brown adipocyte markers (Murholm et al 2009). Silencing of PRDM16 within brown precursor cells has been shown to blunt the induction of typical brown fat marker genes, like UCP1, and PGC-1α (Murholm et al 2009). PRDM16 also acts as a co-regulator to activate PGC-1α and together they drive the molecular phenotype of brown fat cells (Seale et al 2007). Furthermore, expression of PRDM16 is correlated with mitochondrial biogenesis, and uncoupled cellular respiration (Seale et al 2007).

Over-expression of PRDM16 leads to protection from obesity on a high-fat diet due to an increase in energy expenditure which is not related to physical activity, demonstrating a key role for PRDM16 in energy expenditure (Seale et al 2014). Further these mice also demonstrate an increased glucose tolerance and improved insulin sensitivity, in addition they expressed greater levels of brown fat selective genes compared to wild-type (WT) when exposed to a high fat diet (HFD) (Seale et al 2014). Overall this demonstrates that the action of PRDM16 in adipose tissue increases energy expenditure to oppose weight gain and increase the disposal of glucose (Seale et al 2014).

1.2.2.2 PPARγ

The transcription factor PPARγ is highly expressed in both BAT and WAT and its expression is further induced upon cold exposure (Kelly et al 1998; Murholm et al 2009). PPARγ is crucial to the differentiation and function of brown and white adipocytes, and expression of

PRDM16 within WAT triggers the formation of brown adipose like cells (Tzu-Ann et al 1996; Guerra et al 1998; Seale et al 2007). Expression of PRDM16 induces a dramatic increase in the volume of interscapular BAT within depots (Tzu-Ann et al 1996). Further to this PPARγ cooperates with PGC1-α to induce the expression of UCP1 and other typical brown adipose markers (Tzu-Ann et al 1996; Tiraby et al 2003; Pisani et al 2011; Watanabe et al 2008; Murholm et al 2009).

1.2.2.3 PGC-1α

PGC-1α is a cold inducible co-activator of nuclear receptors (Wu et al 1998; Puigserver et al 1998). PGC-1α is crucial to brown adipogenesis, where it is involved in UCP1 expression and mitochondrial biogenesis (Murholm et al 2009). Adipocytes which express PGC-1α demonstrate an elevated expression of key genes pivotal to mitochondrial function and adaptive thermogenesis, which includes an elevation in the expression of genes central to oxidative phosphorylation (Puigserver et al 1998; Tiraby et al 2003; Wu et al 1998; Uldry et al 2006).

It has been demonstrated that with insulin resistant states there is a decrease in both PPARγ and PGC-1α and a resultant reduction in adipose tissue mitochondrial content (Tiraby et al 2003). PGC-1α is also reduced by high fat feeding, indicating PGC-1α's involvement in mediating high-fat diet-induced decreases in adipose tissue mitochondrial proteins (Sutherland et al 2008).

Therefore an increase in expression of PGC-1α could lead to both adaptive thermogenesis and fatty acid oxidation. These increases could consequently lead to desirable decrease in adipose mass (Tiraby et al 2003).

1.2.3 Brown adipose tissue (BAT)

In rodents and humans, brown adipose is predominantly found in the interscapular and paraspinal regions, and the quantity of BAT shows a decrease as mammals go from the neonatal to adult stage (Himms-Hagen et al 2001; Rohner-Jeanrenaud et al 2015). Brown adipocytes are multi-ocular adipocytes rich in iron containing mitochondria with densely packed cristae expressing UCP1, which gives brown adipose its characteristic brown colour. BAT also contains a high number of capillaries due to its need for oxygen, and is critical for energy equilibria (Nicjolls et al 1984; Cypress et al 2010). The structure of the brown adipocyte is shown in figure 1-3.

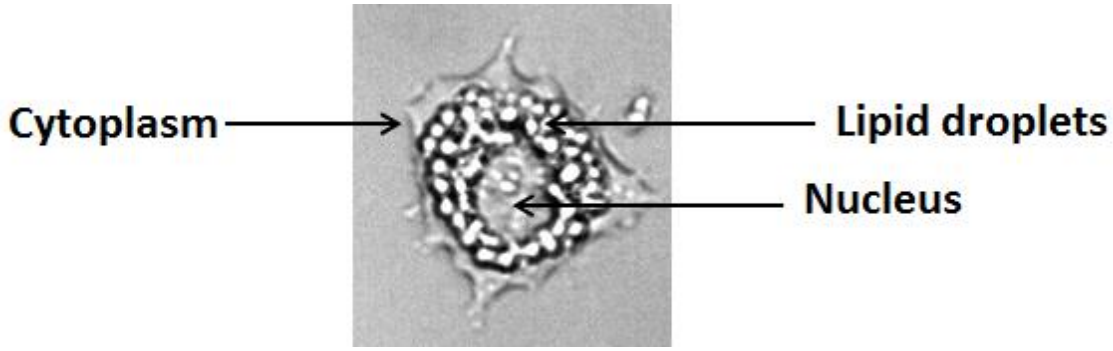

Figure 1-3 Brown adipocyte
Brown adipocytes contain a nucleus and multiple small lipid droplets, encased with cytoplasm. They also have a vast number of mitochondria and are highly vascularised.

BAT utilises lipids and carbohydrates to generate heat by using UCP1 to uncouple electron transport from oxidative phosphorylation, thus playing an active role in energy expenditure and fatty acid oxidation (Nicjolls et al 1984; Cannon et al 2008; Stier et al 2014). Being located in the mitochondrial inner membrane, UCP1 acts as a proton transporter to abolish the proton gradient, the driving force of adenosine triphosphate (ATP) synthesis (Watanabe et al 2008). Thus the free energy conserved in the proton gradient is dissipated as heat (Watanabe et al 2008). This is the mechanism utilised to produce heat in BAT, and is referred to as non-

shivering thermogenesis, which is in contrast to shivering thermogenesis in muscle tissues (Watanabe et al 2008). This non-shivering thermogenesis is highly important in neonatals and small rodents, whose muscle tissues are not well developed (Watanabe et al 2008). It is also utilised in hibernating animals.

BAT plays a pivotal role in the modulation of body fat stores due to its role in energy expenditure. Because of this BAT has potential in combating obesity and diabetes. Further evidence to support this is that mice deficient in BAT develop obesity with insulin resistance and other metabolic abnormalities linked to obesity (Enerback et al 2010; Lowell et al 1993; Hamann et al 1995).

Recent studies using non-invasive imaging technologies such as ^{18}fluoro-labeled 2-deoxy-glucose positron emission tomography (^{18}FDG-PET) scanning and magnetic resonance imagery (MRI) scanning show adult humans possess significant amounts of active brown fat deposits (Cypress et al 2009).

1.2.4 Incidence of brown adipose tissue in humans

BAT is found mostly in new-borns and hibernating animals. It was initially thought that BAT was only evident within new-borns and that its presence decreases with age as a consequence of a decreased need for thermogenesis. However recent studies have conflicted with this initial thought and have shown the existence of BAT within adult humans, Cypress et al 2009 demonstrated this using 18F-FDG PET-CT scans, and showed that BAT was most commonly located in the cervical-suprasclavicular depot. Estimates of BAT mass and activity from FDG-PET studies suggest that humans have, on average, 50–80 g of BAT (Peirce et al, 2014). In addition Sharp et al 2012 have shown that these UCP1-positive brown fat cells located within adult humans have molecular characteristics more common to murine beige cells rather than classical brown adipose cells. This therefore demonstrates conflicting opinions on the true

nature of the UCP1 positive cells discovered, with some sources claiming them to be true BAT cells whereas other sources claiming they are brite cells. It may well transpire that there are detectable levels of BAT within adult humans and that the presence of brite cells can be induced either environmentally or experimentally.

Recent studies have demonstrated that chronic cold acclimation in human subjects increased the quantity of metabolically active BAT, thus leading to an increase in the oxidative capacity of the BAT (Blondin et al 2014; Lichtenbelt et al 2015). Further to this it has been shown that cold-induced increases in thermogenesis have been accompanied by a decrease in body weight (Yoneshiro et al 2013). These results are in keeping with data showing a physiological role of BAT in whole-body energy expenditure, glucose homeostasis, and insulin sensitivity in humans during prolonged cold exposure (Chondronikola et al 2014). This leads brown adipose tissue to have a potential in protecting against obesity (Cypress et al 2009).

1.2.5 Thermogenesis

BAT represents the main site for adaptive thermogenesis. BAT responds to changes in environmental factors, for example diet and cold temperatures, where it increases energy expenditure. An increase in diet is sensed by the brain, which, to avoid excessive weight gain triggers a decrease in food intake and an increase in energy expenditure (Lowell et al 2000). When exposed to a cold environment there is uncoupling of ATP synthesis and the production of heat.

The two most common forms of thermogenesis seen within BAT are non-shivering thermogenesis and diet-induced thermogenesis, which are induced initially by the sympathetic nervous system (SNS) (Rothwell et al 1979). BAT requires ATP for cell maintenance,

therefore thermogenesis in BAT must be controlled in response to necessity (Watanabe et al 2008).

1.2.6 Non-shivering thermogenesis

The action of non-shivering thermogenesis occurs within thermogenically active target tissues, and is initiated by exposure to cold temperatures (Landsberg et al 1984; Himms-Hagen et al 1984; McMillian et al 2015). During active thermogenesis BAT becomes highly vascularised, due to the increased need for blood supply (Timmons et al 2007). The initial stimulus of the cold activates the sympathetic nervous system (SNS) which leads to the release of catecholamines including neural-derived noradrenaline which stimulates the predominantly fat specific β3-adrenergic receptor, and α2 adrenergic receptors (Himms-Hagen et al, 1991; Collins et al 1997; Cannon et al 2004). These receptors signal through cyclic-AMP (cAMP), which is produced via adenylate cyclase, leading to activation of protein kinase A (PKA) (Watanabe et al 2008; Valente et al 2014). PKA up regulates the expression of PGC1-α, which is highly expressed in brown adipose and activates the adaptive thermogenic gene programme which includes up regulation of UCP1, genes in the mitochondrial transport chain, mitochondrial biogenesis, inhibition of apoptosis, fatty acid oxidation, and increased oxygen consumption through co-activation of transcription factors such as PPARγ and NRF1, and an overall increase in thermogenic capacity of the animal (Puigserver et al 1998; Himms-Hagen et al 1990; Cannon et al 2004; Wu et al 1999; Collins et al 1997). Further to this, activation of PKA also leads to the activation of lipolysis and the release of free fatty acids (FFAs) which enter the mitochondria where they act as an energy source. FFAs are used as fuel to supply free energy for thermogenesis and also to activate the function of UCP1 (Krauss et al 2005).

Early on following cold exposure, mobilisation of fatty acids from WAT is used as a primary source for activation of BAT as opposed to the breakdown of fat depots within BAT (Deiuliis et al 2010). Under conditions of long-term cold exposure where there is limited dietary glucose and circulating fatty acids, the TAG in BAT will likely be hydrolysed by ATGL to generate the required energy substrate for non-shivering thermogenesis (Deiuliis et al 2010).

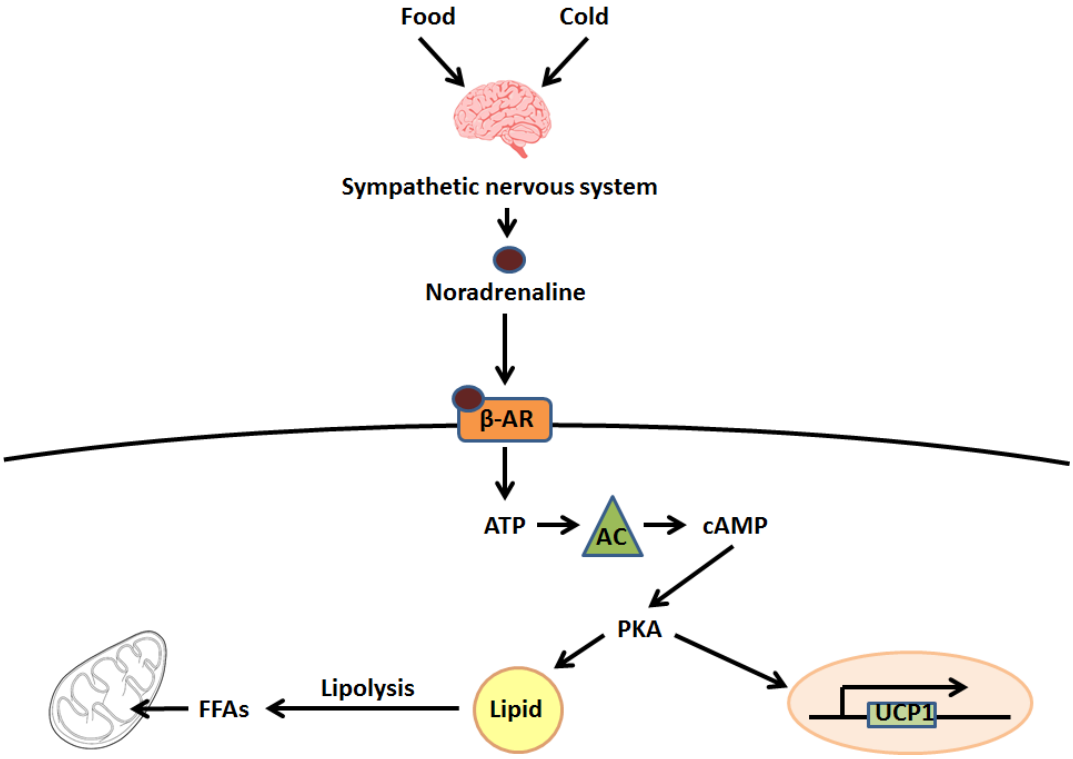

Figure 1-4 Non-shivering thermogenesis
When exposed to cold or following a meal there is activation of the sympathetic nervous system. This leads to the release of noradrenaline which is able to bind to the B-adrenergic receptor. This binding stimulates the production of cAMP via adenylate cyclase. cAMP then activates protein kinase A which is able to activate lipolysis and result in the release of free fatty acids which are transported into the mitochondria, or increase the expression of UCP1.

In non-shivering thermogenesis there is also an increase in BAT cell proliferation and differentiation following cold stimulation (Cannon et al 2004; Himms-Hagen et al 1990; Bukowiecki et al 1982). Several reports demonstrate that enlargement of BAT induced by

cold exposure is caused by an increase in the number of mature brown adipocytes, these results indicate that cold stimulation induces a series of gene expression changes that are needed for proliferation and differentiation of brown adipocytes precursor cells in BAT (Bukowiecki et al 1982).

The mitochondrial UCP1 expressed in adaptive thermogenesis is a proton channel protein in the inner mitochondrial membrane which functions to uncouple electron transport from oxidative phosphorylation by promoting a proton leak across the mitochondrial membrane, and utilising lipids and carbohydrates to generate heat by converting the electrochemical potential of the mitochondria into heat instead of ATP (Houstek et al 1978; Rousset et al 2004; Nicjolls et al 1984). When BAT generates heat it leads to unrestrained oxidation by drawing lipids and carbohydrates from outside the cell (Watanabe et al 2008). The idea that UCP1 might be involved in regulating energy balance and the abnormalities in brown fat might result in obesity was first proposed by Rothwell and Stock in 1979 (Rothwell et al 1979).

Reflecting the physiological uncoupling of oxidative phosphorylation BAT mitochondria have a high oxidative capacity, a high content of cytochrome oxidase, but a low content of ATP synthetase (Houstek et al 1978).

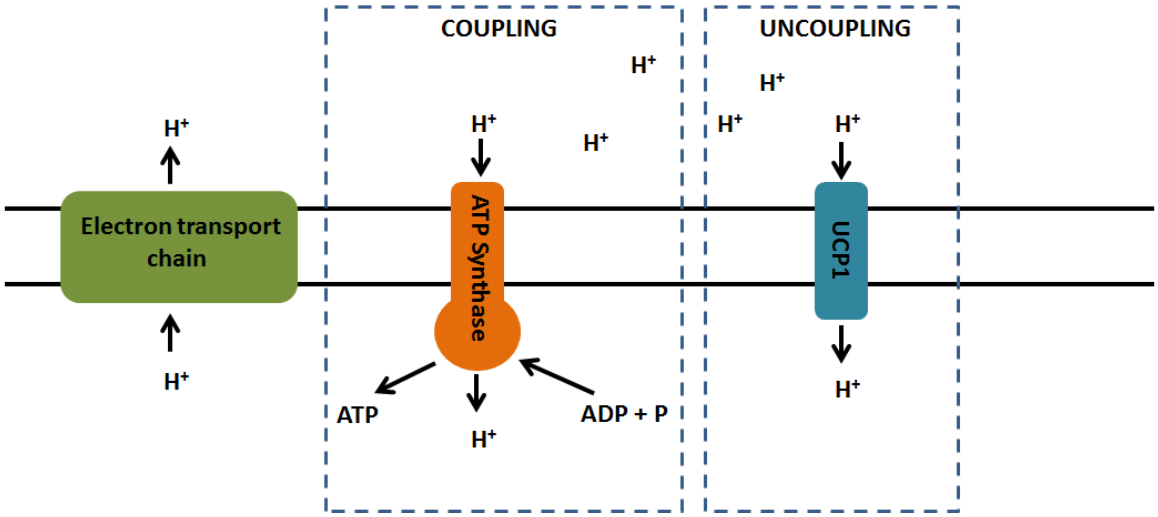

Figure 1-5 Uncoupling of ATP synthesis by UCP1
UCP1 is able to uncouple ATP production via ATP synthase into the production of heat. In doing so it utilises the proton gradient set up by the mitochondrial electron transport chain to produce heat.

The key role of PGC1-α in adaptive thermogenesis has been shown in PGC-1α deficient mice that cold sensitive with poor expression of UCP1 (Lin. J et al 2004). In addition the BAT appears morphologically abnormal, with abundant accumulation of large lipid droplets, reminiscent of WAT (Lin et al 2004).

An important issue which is often overlooked is that normal animal house conditions (18-22°C) are a chronic thermal stress to mice (Golozoubova et al 2004). To maintain their body temperature in these conditions the mice increase their metabolism and thus their food intake to around 50%-60% above basal levels. It is hypothesised that this thermal stress induced by standard animal house conditions may influence the outcome of metabolic studies (Golozoubova et al 2004).

Many studies have been conducted over the past three years on BAT which have led to vast increase in our understanding of the role of BAT in adult humans. This has led to a substantial increase in belief for the possible use, or manipulation of BAT to increase energy

expenditure and thereby aid in regulating body weight and ameliorating metabolic diseases (Tam et al 2012).

1.2.7 Effect of age on brown adipose tissue

It is not disputed that ageing is strongly negatively correlated with BAT mass. Using immunohistochemistry or PET/CT it can be seen that there is a decrease in BAT following adolescence and a vast decrease after 80 years of age (Sacks et al 2013). However whether BAT function deteriorates with age is an area of debate. It has been proposed that with increasing age there is a decline in the metabolic effect of BAT (Tam et al 2012). This has been further demonstrated by Saito et al 2009, who showed that there is a higher incidence of BAT following cold exposure in a younger cohort. The decrease in mitochondrial biogenesis and function with age has been linked to a decrease in BAT activity (Graja et al 2015). Contradictory to these findings is a study by Van Marken Lichtenbelt et al 2009, who showed that there was no correlation between BAT activity and age, the study showed that the decrease in BAT activity which is seen with increased age is related to a decrease in the quantity of BAT rather than a decrease in activity. It is clear that with age there is a marked decrease in BAT and this will consequentially lead to a decrease in BAT activity, however there is conflict as to whether the decrease in BAT activity seen with age is solely attributable to a decrease in BAT mass or whether there are other factors which play a part. This is an area which requires further investigation and offers up the potential of a therapeutic if it transpires that BAT function is impaired with age, as preventing this may prove metabolically beneficial.

1.2.8 Potential role of brown adipose tissue in obesity prevention/therapeutic target

BAT has emerged as a possible therapeutic target in the prevention and treatment of obesity, type 2 diabetes and the MS. This is due to its role within energy expenditure, where it utilises

fatty acids for the production of heat. In support of this Vijgen et al 2001 reported an increase in BAT activity after weight loss in obese patients given gastric banding surgery, suggesting the effectiveness of successful recruitment of BAT in body fat reduction in humans.

Within rodent studies an increase in BAT has been shown to promote energy expenditure, reduce adiposity, and protect mice from diet-induced obesity (Almind et al 2007). Furthermore BAT ablation leads to obesity in response to a HFD (Tateishi et al 2009). The interest in BAT is of further importance due to the recent discovery of its presence within adult humans, although there is conflicting data as to whether it is true BAT within humans or brite cells.

1.3 White adipose tissue (WAT)

WAT represents the majority of the adipose tissue in the human body, within humans it constitutes up to 20% of the body weight in men and around 25% of the body weight in women. White adipocytes consist of a large lipid droplet surrounded by a thin layer of cytoplasm and the nucleus, thus demonstrating uni-ocular morphology. The primary use of WAT is in the storage of energy where it stores triglycerides within individual adipocytes for use in times of nutrient shortage, this has evolutionary enabled humans to survive for longer periods between meals, where energy is stored mainly as triglycerides and released as fatty acids during fasting periods (Rohner-Jeanrenaud et al 2015). Further WAT contributes to whole body insulation, and is also an active endocrine organ where it releases FFAs and adipokines such as leptin, resistin and adiponectin.

There are many white adipose depots within the body, which can be separated into two areas with highly varying biology; visceral and subcutaneous adipose. An increase in visceral adipose tissue leads to an increased potential for cardiometabolic disease, conversely accumulation of peripheral adipose offers relative protection (Wamill et al 2011). Further

visceral adipose tissue is likely to contribute most strongly to the metabolic consequences of obesity and has a higher density of GC receptors, which may contribute to visceral adipose tissue being less insulin sensitive (Morton et al 2004; Wamil et al 2011; Jensen et al 2008). There are also differences between these two locations in their response to GCs where visceral adipose has an increased expansion in response to increased plasma cortisol or adipose 11β-HSD1 (Wamill et al 2011).

1.3.1 Differences between white adipose tissue depots

Studies have shown that there are profound differences between subcutaneous and visceral adipose, in both the functioning of these depots and also the metabolic consequences of their accumulation. There is a different expression of genes in subcutaneous and mesenteric fat depots, showing differences between the depots on a molecular scale (Wamil et al 2011). After the initial observations of Vague et al in 1947, it is well known that the distribution of adipose tissue is central in determining the risk of developing metabolic complications, more so than the degree of excess adipose tissue. This is supported by Fujioka et al 1991 who showed that reduction of visceral adiposity is accompanied by improvements in intermediary metabolism.

Studies by Gabriely et al 2002 showed that the removal of two intra-abdominal adipose depots prevents age-dependent insulin resistance and substantially delays the onset of glucose intolerance and diabetes, whereas removal of subcutaneous adipose tissue does not demonstrate this effect.

When compared to subcutaneous adipocytes, visceral adipocytes have higher basal and adrenaline-stimulated levels of intracellular cAMP (Ostman et al 1979; Wahrenberg et al 1989; Richelseon et al 1991). In addition to classical white cells described earlier WAT also

contains cells which have a brown-like characteristic and have been termed brite cells. These cells have been shown to be up regulated following exposure to cold.

1.4 Brite adipose tissue

The term brite (brown in white) adipose tissue has been coined to describe a subset of cells situated within classical WAT depots that show a genetic fingerprint similar to that of classic brown adipocytes. These multi-ocular brown fat-like cells are detectable at low numbers within subcutaneous and visceral WAT depots of primary cultures of mice maintained at ambient temperatures with no prior cold exposure, and show levels of mRNA encoding UCP1, Cidea, Cox7a1 and PGC1-α, although their expression is at lower levels than that seen in classical BAT tissue (Wu et al 2012), this has also been demonstrated in humans (Tiraby et al 2003). However once stimulated via cAMP stimulation, brite significantly increases its levels of thermogenic genes including; UCP1, mitochondrial genes Cox7a1 and Cox8b, and transcriptional regulators PRDM16 and PGC-1α, to similar levels as that seen in classical brown fat cells (Wu et al 2012; Barbatelli et al 2010; Walden et al 2012; Cousin et al 1992). This change is more prominent in subcutaneous adipose compared to visceral adipose following cold exposure (Barbatelli et al 2010; Lee et al 2012). This ability of cells within classical WAT depots to express UCP1 has been recognised for 30 years as first demonstrated by Young et al in 1984.

Cold-acclimatised pre-adipocytes in WAT have slightly more pronounced brown characteristics often with large mitochondria (Barbatelli et al 2010). This demonstrates that brite cells have the capacity to switch between an energy storage and energy dissipation phenotype (Wu et al 2012). Further Young et al 1984 showed similar changes in brite cells following cold acclimatisation, and also that in mice maintained at standard animal house

conditions (23°C) there was existence of brown fat cells in traditional white fat cell pads in mice, due to chronic stimulation by cold exposure (Young et al 1984; Seale et al 2014).

Brite cells however differ from classical BAT cells in that they are not derived from a myf5-positive lineage, and their appearance is through a process known as browning, and are termed beige or brown-in-white (brite) cells (Seale et al 2011).

A strong characteristic of brite cells is its high levels of UCP1 expression, where the induction of UCP1 mRNA upon browning can be up to 100-fold increase (Nedergaard and Cannon et al 2013). Despite this strong induction of UCP1 expression the expression levels are still <20% of those found within classical brown depots (Nedergaard and Cannon et al 2013; Walden et al 2012). It has therefore been questioned whether there will be sufficient UCP1 within brite depots to mediate significant thermogenesis as the amount of UCP1 is the rate-limiting for thermogenesis as studied in brown-fat mitochondria (Shabalina et al 2010). However Nedergaard et al 2013 demonstrated that the UCP1 was thermogenically functional, however the contribution from inguinal mitochondria was one-third that of interscapular brown-fat mitochondria, indicating that classical BAT would still predominate in thermogenesis.

The expression of certain genes is also influential in the development of brite adipocytes. Firstly in the absence of PRDM16 the differentiation of most brown fat-selective genes, including UCP1, Cidea and Cox8b are decreased (Seale et al 2011). These results indicate that PRDM16 is required in mature adipocytes for the full activation of the brown fat-like gene program (Seale et al 2011). Further PPARγ activation enables white preadipocyte cultures to acquire brown adipocyte-like characteristics. (Petrovic et al 2010). This activation is achieved through PGC-1α which coactivates the PPARγ2/RXRa heterodimer to stimulate

UCP1, cytochrome c, fatty acid oxidation enzyme and other brown adipocyte marker expression (Tiraby et al 2003).

These brite cells have been clearly distinguished from classical brown cells: their expression pattern of various genes, including the absence of myogenic factors, clearly distinguishes them from the classic brown adipocytes where they have a molecular signature distinct from those of genuine brown adipocytes (Petrovic et al 2010; Pisani et al 2011).

Due to their gene expression pattern varying from that of classical brown adipocytes, it is clear that brite cells do not emanate from a few precursors of classic brown adipocytes resident in the precursor population, (Tiraby et al 2003), it has been shown that cold acclimatisation favours direct transformation of white to brown adipocytes in WT, a process termed trans differentiation (Barbatelli et al 2010). However only a subset of cells within WAT can attain brown characteristics (Petrovic et al 2010). This has led to the hypothesis that brite cells are the result of precursor cells within WAT which are able to differentiate following a given stimulus (Tiraby et al 2003).

1.4.1 Potential role of brite as a therapeutic target

The white/brown plasticity of adipose tissues may have considerable medical implications, since the brown-like phenotype seems to correlate with a reduction in the development of obesity and diabetes in rodent models (Murano et al 2009; Bachman et al 2002). Further an increase in expression of PGC-1α within white adipose may lead to expression of UCP1 and fatty acid oxidation (Tiraby et al 2003). This may consequently lead to a desirable decrease in adipose mass. Further Tsukiyama-Kohora et al 2001 have shown that there is an increase in brite cells within WAT in KO strains. These strains also demonstrate resistance to diet induced obesity and also an improvement in their glucose metabolism. Similar studies by

Hansen et al 2004; Christian et al 2005; Wang et al 2008; Romanatto et al 2009 have shown much the same.

Glucocorticoids (GC) have also been demonstrated to have a role in obesity where when in excess they have been shown to contribute towards metabolic syndrome, Cushing's disease and insulin resistance.

1.5 Glucocorticoids

Since their elucidation (Nobel Prize 1950) as potent anti-inflammatory agents (Hench et al 1950), GCs have become one of the most widely prescribed drugs in medical practice for many conditions including asthma, chronic obstructive pulmonary disease and inflammatory disease. Further, GCs have also been exploited for their antiproliferative and antiangiogenic actions for the treatment of cancers (Vilasco et al 2011). GCs also perform a regulatory role within metabolic and homeostatic processes where they are central in the stress response. However GCs also have many diverse side effects such as contribution to obesity, Cushing's syndrome, metabolic syndrome and insulin resistance.

GCs are an important class of adrenocorticosteroids that effect their action by binding to the GR. Within humans cortisol represents the active GC with cortisone its inactive precursor. Both cortisol and cortisone are synthesised from cholesterol within the Zona fasciculate (zF) of the adrenal cortex, where cortisol is released in circadian and ultradian rhythms (Russell et al 2010). The release of cortisol from the adrenal glands is regulated by the hypothalamic-pituitary-adrenal (HPA) axis. The availability of natural GCs in tissues is also regulated locally by the 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) enzyme, which was first discovered by Amelung et al 1953.

1.5.1 Cushing's syndrome

Cushing's syndrome (CS) is named after Harvey Cushing, who documented a patient with an excess of GC in 1912. Greater than 99% of the cases of CS that are diagnosed are a consequence of the prescription of excessive quantities of exogenous therapeutic GC. Classic signs and symptoms of CS include weight gain, menstrual irregularity, depression, decreased memory, bruising, facial plethora, abdominal striate, and increased centripidal, supraclavicular, or dorsocervical fat.

1.5.2 Therapeutic use of glucocorticoids

Synthetic GCs are frequently prescribed to treat a myriad of ailments and resemble natural GCs. Prednisolone, dexamethasone, and budesonide are among the commonly prescribed GCs within medical practice. Synthetic GCs differ from natural GCs by both their potency and metabolic clearance, furthermore unlike natural GCs, synthetic GCs do not bind corticosteroid-binding globulin and are thereby not susceptible to their regulation of available levels, further they are not susceptible to inactivation by 11β-HSD2, therefore there is an increase in GC availability (Krasner et al 1999; Clark et al 2012).

Adverse effects of long-term GC therapy in supraphysiological doses include suppression of the HPA axis, and GCs represent the most common cause of drug-induced diabetes (Krasner et al 1999; Lansang et al 2011). The mechanism by which GCs cause diabetes is predominantly through insulin resistance rather than decreased insulin production (Lansang et al 2011).

1.5.3 Adrenal glands and glucocorticoid production

The adrenal glands are an endocrine gland located at the top of the kidneys, and weigh about 10-15g in humans (Mitani et al 2014). The first report on the physiological role of the adrenal gland was presented by Thomas Addison in 1855 (Addison et al 1855). Their primary

function is in the production of hormones in response to stress where they release corticosteroids such as cortisol, and catecholamine's such as adrenaline and noradrenaline. Further to this they also produce androgens within their innermost cortical layer.

Both of the adrenal glands have a distinct structure composing of two structurally and functionally distinct endocrine tissues, the outer adrenal cortex and the inner medulla (Orth et al 1992). The adrenal cortex is mainly responsible for the production of corticosteroids while the medulla is mainly responsible for the production of adrenaline and noradrenaline. The cortex is further subdivided into concentric zones, the Zona glomerulosa (zG), the zone fasiculate (zF) and the Zona reticularis (zR), the zG is responsible for the secretion of mineralocorticoids, the zF for glucocorticoids, and the zR for androgens (Arnold et al 1866; Mitani et al 2014). The structure of the adrenal gland is shown in figure 1-6.

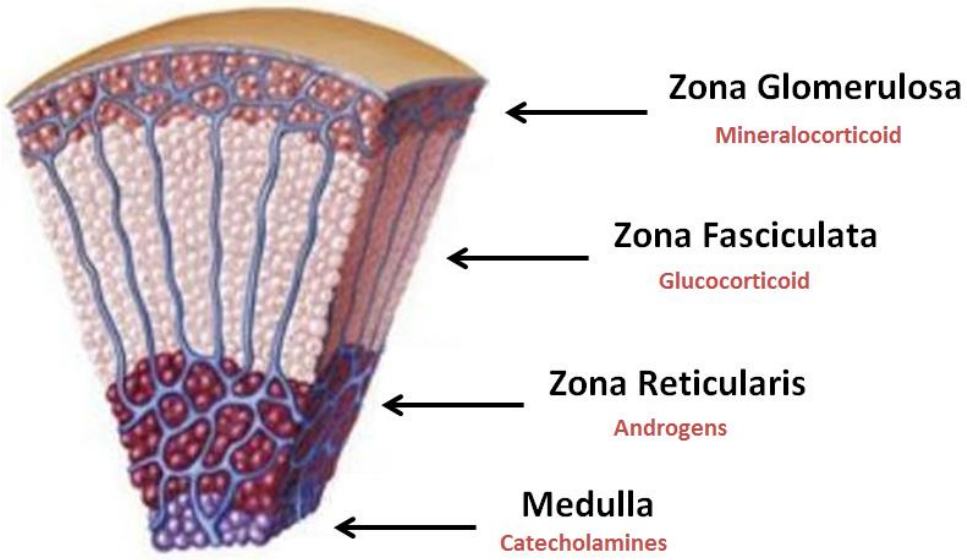

Figure 1-6 Structure of the adrenal gland
The adrenal gland is split into two distinct sections, the adrenal cortex and the adrenal medulla. The adrenal cortex can be further subdivided into the Zone Reticularis, the Zona Fasiculate and the Zona Glomerulosa. The Zona Reticularis is primarily responsible for the production of androgens, the Zona Fasiculate for the production of glucocorticoids and the Zona Glomerulosa for the production of miaralocorticoid. The adrenal medulla is responsible for the production of catecholamines. Adapted from medicalook.com.

1.5.4 Cortisol and cortisone

Cortisol and cortisone are central GCs within humans where they impact a variety of cardiovascular, metabolic, immunologic, and homeostatic functions. Cortisol is released following stimulation of the HPA axis and also via the oxo-reductase action of 11β-HSD1. Cortisone is primarily generated within the kidneys by 11β-HSD2 from active cortisol.

Cortisol is secreted at levels of around 15 mg/day into the blood stream, when in the blood stream 96% of cortisol exists bound to plasma proteins; 90% to corticosteroid binding protein (CBP) and 6% to serum albumin, with only a small amount being unbound and therefore biologically active (Koteleytsev et al 1997; Lepsin et al 2011). The levels of cortisol are highest in the morning and following a meal and are at their lowest in the evening (Rosmond et al 1998). Cortisone and 11-DHC have low affinities to bind to CBG and albumin, and therefore exist mostly in the free form. This enables them to enter target cells and undergo conversion to their active 11b-hydroxy forms via 11β-HSD1 (Lepsin et al 2011; Slaunwhite and Sandberg et al 1959; Cope and Black et al 1958).

1.5.5 Hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis is a complex set of interactions between the hypothalamus, the pituitary gland and adrenal glands, controlled via ultradian and circadian rhythms, with the purpose of regulating the levels of cortisol within the body (Russell et al 2010; Carnes et al 1989).

Central regulation of the HPA axis occurs within the hypothalamus where a given stressor initiates the release of corticotrophin-releasing factor (CRF) and arginine vasopressin (AVP), which are produced within the paravocellular neurons of the hypothalamic periventricular nucleus (PVN). These act upon the corticotrophs of the anterior pituitary gland (Warne et al 2009) to initiate the secretion of adrenocorticotrophic hormone (ACTH) into the

adrenal circulation (Carnes et al 1989). ACTH acts on the adrenal cortex to promote the biosynthesis of cortisol from cholesterol, and its subsequent release into the circulation (Chrousos et al 1995). The HPA axis is shown is figure 1.7.

The HPA axis is tightly regulated by negative feedback mechanisms. Binding of GCs to central GRs, leads to inhibition of the HPA axis (Rosmond et al 1998). This inhibition involves signalling networks at the pituitary, hypothalamus and hippocampus, resulting in inhibition of CRH, AVP, and ACTH secretion in a negative feedback loop by which the HPA axis inhibits its own secretion (Watts et al 2005; Eijsbouts and Murphy et al 1999). The importance of this negative feedback loop is highlighted in patients treated with exogenous GCs, where there is inhibition of endogenous GC release resulting in atrophy of the adrenal cortex (Rang et al 2001).

Obesity leads to dysregulation of the HPA axis, resulting in lower morning cortisol levels (Wajchenberg. B. L. 2000; Rosmond. R. 1998). This is due to an increase in output of cortisol due to central obesity which activates the negative feedback loop.

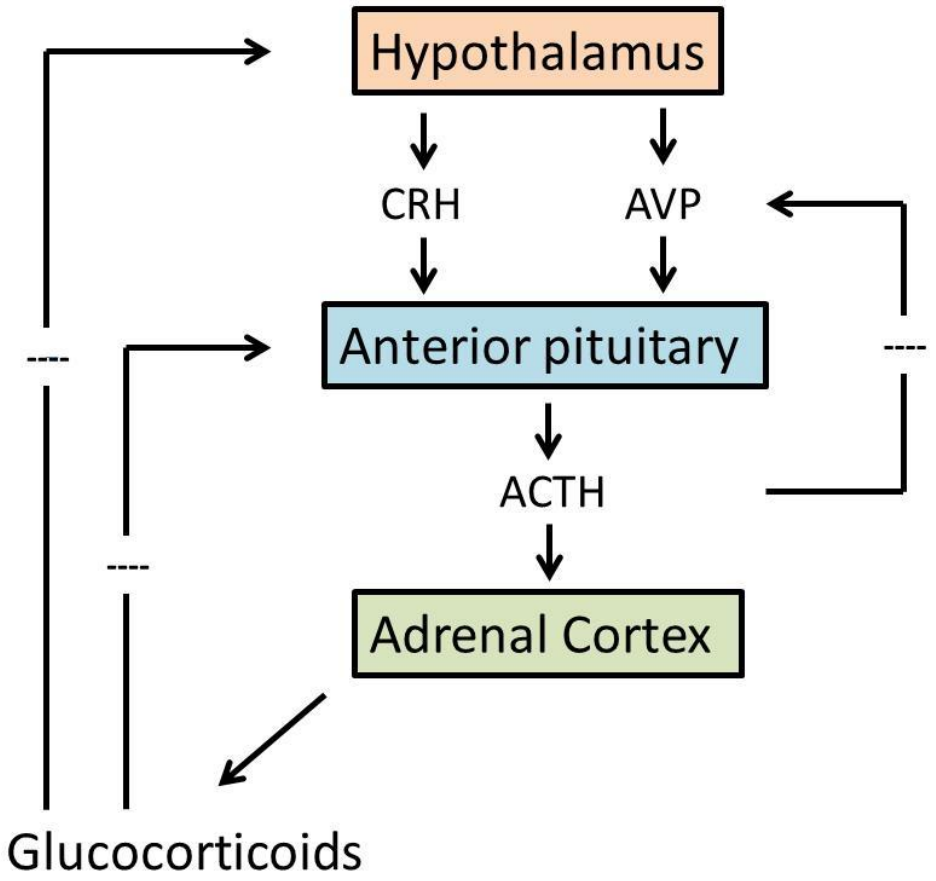

Figure 1-7 The hypothalamic-pituitary-adrenal axis
The hypothalamus produces CRH and AVP in response to a given stimulus. These induce the production of ACTH from the anterior pituitary. ACTH then stimulates the adrenal cortex to release glucocorticoids. These glucocorticoids produced act as part of a negative feedback loop to inhibit the hypothalamic-pituitary-adrenal axis and thus prevent the over production of glucocorticoids.

1.5.6 Glucocorticoid receptor

The biological action of GCs is mediated through intracellular glucocorticoid receptors (GR). The GR is expressed in almost every cell and regulates genes controlling development, metabolism, and the immune response (Pujols et al 2002). Therefore appropriate control of GR expression is critical for maintenance of cellular and organismic homeostasis.

The GR is the product of a single gene; nuclear receptor superfamily 3, group C, member 1 (nr3c1), which is located on chromosome 5q31-32 in humans (Kadmiel et al 2013).

Two human isoforms of the GR have been identified, termed GR-α and GR-β, which originate from the same gene by alternative splicing of the GR primary transcript. GR-α is the predominant isoform of the receptor and the one that shows steroid biding activity, GR-β is located within inflammatory cells (Hollenberg et al 1985; Pujols et al 2002).

Unbound GR is located within the cytoplasm of the cell, where it is bound to chaperone proteins such as heat shock protein 90 (Kadmiel et al 2013). Upon the binding of GC to the receptor the GR undergoes a conformational change and the GR-GC complex is translocated to the nucleus. Within the nucleus the GR-GC complex mediates either transactivation or transrepression of target genes, by binding to specific DNA motifs termed glucocorticoid response elements (GREs) (Pujols et al 2002). Once bound to GREs the GC/GR complex is able to recruit and activate transcriptional co-activator molecules such as CREB binding protein (CBP), steroid receptor coactivator-1 (SRC-1) and other factors, allowing for the transcription of GC responsive genes (Adcock et al 2004). The mechanistic action of the GR is shown in figure 1-8.

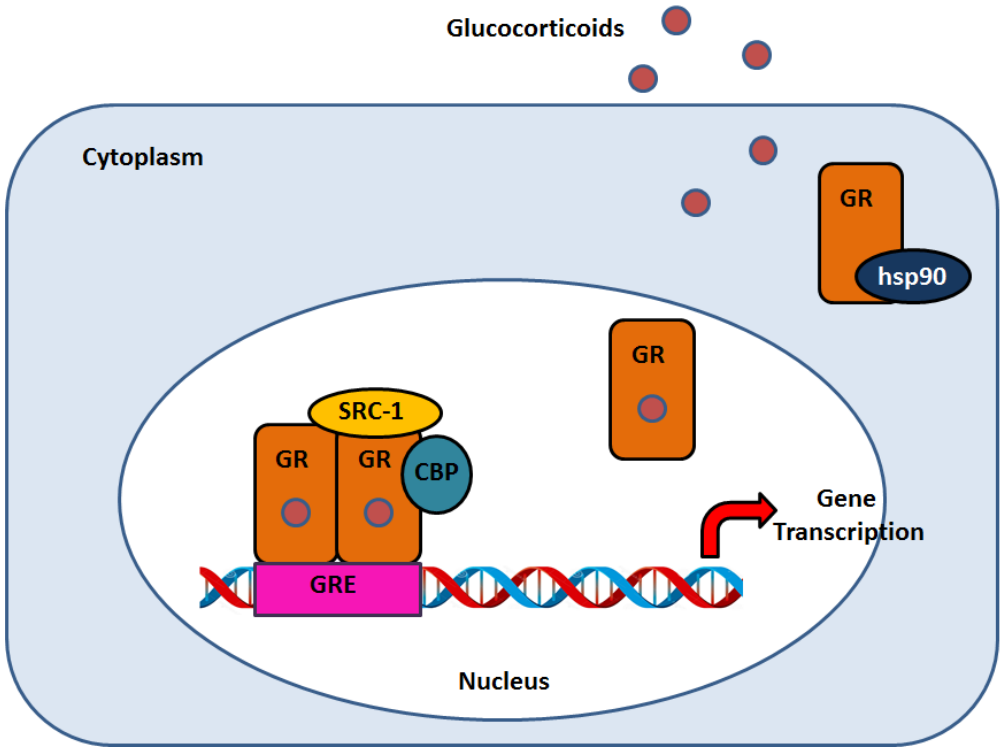

Figure 1-8 Action of the glucocorticoid receptor
Upon the binding of glucocorticoid to the glucocorticoid receptor there is dissociation of hsp90 and the glucocorticoid: glucocorticoid receptor complex is then translocated into the nucleus. Once within the nucleus the GR-GC complex binds to GREs. From here it recruits an array of transcriptional co activators including SRC-1 and CBP which aid leads to gene transcription.

1.5.7 Action of glucocorticoids

GCs may exert their effects by increasing the expression of anti-inflammatory genes, including interleukin-10 and interleukin-1 receptor antagonist. This increase in transcription is likely to be the result of an inhibitory relationship between activated transcription factors and activated GRs (Barnes et al 1998).

GCs are catabolic in nature, they release energy in response to stress in order to service the increase in metabolic demand (Peckett et al 2011; Hermanowski-Vosatka et al 2005). GCs exert multiple effects on lipid metabolism within adipose tissue, where they enhance adipose lipolysis. GCs lead to an increase the quantity of fatty acids in circulation by increasing caloric and dietary fat intake and the hydrolysis of circulating lipids via lipoprotein

lipase activity. This leads to patients with CS demonstrating elevated serum free fatty acids, triglycerides, and cholesterol. The increase in fatty acid content that results is then available for ectopic fat distribution (Hermanowski-Vosatka et al 2005; Peckett et al 2011).

In addition GCs increase hepatic gluconeogenesis and antagonise the effects of insulin. This is achieved by directly inhibiting β-cell insulin secretion in the pancreas and peripheral glucose uptake in muscle. This results in a decrease in glucose utilisation by cells and leads to elevated circulating levels of glucose, and insulin resistance (Andrew et al 1999; Lepsin et al 2011; Hermanowski-Vosatka et al 2005). Further to this GCs promote pre adipocyte conversion to mature adipocytes, resulting in hyperplasia of the adipose tissue (Peckett et al 2011).

1.5.8 Glucocorticoids in disease

GC excess confers a negative phenotype, including the manifestation of central obesity and type 2 diabetes. GCs lead to an increase in lipolysis and therefore an increase in circulating fatty acids. These fatty acids are therefore available for deposition around the body leading to an increase in adiposity (Peckett et al 2011). There is a preference for deposition centrally leading to central obesity, which is metabolically less favourable. This is due to their being an increased level of GC receptors within visceral adipose (Lee et al 2014). In addition to this an increase in GCs leads to an increase in hepatic gluconeogenesis and antagonistic effects on insulin secretion. This leads to a decrease in glucose utilisation, an increase in the circulatory levels of glucose and ultimately the potential for the development of type 2 diabetes (Londero et al 2015).

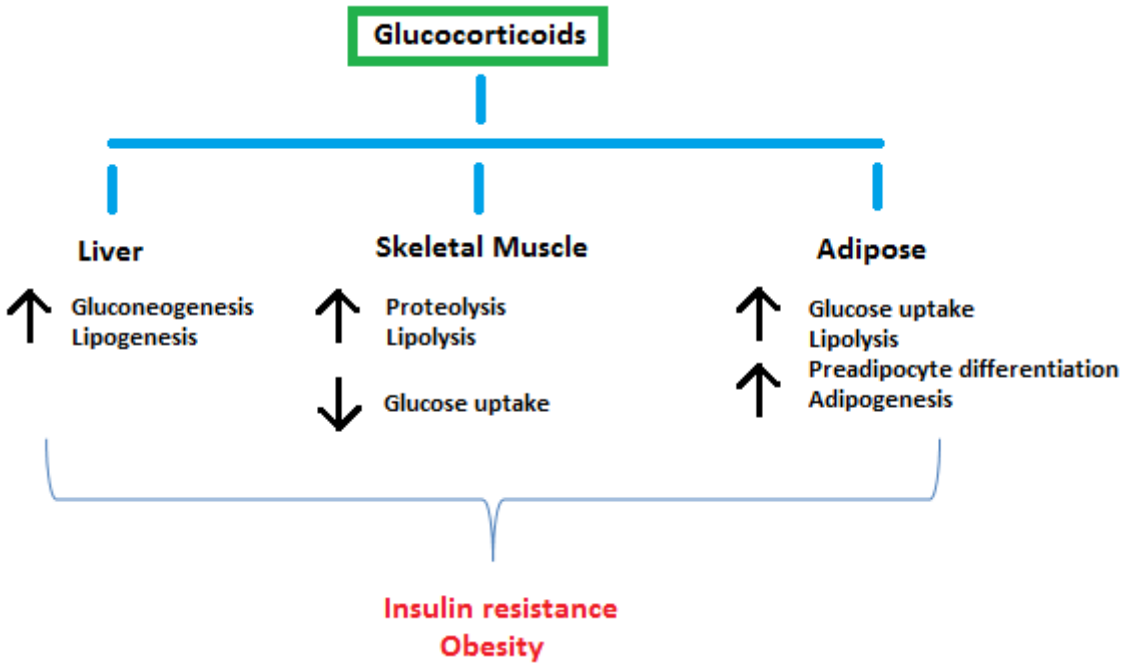

Figure 1-9 Effects of glucocorticoids on the body
An excess of glucocorticoids affects the liver by increasing gluconeogenesis and lipogenesis, in addition it leads to an increase in proteolysis and lipolysis in skeletal muscle with a decrease in glucose uptake. Within adipose an increase in glucocorticoids leads to increases in glucose uptake, lipolysis, preadipocyte differentiation and adipogenesis.

1.6 Pre-receptor glucocorticoid metabolism

Biochemical evidence indicates the existence of two distinct forms of 11β-HSD, one with a higher Km for GC substrates and a preference for $NADP^+$ as a cofactor (11β-HSD1) and a second (11β-HSD2) with a lower Km and preference for NAD^+ as a cofactor (Naray-Fejes-Toth et al 1991). Studies have revealed that certain human tissues were able to inter-convert cortisol and cortisone utilising the 11β-HSD enzymes, including the kidney, placenta and liver (Amelung et al 1953; Bush et al 1968). Continuing observation revealed that the direction of GC inter-conversion varied between tissues, with kidney and placenta possessing greater dehydrogenase (cortisol to cortisone) activity, whilst the liver presented greater reductive (cortisone to cortisol) activity (Tomlinson et al 2004). The inter-conversion of active and inactive GCs by 11β-HSD1 and 11β-HSD2 are shown in figure 1-9.

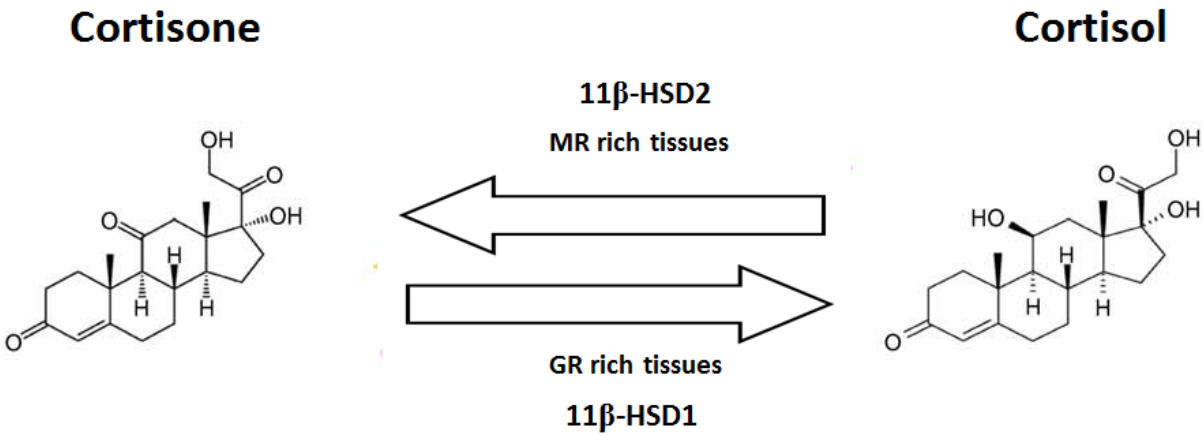

Figure 1-10 11β-HSD1 and 11β-HSD2 activity
11β-HSD1 is a bi-directional enzyme which functions to interconvert inactive cortisone to active cortisol and is located in glucocorticoid receptor rich tissues. It primary functions as an oxo reductase, where it produces active glucocorticoid cortisol in the presence of NADPH. 11β-HSD2 is a uni-directional enzyme which functions to convert active cortisol to inactive corticosterone and is located within mineralocorticoid rich tissues.

1.6.1 11β-hydroxysteroid dehydrogenase type 1

Intracellular GC metabolism is regulated at the pre-receptor level by 11β-HSD1 (Rabbitt et al 2002). 11β-HSD1 mRNA is highly expressed in mouse liver, kidney and lung, with lower expression in brain sub regions and gonads (Rejan et al 1995). It is a 34kD NADPH dependent bi-directional enzyme anchored in the endoplasmic reticulum (ER) membrane, with its catalytic domain projecting into the ER lumen. 11β-HSD1 is a member of the short-chain dehydrogenase/reductase (SDR) super-family, possessing both oxo reductase and dehydrogenase activity.

11β-HSD1 can be divided into four functionally important regions (Hosfield et al 2005). The trans-membrane domain (residues 1-23 in humans), which allows the enzyme to attach to the ER lumen, the nucleotide binding domain (Rossmann fold), which represents the catalytic site, and the c-terminal domain which is involved in the oligomerisation of the enzyme (Hosfield et al 2005).

11β-HSD1 acts predominantly as an oxo reductase, thus increasing intracellular GC levels by converting circulating inert 11-dehydrocorticosterone (11-DHC) (Cortisone in man (Seckl et al 2001)) into active corticosterone (Cortisol in man (Seckl et al 2001)) through oxo-reductase activity in most intact cells, thus amplifying intracellular GC levels in select tissues and increasing local GC action (Tomlinson et al 2001; Napolitano et al 1998).

11β-HSD1 activity requires a supply of NADPH which acts as a rate limiting cofactor, and thus the direction of catalysis is primarily driven by the intraluminal concentration of $NADP^+$ and NADPH. The NADPH utilised by 11β-HSD1 is supplied by Hexose-6-phosphate dehydrogenase (H6PDH), therefore the functional direction of 11β-HSD1 is dependent on the action of H6PDH that supplies the reduced cofactor, driving the oxo reductase activity of 11β-HSD1 (Atanasov et al. 2004b). Figure 1-10 illustrates the control of 11β-HSD1 activity.

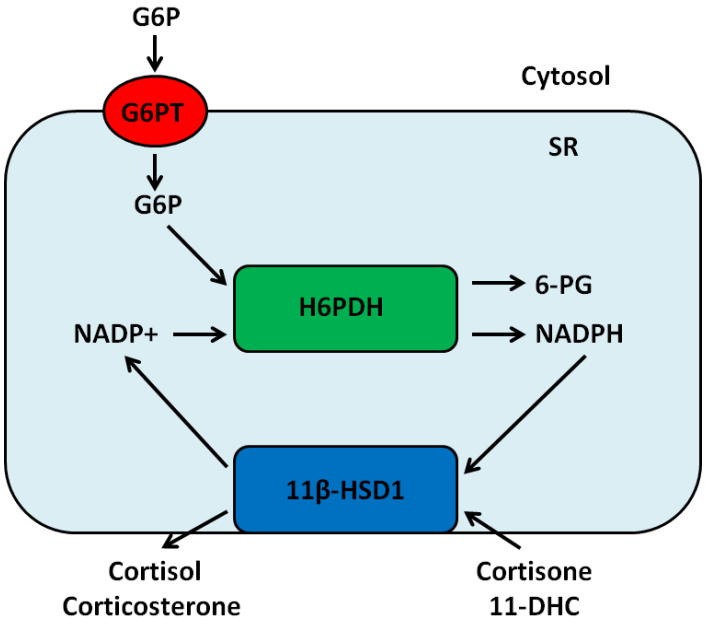

Figure 1-11 Pre-receptor glucocorticoid metabolism
Glucose-6-phosphate (G6P) is translocated into the ER by the Glucose-6-phosphate transporter (G6PT). This G6P is then utilised along with $NADP^+$ by H6PDH to produce 6-phosphoglutante (6-PG) and NADPH. This NADPH is then used as a cofactor by 11β-HSD1 to perform its oxo reductase action by converting inactive cortisone into active cortisol, along with the production of $NADP^+$.

1.6.2 Transcriptional control of 11β-hydroxysteroid dehydrogenase type 1

Transcription of 11β-HSD1 is directly regulated by members of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors: C/EBPa, b, c, d, e, and f (or CHOP) (Williams et al 2000) (Gout et al 2006; Cao et al 1991). C/EBP a and b each occur as distinct isoforms arising from differential translation initiation or proteolysis (Ossipow et al 1993). C.EBPa produces 42kDa and 30kDa isoforms, and C/EBPb exists as three major isoforms; the 38kDa liver-enriched activator protein (LAP*), 35kDa liver-enriched activator protein (LAP), which stimulate transcription, and 20kDa liver-enriched inhibitor protein (LIP) (Calkhoven et al 2000). LIP lacks the transcription activation domain of C.EBPb and is typically a dominant-negative regulator of C/EBP function (Calkhoven et al 2000). The LIP:LAP ratio is thus an important determinant of C/EBPb action (Duong et al 2002). It has since been shown that C/EBPa and C/EBPb play central roles in regulating the expression of 11β-HSD1 (Esteves et al 2012). Both C/EBPa and C/EBPb play a direct and positive role in regulation of 11β-HSD1 transcription and differentiation in adipocytes, but C/EBPb alone is crucial for differentiating cells by activating PPARγ and also functions synergistically with PPARγ to promote the expression of genes found in both brown and white adipocytes (Esteves et al 2012; Tanaka et al 1997; Wu et al 1999). Conversely in liver C/EBPa is the major known inducer of 11β-HSD1 transcription within the liver, where C/EBPb acts as a repressor (Williams et al 2000).

1.6.3 11β-hydroxysteroid dehydrogenase type 1 in disease

11β-HSD1 has been implicated in the pathogenesis of several human diseases. This is due to the physiological role played by 11β-HSD1 where it converts inactive cortisone to active cortisol thereby increasing the intra-cellular concentration of GC (Koteleytsev et al 1997). Increased 11β-HSD1 activity and expression are closely associated with fasting plasma

glucose, adiposity, insulin resistance and hyperglycaemia (Lindsay et al 2003; Kannisto et al 2004; Baudrand et al 2010). Further, adipose tissue 11β-HSD1 expression is higher in metabolic disease prone mice than in metabolic disease resistant mice, and conversely to this metabolic disease resistant mice have lower basal adipose tissue 11β-HSD1 levels than disease prone mice, showing that an increase in 11β-HSD1 may be responsible for the metabolic complications (Morton et al 2004).

1.6.4 Metabolic syndrome

The metabolic syndrome (MS) is characterised by visceral obesity, insulin resistance, type 2 diabetes mellitus, dyslipidaemia, hypertension, and an increased cardiovascular risk profile (Walker et al 2000). Chronic exposure to increased circulating GCs is causative of CS, however there is no evidence that plasma cortisol excess leads to MS. However there is an increase in 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) levels within the adipose of patients suffering with MS, which will lead increases in the regeneration of active GC (Rosmond et al 1998; Walker et al 2000).

It is therefore hypothesised that reducing the local activity of GC within the liver and adipose tissue may offer protection against the development of MS. Therefore 11β-HSD1 has developed into a major target for pharmaceutical research as a viable target for the treatment of MS.

1.6.5 Effect of 11β-hydroxysteroid dehydrogenase type 1 on adipose tissue

11β-HSD1 located within adipose tissue is involved in modulating metabolism and function. An increase in 11β-HSD1 activity within adipose tissue results in MS, visceral obesity, diabetes, dyslipidaemia and hypertension (Masuzaki et al 2001). Following an increase in 11β-HSD1 expression the effects are seen strongest within visceral adipose depots. This is because visceral adipose possesses high levels of GC receptor.

There is an increase in the expression of 11β-HSD1 within the adipose tissue of obese humans and rodents, however hepatic expression is reduced or remains constant (Masuzaki et al 2001; Paulmyer-Lacroix et al 2002). This increase in 11β-HSD1 expression causes an increase in the concentration of corticosterone within adipose despite no change in plasma levels (Masuzaki et al 2001). This is likely to have a pivotal role in the pathogenesis of MS suggesting that an increase in GC action in adipose mediated by 11β-HSD1 activity may offer an explanation as to the resemblances seen with both CS and MS.

Overexpression of 11β-HSD1 selectively within adipose leads to obesity, hypertension, and insulin-resistance; however transgenic mice overexpressing 11β-HSD1 within liver do not demonstrate obesity, but still show other side effects (Masuzaki et al 2001). It is therefore hypothesised that increased 11β-HSD1 in adipose increases intra-adipose GC action which has causative effects on; MS, visceral obesity, insulin resistance, and cardiovascular disease.

Overexpression of 11β-HSD1 has stronger effects in the visceral adipose tissue due to it possessing a higher quantity of GR (Bonnegard et al 1995; Masuzaki et al 2001). This observation adds further weight to the hypothesis that GCs possess a pivotal role in the pathogenesis of central obesity.

In contrast, mice lacking 11β-HSD1 expression are insulin sensitised and demonstrate a resistance to the undesirable metabolic effects of a HFD, showing a cardio-protective phenotype (Morton et al 2004). This has been demonstrated in various transgenic models (Kotelevtsev et al 1997; Morton et al 2001, 2004). These 11β-HSD1 deficient mice oppose diet-induced obesity and diabetes via an improvement in adipose and liver function. This is a consequence of a decrease in both the production and secretion of triglycerides and by increasing the rate of fatty acid oxidation. Transgenic 11β-HSD1 KO mice maintained on a

HFD accumulate adipose within peripheral areas preferentially to visceral depots. This is of benefit to health as peripheral areas are metabolically safer. Overall a decrease in 11β-HSD1 expression within adipose tissue leads to a preferential metabolic phenotype. This supports the role of 11β-HSD1 as a potential target for the treatment of MS.

1.7 Manipulation of the 11β-hydroxysteroid dehydrogenase type 1 enzyme

Transgenic mouse models involving genetic manipulations of the 11β-HSD1 gene have allowed for a greater understanding into the effects of this enzyme on whole body functioning. Thus allowing for the potential development of novel ways to target and manipulate the workings of the enzyme in the treatment of metabolic diseases.

1.7.1 11β-hydroxysteroid dehydrogenase type 1 knock-out

Various rodent studies involving mice with 11β-HSD1 GKO have shown that they have favourable metabolic profiles (Morton et al 2001). In the absence of 11β-HSD1 inert 11-keto corticosteroids cannot be reduced to active 11-hydroxy forms (Kotelevtsev et al 1997). Knock-out of 11β-HSD1 leads to protection against diet-induced visceral obesity and its metabolic consequences, probably via a decrease in the regeneration of intracellular GC and in part through insulin sensitisation and redistribution of adipose tissue to metabolically safer sites (Morton et al 2001, 2004).

Further 11β-HSD1 GKO mice demonstrate improved insulin sensitivity, lipid profiles, lower fasting plasma glucose and lipid levels, protection from hyperglycaemia, lower plasma triglyceride levels, increased hepatic insulin sensitivity, reduced fat mass, higher oxygen consumption rate, increased hepatic lipid catabolism, while synthesis is normal, resulting in reduced serum triglycerides and total cholesterol, higher PPARγ expression and higher energy dissipation (Wamil et al 2011; Liu et al 2013).

Knock-down of 11β-HSD1 has profound effects on the functioning of adipose tissue. There is reduced subcutaneous adipocyte hypertrophy in 11β-HSD1 GKO mice, which may be explained by enhanced β3-adrenergenic remodelling (Wamil et al 2011; Ataab et al 2010). A decrease in lipid accumulation within adipocytes decreases expression of lipoprotein lipase (LPL) and fatty acid synthetase, a decrease in visceral adipose preadipocyte differentiation, and increased glucose uptake, suggesting greater insulin sensitivity (Liu et al 2007; De Sousa Peixoto et al 2008; Wamil et al 2011; Morton et al 2004). Further a decrease in adipose 11β-HSD1 is linked to a favourable distribution of adipose tissue and opposition to metabolic disease caused by a high fat diet, including insulin sensitivity, this involves an increase in subcutaneous adipose which demonstrates higher insulin sensitivity than visceral adipose (Morton et al. 2004; Wamil et al 2011).

Knockdown of 11β-HSD1 aids brown adipocyte function, and there is an inverse relationship between mRNA expression of 11β-HSD1 and BAT signature genes UCP1, Cidea, Cox7a1 and Cox8b in brown adipocyte differentiation. This suggests that 11β-HSD1 is detrimental to brown adipocyte differentiation (Liu et al 2013). In addition11β-HSD1 knockdown cells possess smaller lipid droplets than control cells. This decreased lipid droplet size is linked to an increase in lipid oxidation and energy expenditure (Liu et al 2013).

Overall these studies demonstrate that the pharmacologic inhibition of 11β-HSD1 is a promising strategy to treat metabolic diseases.

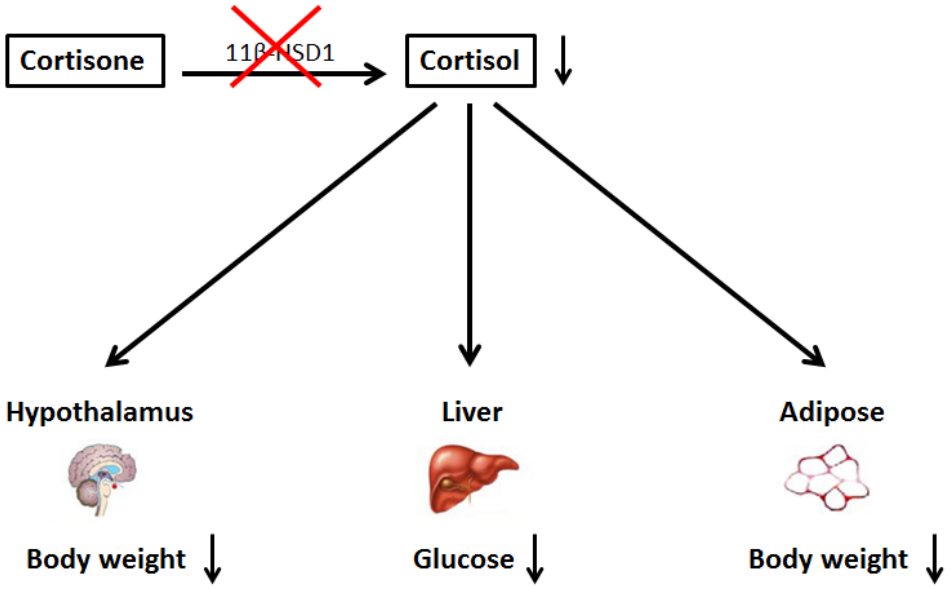

Figure 1-12 Effect of 11β-HSD1 knock-out
In the absence of 11β-HSD1 there is no tissue specific regeneration of cortisol from cortisone. This has effects on the hypothalamus, the liver, and adipose tissue, leading to an overall decrease in glucose and body weight.

1.7.2 11β-hydroxysteroid dehydrogenase type 1 over expression

As a contrast to 11β-HSD1 GKO experiments, models of 11β-HSD1 over expression have also been generated. Over expression of 11β-HSD1 leads to increased weight gain (predominantly visceral), dyslipidaemia, hyperglycaemia, hypertension and insulin resistance (Masuzaki et al 2003). It is hypothesised that this is due to increased FFA draining from visceral adipose tissue into the portal circulation, adding weight to this is that FFA levels were shown to be increased in 11β-HSD1-over-expressing mice (Masuzaki et al 2001). Further the adipose tissue accumulation is located at the more therapeutically damaging visceral sites are as opposed to the more favourable subcutaneous depots. This could be due to GR mRNA expression being higher in visceral than subcutaneous adipose and therefore the increase in GC levels would have a more prominent effect on this adipose depot, due to GCs promoting adipocyte differentiation (Masuzaki et al 2001).

In addition to this, the mRNA levels of BAT specific genes are down regulated with 11β-HSD1 overexpression, and BAT cells contain larger lipid droplets (Liu et al 2013). In addition UCP1 expression in BAT has been shown to be significantly decreased in 11β-HSD1 over-expressing mice, indicating a possible role for decreased BAT function in energy dyshomeostasis (Masuzaki et al 2001).

1.7.3 11β-hydroxysteroid dehydrogenase type 1 inhibitors

11β-HSD1 has been demonstrated as a potential novel drug target in the suppression of the action of GCs. This would offer a potential therapy to combat the disorders linked with cortisol excess, for example obesity, insulin resistance, and type 2 diabetes.

Inhibitors of 11β-HSD1 decrease the level of cortisol generated in the liver and adipose tissue, and decrease tissue-specific gluconeogenesis and fatty acid metabolism (Harno et al 2010). The potential adverse effects linked to up regulation of the HPA axis are characterised by osteoporosis, immunosuppression, hypertension and glucose intolerance. Therefore the selectivity of inhibitors is crucial to ameliorating the negative effects linked with non-selective binding to 11β-HSD2, leading to over activation of MRs, thus causing hypokalemia and hypernatremia, which in turn would lead to hypertension (Lepsin et al 2011).

Administration of 11β-HSD1 inhibitors results in a lower body mass, fasting glucose, insulin, and cholesterol in diet-induced obese mice (Vaeniant et al 2010). Further non-selective 11β-HSD1 inhibitors result in a decrease in human adipocyte differentiation and cellular lipid content (Bujalska et al 1999; Bujalska et al 1998; Walker et al 1995). In addition, inhibition of 11β-HSD1 activity by BVT.2733 results in an increase in brown adipocyte function (Liu et al 2013).

1.8 Hypothesis

GCs play a key role within the body and can be reactivated at the tissue specific level by 11β-HSD1, thus giving a high available concentration of active GC within the body. I hypothesise that a genetic depletion of 11β-HSD1 activity in adipose will alleviate the negative effects of exposure to exogenous GC excess. This includes an improvement in the functioning of adipose tissue, inducing an increased capacity for non-shivering thermogenesis, which can lead to an improved metabolic and physiological phenotype.

1.9 Aims

- To generate and characterise a mouse model of 11β-HSD1 knock-out specifically within adipose tissue and to assess the effects of administration of exogenous glucocorticoids.
- To assess the effect of exogenous 11β-HSD1 on BAT and WAT tissue in a mouse model of ageing.
- To assess the effects of administration of exogenous glucocorticoids on BAT and WAT thermogenic programmes.

2. Chapter 2 - Materials and Methods

2.1 RNA extraction

2.1.1 Method

Around 20 mg of tissue was homogenised in 1 ml of TRI reagent, or 1 ml of TRI reagent was added to each well of cells and the cells manually dislodged from the wells. Samples were left at room temperature for 5 minutes. 200 µl of chloroform was added and the solution vortexed and placed at room temperature for 10 minutes. The solution was centrifuged at 4°C, at 14,000g for 15 minutes and the aqueous phase was transferred to a fresh 2 ml micro centrifuge tube. 500 µl of isopropanol was added and the solution vortexed and left at room temperature for 15 minutes. The samples were then centrifuged at 4°C, at 14,000g for 15 minutes. The RNA pellet was then washed with 75 % ethanol, the ethanol was then aspirated and the RNA pellet left to air-dry at room temperature. The pellet was then re-suspended in 20 µl of nuclease free water and stored at -80°C.

2.2 RNA quantification

2.2.1 Method

RNA concentration was determined using the Nano Drop ND-1000 UV-Vis Spectrophotometer (Thermofisher, Surrey, UK). The OD260/280 ratio was used as an indication of the RNA purity, and only samples whose ratio fell between 1.8 and 2.0 were used. Measurements used nuclease free water as a blank and were made using 1.5 µl of RNA sample.

2.3 Reverse transcription of RNA

2.3.1 Method

This method leads to the generation of stable complementary DNA (cDNA) from RNA previously extracted from tissues.

Reverse transcriptase reactions were undertaken using Applied Biosystems High-Capacity Reverse Transcription Kit (Applied Biosystems, Warrington, UK). 1ug of RNA was added to a reaction mix with a final volume 25 µl. The reaction mix consists of: 2.0 µl 10x reaction buffer,0.8 µl 25x dNTPs (100mM), 2.0 µl 10x RT Random Primers, 1.0 µl RNase inhibitor, 1.0 µl Multiscribe Reverse transcriptase and made up to 25 µl with RNA free water. The reaction was carried out using a thermal cycler (Applied Biosystems, Warrington, UK) as follows: 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes. The cDNA generated was stored at -20°C.

2.4 DNA extraction

2.4.1 Method

Around 20 mg of tissue was digested in 100 µl of digestion buffer at 56°C overnight. 400 µl of nuclease free water and 500 µl of phenol was added and the solution vortexed. The solution was centrifuged at 4°C, at 14,000g for 15 minutes and the aqueous phase was transferred to a fresh 2ml micro centrifuge tube. 500 µl of 50:50 phenol/chloroform was added and the solution vortexed. The samples were then centrifuged at 4°C, at 14,000g for 15 minutes and the aqueous phase was transferred to a fresh 2ml micro centrifuge tube. 10 µl of sodium acetate was added along with 300 µl of 100% ethanol. The samples were then vortexed and left on ice for 30 minutes. The solution was then centrifuged at 4°C, at 14,000g for 15 minutes. The supernatant was then removed and the DNA pellet was then washed with 75 %

ethanol, the ethanol was then aspirated and the DNA pellet left to air-dry at room temperature. The pellet was then re-suspended in 100 µl of nuclease free water. The extracted DNA was stored at -20C.

2.5 DNA quantification

DNA concentration was determined using the NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermofisher, Surrey, UK).

2.6 Polymerase chain reaction (PCR)

2.6.1 Method

Polymerase chain reactions (PCR) is used to amplify specific regions of DNA or cDNA, using oligonucleotide primers which are complementary to the 3' and 5' regions of the target sequence. PCR was carried out using New England Biolabs reagents (Biotaq, NEB, Herts, UK). A 20 µl reaction was carried out using the following reagents: 10 µl Master Mix, 0.5 µl forward and reverse primer, 8 µl nuclease free water and 1 µl of DNA template (200 ng/ml). The samples were run using a thermal cycler with the following reaction: 94°C 2 minutes then cycled 35 times at 94°C for 30 seconds, 57°C for 30 seconds, then 72°C for 1 minute. Finally samples were incubated at 72°C for 5 minutes. DNA product was visulaised under UV conditions using a 2% agarose gel containing SYBRSAFE Dye (x10,000) (Invitrogen).

2.7 Quantitative polymerase chain reaction

2.7.1 Method

Relative quantification real-time polymerase chain reaction (RT-PCR) enables the accurate measurement of selected DNA sequences from cell and tissue samples. Real-time quantitative PCR was performed using ABI Prism 7,500 Sequence Detection System (Applied Biosystems, Warrington, UK). Primers were ordered pre-designed and optimised from

Applied Biosystems, Warrington, UK. Reactions were carried out in duplicate in 96-well plates (Applied Biosystems, Warrington, UK). The reactions contained the following reagents: 10 µl 2x Master mix, 1 µl of 18S or 20x expression assay, 100 ng of cDNA and nuclease free water to give a final volume of 20 µl. The plates were sealed using a clear adhesive film (Applied Biosystems, Warrington, UK). The results were given as Ct values and the ΔCt values were calculated (Ct of gene of interest-Ct of 18S) and given as a fold change ($2^{-\Delta\Delta Ct}$).

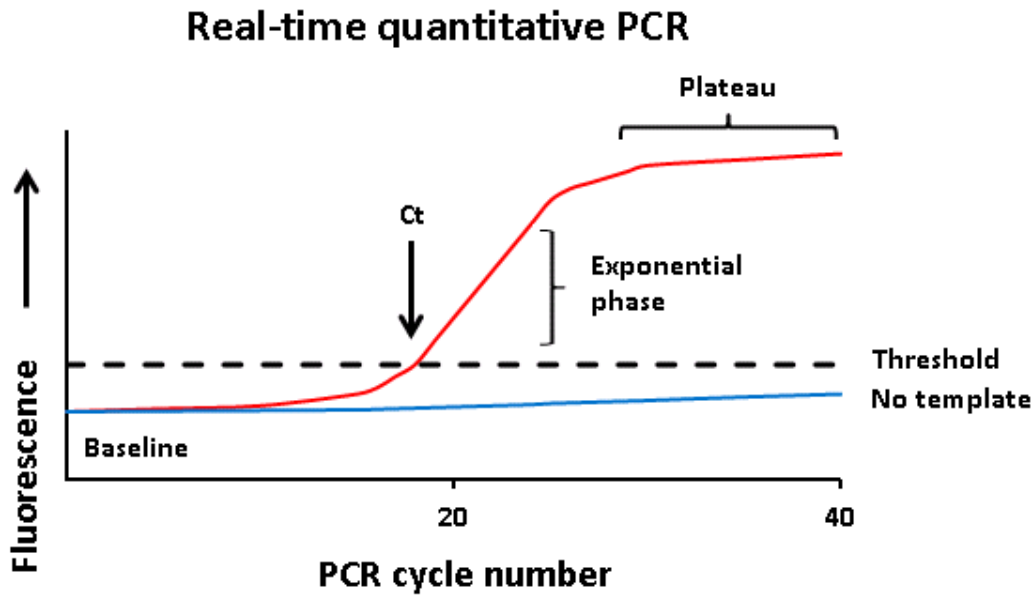

Figure 2-1 Relative quantification polymerase chain reaction
Once reaching the exponential phase the cycle threshold (Ct) value is recorded. This value is inversely proportional to the copy number of the target DNA template.

2.7.2 Validation and calibration

To ensure that the 18S internal reference primers did not interfere with the target gene primers in multiplex reactions, both target gene and 18S primer and probes were run in singleplex. All TaqMan Gene Expression Assays have been designed using a validated bioinformatics

pipeline, and run with the same PCR protocol, eliminating the need for primer design or PCR optimisation.

2.7.3 Primer and probe sequences and details

All primers used in Taqman Gene Expression Assays were provided by Applied Biosystems, California, USA.

11β-HSD1	Assay ID Details:Mm00476182_m1 TaqMan Gene Expression Assay
UCP1	Assay ID Details:Mm01244861_m1 TaqMan Gene Expression Assay
Cox8b	Assay ID Details:Mm00432648_m1 TaqMan Gene Expression Assay
Cox7a1	Assay ID Details:Mm00438297_m1 TaqMan Gene Expression Assay
Nr3c1	Assay ID Details:Mm00433832_m1 TaqMan Gene Expression Assay
PPARγ	Assay ID Details:Mm01184322_m1 TaqMan Gene Expression Assay
PRDM16	Assay ID Details:Mm01184322_m1 TaqMan Gene Expression Assay
PGC-1α	Assay ID Details:Mm01208835_m1 TaqMan Gene Expression Assay
NRF1	Assay ID Details:Mm00548493m1 TaqMan Gene Expression Assay
TFAM	Assay ID Details:Mm00235765m1 TaqMan Gene Expression Assay
CoxIV	Assay ID Details:Mm01250094m1 TaqMan Gene Expression Assay

Table 2-1 Primer and probe sequences
Table showing the primers and probes used for polymerase chain reaction experiments.

2.8 ^{3}H labelled 11β-HSD1 activity assays

2.8.1 Method

This technique is used to measure the inter conversion of active and inactive glucocorticoids by 11β-HSD1. Extracted tissue samples (~20 mg/well) were incubated in serum free media enriched with 20,000 cpm/reaction of ^{3}H-11-DHC to allow for the assessment of 11β-HSD1

activity. During glucocorticoid substrate metabolism by 11β-HSD1 there was a proportional amount of the labelled tracer metabolised also. Following incubation the steroids were extracted using dichloromethane and separated via TLC. The percentage of active and inactive tracer labelled glucocorticoids was determined and from this the quantity of steroid conversion in pmoles per g of sample can be calculated.

To measure the oxo-reductase activity of 11β-HSD1, 100 nM of 11-DHC was added to the serum free media along with 20,000 cpm/reaction of ^{3}H-11-DHC. Samples were incubated at 37°C in air/5 % CO_2 for 4 hours (adipose) and 30 minutes (liver). The media was transferred into 10ml glass tubes and 5 ml of dichloromethane was added. The remaining tissue samples were weighed and the weights recorded. The media was vortexed for 15 minutes at 15,000 rpm to separate the organic and aqueous phase. The aqueous phase containing proteins was aspirated, leaving the steroid containing organic phase. The steroids were concentrated by the evaporation of the dichloromethane at 50°C for 30 minutes under an air blowing sample concentrator (Techne, New Jersey, US). The remaining steroids were re-suspended in 70 µl of dichloromethane and spotted onto pre-labelled silica thin layer chromatography plates (thermofisher, Surrey, UK). 1 µl of standard steroid solution (10 mM of 11-DHC or corticosterone) was also spotted on the plates. The steroids were separated by TLC in ethanol: chloroform (8:92) as the mobile phase for 1.5 hours. The TLC plates were then analysed using Bioscan imaging detector (LabLogic, Sheffield, UK) and the conversion of inactive to active steroid was determined. Results were expressed as pmol of steroid converted per g of tissue per hour (pmol/g/h), and experiments were carried out in triplicate.

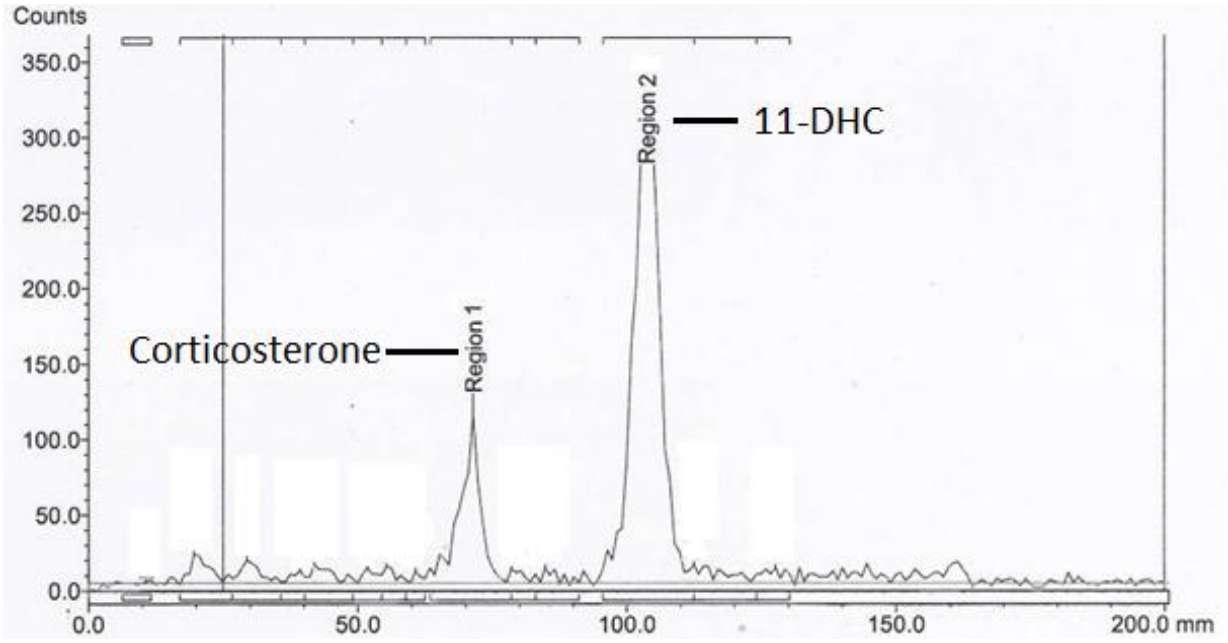

Figure 2-2 ^{3}H labelled 11β-HSD1 activity assay data read out
Region 1 represents the amount of radio-active corticosterone present and region 2 represents the amount of 11-dehydrcorticosterone present. From the peak size the % conversion of 11-dehydrocorticosterone to corticosterone can be calculated.

2.9 Generation of ^{3}H-11-dehydrocorticosterone

For the 11β-HSD1 conversion assays, inactive tritium labelled tracer substrate of 11-DHC was required, however this is not commercially available. This technique provides a means by which ^{3}H-11-DHC was produced in-house through the conversion of 3H-corticosrterone (GE healthcare, Bucks, UK) by the 11β-HSD2 enzyme.

20µl of ^{3}H-corticosterone (1mCi/mL) was incubated with 250 mg of human placenta in 500 µl of 0.1 M potassium phosphate buffer (pH7.4) containing 500 µM NAD^{+}, at 37°C for 18 hours in 1 ml glass tubes in a shaking water bath. ^{3}H-GC were extracted by addition of 5mL dichloromethane and vortexed for 10 seconds to extract the steroids. The solution was then centrifuged for 15 minutes at 15,000 rpm to separate the organic and aqueous phase. The steroids were concentrated by evaporating the dichloromethane at 50°C for 30 minutes using an air blowing sample concentrator (Techne, New Jersey, US). Steroids were then re-

suspended into 70 µl of dichloromethane and spotted onto silica coated thin layer chromatography plates (Thermofisher, Surrey, UK), using a Pasteur pipette. The steroids were separated by thin-layer chromatography (TLC) using ethanol: chloroform (8:92) as the mobile phase along with 1 mg/ml of 11-DHC and corticosterone. TLC plates were analysed using Bioscan imaging detector (Bioscan, Washington, DC, USA). The 11-DHC and corticosterone fluoresce under UV light which allows for the localisation of the labelled steroid on the TLC plate (+/- 0.5 cm). The silica is then scraped into a fresh 10 ml tube and the steroids are eluted in 300ul of ethanol overnight at 4°C. The tube was spun at 15,000 rpm for 10 minutes and the liquid phase was transferred to a fresh tube. The remaining silica was re-suspended into 300 µl of ethanol and the elution process repeated. The ^{3}H-11-DHC was tested for purity by running 1 µl on a TLC plate and analysed for 1 minute. The number of counts recorded was analysed and the level of ethanol was altered by either addition or evaporation to give a concentration of 1,000 counts/µl.

2.10 Protein extraction

2.10.1 Methods

For tissue samples the tissue was homogenised in 100 µl of RIPA buffer (50 mmol/l Tris-HCl pH 7.4, 1% NP40, 0.5% sodium deoxycholate, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l, 0.1 % SDS, and protease inhibitor cocktail (Roche, Lewes, UK), and placed at -80°C for 1 hour. The tissue was then further homogenised at left at -80°C for a further hour. The samples were then centrifuged at 4°C for 5 minutes at 14,000 rpm. For primary cell culture, 100 µl of RIPA buffer was added to the well and the protein manually removed from the surface of the well.

2.11 Protein estimation

2.11.1 Method

The soluble protein concentrations of tissue homogenates or primary cell culture were measured using Bio-Rad protein assay dye (Bio-Rad Laboratories, GmbH, Germany) using varying concentrations of BSA standards. Protein assays were performed using BIO-RAD protein assay kit (BIO-RAD laboratories, USA), according to the manufactures protocol. Samples were run in parallel with a range of concentrations of BSA standard (0, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8 mg/ml) in 96 well plates. To these 25 µl of reagent A containing Coomassie Brilliant Blue G-250 dye were added, followed by 200 µl of reagent B. Plates were incubated at room temperature for 15 minutes and their optical density read at 595 nm (OD_{595} nm). A standard curve of mg protein/ml against OD_{595} nm was then plotted. From this curve a line of best fit is added. From this line of best fit, protein concentration of samples can be determined for sample OD_{595} nm values. As all sample concentrations are taken from the same graph any fluctuations from the line of best fit are consistent throughout the samples and therefore do not affect the results.

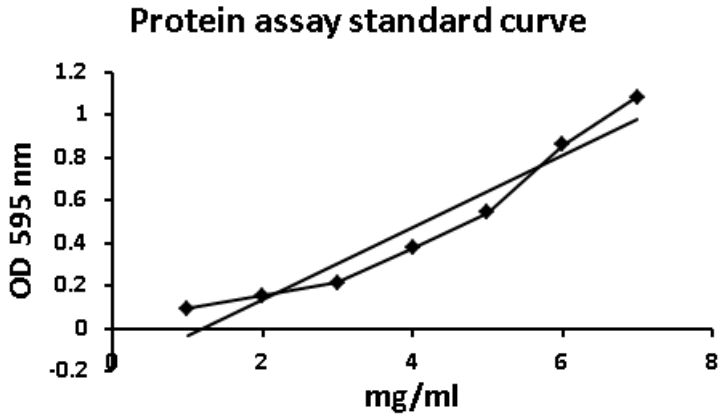

Figure 2-3 Protein assay standard curve
Protein samples of known concentration were used to produce a standard curve with a line of best fit. This allowed for the concentration of the proteins with unknown concentration to be elucidated.

2.12 Western Blot analysis

2.12.1 Principles

Western blotting allows for target proteins from cell or tissue homogenate to be visualised as a band on a nitrocellulose membrane. Proteins are denatured and separated by electrophoresis on an SDS PAGE gel. The proteins are then transferred to a nitrocellulose membrane which is incubated with a primary antibody targeted to the protein of interest and then secondary antibody which is targeted to the primary antibody. The secondary antibody is horseradish peroxidase-linked and used together with a chemiluminesent agent to produce luminescence in proportion to the quantity of protein. A sheet of photographic film is placed against the membrane, and exposure to the light creates an image of the antibodies present on the blot.

2.12.2 Solutions

Stacking gel	625 µl Acrylamide, 1.25 ml stacking buffer, 25 µl 20 % SDS, 250 µl 1.5% ammonium persulphate, 2.8 ml dH_2O, 5 µL TEMED.
Resolving gel	2.08 ml Acrylamide, 0.625 ml resolving buffer, 25 µl 20% SDS, 250 µl 1.5% ammonium persulphate, 2 ml dH_2O, 5 µl TEMED.
SDS PAGE running buffer	25 ml Tris base pH 7.4, 14 g glyceine, 1 g SDS, 975 ml dH_2O

Table 2-2 Solutions used in Western Blot analysis
Table showing solutions used during Western Blot analysis. Staking gel and resolving gel were used when running the proteins and SDS PAGE running buffer was used as the solution.

2.12.3 Method

25 µg of protein solution (protein, LB and nuclease free water) was loaded into a 10 % SDS PAGE gel containing a resolving gel and a stacking gel, and run at 130 volts for 2 hours, covered by SDS PAGE running buffer. Proteins were then transferred onto a nitrocellulose

membrane (Amersham, Buckinghamshire, UK) using an iBLOT. The membrane was blocked for 1 hour in 5 % milk in PBS at room temperature on a shaker. The membrane was washed 4 times in PBST for 15 minutes each. The membrane was then incubated with primary antibody overnight at 4°C on a shaker. The membrane was then washed 4 times in PBST for 15 minutes each. The membrane was then incubated with secondary antibody for 1 hour at room temperature on a shaker. Finally the membrane was incubated with ECL for 5 minutes and the luminescence measured using a light sensitive Kodak MXB photographic film, in a dark room. Membranes were re-probed using antibodies against β-actin, which was used as an internal reference control. The bands were quantified using imageJ and expressed relative to β-actin to normalise for gel loading.

All primary antibodies used were obtained from abcam; all secondary antibodies were obtained from Dako.

2.12.4 Primary and secondary antibody details

Antibody	**Order reference**
UCP1	Ab23841
β-actin	Ab8227
11β-HSD1	Ab39364
Polyclonal Goat Anti-rabbit immunoglobulins HRP	P0448
Polyclonal Goat Anti-mouse immunoglobulins HRP	P0447

Table 2-3 Primary and secondary antibodies
I tested UCP1 and 11β-HSD1 protein expression and utilised β-actin as a control when performing Western Blot analysis. Anti-goat and anti-rabbit immunoglobulins were used as secondary antibodies.

2.13 Primary adipose tissue culture

White adipose tissue (Subcutaneous, Mesenteric, and Gonadal), and brown adipose tissue (Intrascapluar) was extracted from wild-type and 11β-HSD1 global knock-out mice. The extracted tissue was manually digested, placed in 10ml of collagenase, and incubated in a 37°C water bath for 1 hour (WAT) or 30 minutes (BAT). Following incubation the sample was vortexed for 10 minutes at 10,000 rpm and the supernatant discarded. The pellet was then re-suspended in 8 mls (WAT) or 4 mls (BAT) of proliferation media (DMEM/F12 culture media supplemented with 10% FCS and 1% penstrep) and 1ml aliquots were placed in a 12-well plate. The cells were incubated at 37°C and 5% CO_2 for 24 hours. The proliferation media was removed and the cells washed with 1 ml of proliferation media. 1 ml of proliferation media was then added to each well and the cells were placed in the incubator to proliferate for 2 days with the proliferation media changed daily. The proliferation media was then replaced with 1ml of differentiation media (DMEM/F12 culture media supplemented with 17 µM D-panthotenic acid, 33 µM biotin, 1 nM triiodothyronine, 166 nM human insulin, and 1 µM dexamethasone). The cells were placed in the incubator for 9 days to differentiate with the differentiation media changed daily.

2.13.1 Statistical analysis

Statistical analysis was used to test the significance levels of the data produced. A paired student T-test was used predominantly throughout the work to test for statistical significance. In addition, densitometry was used when assessing the significance levels of a Western blot analysis, this involved a quantitative measurement of optical density on photographic paper. Two-way ANOVA followed by Bonferroni's multiple comparison post hoc test was used. Data are presented as mean ± SEM and data was deemed statistically significance if the p value was <0.05.

3. Chapter 3 – Role of adipose 11β-hydroxysteroid dehydrogenase type 1 and metabolic phenotype in glucocorticoid excess

3.1 Methods

3.1.1 Histological analysis of tissues

3.1.1.1 Tissue preparation

Samples were kept in 4 % formalin for at least 24 hours at room temperature to ensure the tissue is fixed. The sample is then placed into a cassette and immersed in 99 % industrial methylated spirits (IMS) (Leica biosystems, Peterborough, UK) for 1 hour. This was repeated two further times. The cassette containing the sample was then placed into xylene (Leica biosystems, Peterborough, UK) for 30 minutes and this was repeated a further two times. The cassettes were then removed from the xylene and placed in molten paraffin wax (Leica biosystems, Peterborough, UK) in a 65°C oven for 1 hour. This was repeated for a further hour and then again to be left in molten paraffin wax overnight. The tissue was then embedded in a mould with the use of molten paraffin wax, once the wax has set the cassette was separated from the base and the tissue was sectioned. The paraffin block was clamped into the microtome (Leica RM2125 RTS, Germany) and a mechanical blade (Leica DB80 LS, Germany) was used to cut 5 µm sections. These sections were then transferred to a 45°C water bath for 1 minute and collected onto Superfrost plus slides (Surgipath, Peterborough, UK). The slides were then placed into an incubator set at 40°C for 1 hour.

3.1.1.2 Haematoxylin and Eosin (H & E) staining

Slides were placed in clearene for 2 minutes, and this was repeated two further times. The slides were then transferred to 99 % IMS for two separate 2 minute intervals. Following this slides were placed in dH_2O for two sets of 2 minutes and then placed into Haematoxylin stain for 5 minutes. The slides were then transferred to Scott's tap water substitute (Leica

Biosystems) for 2 minutes and then dipped for 2 seconds in acid alcohol and transferred to Eosin stain for 5 minutes. Following this the slides were dipped in 99 % IMS for 2 seconds and then transferred to 99 % IMS for two sets of two minutes, before being transferred to clearene for three sets of 2 minutes. The slides were then mounted using duplex and covered with a cover slip.

3.1.2 Hepatic TAG quantification

Hepatic TAG content was measured using a colorimetric assay (Bio Vision, Inc.). 100mg of liver tissue was homogenised in 1 mL of 5% Nonidet P-40 in water. The samples were then heated to 80°C in a water bath for 5 minutes. The samples were then left to cool to room temperature. This heating step was then repeated to ensure all TAGs were solubilised. The samples were then centrifuged for 2 minutes to remove any insoluble material. The samples were then diluted 10-fold with distilled water. The TAG assay was then performed. 10 µl of standard was added to designated wells. The standards were diluted to known concentrations. 10 µl of the sample was added to three wells and the reaction was initiated by adding 150 µl of diluted Enzyme Buffer solution to each well. The plate was then gently shaken for 5 seconds in order to mix the solution. The plate was then incubated for 15 minutes at room temperature. The absorbance was then read at 530-550 nm. The average absorbance of each sample was then calculated. A graph of the standard absorbances was produced, the values of the samples were then calculated using the equation obtained from the linear regression of the standard curve. Triglycerides (mg/dl) = ((corrected absorbance – y-intercept)/slope).

3.1.3 Free Fatty Acid quantification

A standard curve was generated using 0, 2, 4, 6, 8, 10 µl Palmitic Acid Standards which were adjusted to give a total volume of 50 µl per well. Test samples were added in triplicate to the wells. 50 µl of reaction mix was added to each well (44 µl assay buffer, 2 µl fatty acid probe,

2 µl enzyme mix, 2 µl enhancer). The OD_{570nm} was measured and the results averaged for each sample. A standard curve was plotted using the standard values and this was used to read off the values of the samples.

3.1.4 11β-HSD1 adipose specific knock-out allele

The 11β-HSD1 adipose specific knockout mice were purchased from Jackson labs. The following primers were used to confirm the presence of the Cre allele:

Primers	Sequence	Product size
Cre F	GTAGTTATTCGGATCATCAGCTACAC	402
Cre R	GCTGCCACGACCAAGTGACAGCAATG	

Table 3-1 Cre primers
A Cre forward and Cre reverse primer pair, giving a product size of 402bp were used to identify the presence of the Cre allele.

PCR reactions were carried as described previously. The samples underwent gel electrophoresis on a 2 % agarose gel containing 2,000x GelRed (Cambridge BioScience, Cambridge, UK) at 120 volts for 1 hour. 10 µl of hyper ladder 1 was used. The gel was analysed for the presence of amplified genetic products (Syngene, Cambridge, UK).

3.1.5 11β-HSD1 adipose knockout

Cre/LoxP technology was used to delete loxP flanked chromosomal DNA sequences within the transgenic mice. *I* purchased a transgenic mouse which expresses cre recombinase under the control of the adiponectin promoter. The following primers were used to confirm the presence of loxP flanked chromosomal DNA:

Primers	Sequence
P1	GGGAGCTTGCTTACAGCATC
P2	CATTCTCAAGGTAGATTGAACTCTG
P3	TCCATGCAATCAACTTCTCG

Table 3-2 LoxP primers
Three primers were used to confirm the presence of loxP flanked chromosomal DNA. Recombination of P2 and P3 gave a 138bp product and represented WT and recombination of P1 and P3 gave a 172bp product and represented KO.

Recombination of P2 and P3 primers yields a 138bp WT product, recombination of P1 and P3 primers yield a 172bp KO product when P2 has been deleted. The production of two bands represents a heterozygote.

3.1.6 Breeding scheme

Figure 2-4 demonstrates the breeding scheme used to establish an 11β-HSD1 adipose knockout mice.

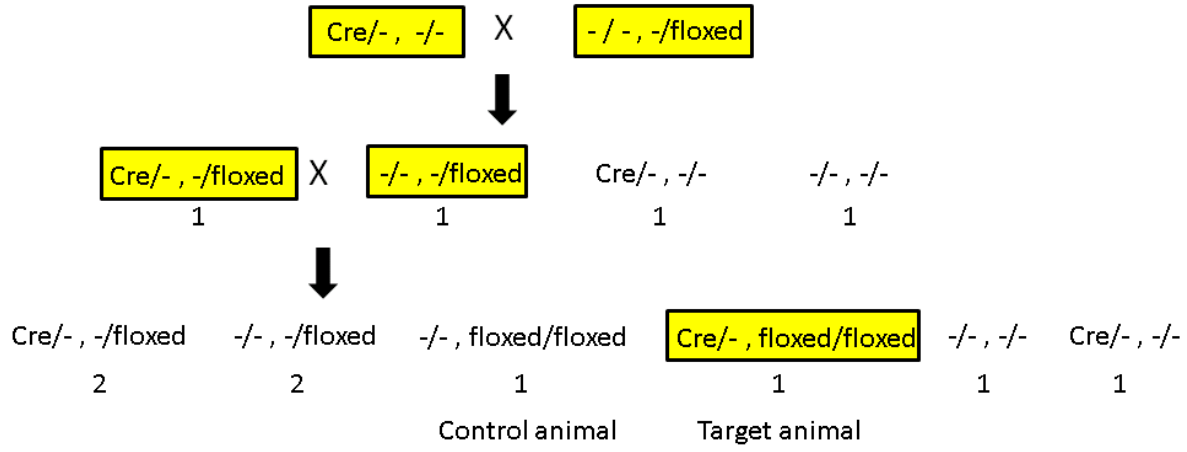

Figure 3-1 Breeding scheme to generate adipose specific knock-out of 11β-HSD1
Initially cre positive mice were crossed with heterozygous floxed mice. From the litters produced a cre positive and heterozygous floxed mouse was crossed with a cre negative and heterozygous floxed mouse. This allowed for the generation of a target adipose specific 11β-HSD1 knock-out animal.

3.1.7 Genotyping

Mouse ear clippings were collected from each animal and a single ear clip was transferred to a 2ml micro centrifuge tube. 100 µl of Proteinase K buffer (96 µl of 10x PCR buffer (Bioline, London, UK), 4 µl of 50 nM MgCl2 (Bioline, London, UK), 5 µl of Tween-20, 885 µl of H_2O and 100 µl of proteinase K (10 mg/ml) (Promega, Southampton, UK)) was added to each tissue sample and incubated in a hot block at 56°C overnight or until the tissue had digested. The samples were vortexed and placed in a hot block at 99°C for 20 minutes to denature the proteinase K enzyme. The samples were then left at room temperature to cool and centrifuged for 1 minute at 14,000g on a desk top centrifuge. The concentration of the sample was found using Nanodrop ND-1000 spectrophotometer (Wilmington, Delaware, USA). A working concentration of around 200 ng/ml was obtained by appropriate dilution in nuclease free water. The DNA was then stored at 4°C until required.

3.1.8 Administration of glucocorticoids

3.1.8.1 Methods

Three week old male adipose specific 11β-HSDS1 knock out mice (FKO) and wild type mice were given drinking water supplemented with either corticosterone (CORT: 100 µg/mL, 0.66 % ethanol), 11-dehydrocorticosteone (11-DHC: 100 µg/mL, 0.66 % ethanol) or vehicle (0.66 % ethanol) for 5 weeks. The supplemented drinking water was changed twice weekly. Following the completion of the experiment, animals were sacrificed by cervical dislocation (schedule 1 procedure) and tissue extracted, weighed and snap frozen in liquid N_2 for future analysis.

3.1.9 Glucose tolerance test

A glucose tolerance test is commonly used to diagnose diabetes and insulin resistance. A dose of glucose is given based on body weight, and blood glucose measurements are taken at set time points up to 2 hours post-administration. This allows for a measure of the rate of glucose clearance by the subject.

The mice were fasted for 5 hours prior to the procedure and glucose was measured from tail vein nicks using a glucometer (Accu-Chek, Roche, Hertfordshire, UK) at 0, 15, 30, 60, 90, and 120 minutes following an IP injection of glucose (2 g/kg).

3.1.10 Insulin tolerance test

An insulin tolerance test is commonly used to assess adrenal and pituitary function and insulin sensitivity. A dose of insulin is administered with the aim of inducing hypoglycaemia. In response to this adrenocorticotrophic hormone (ACTH) and growth hormone (GH) are released. ACTH elevation causes the adrenal cortex to release cortisol to oppose the action of insulin.

The mice were fasted overnight and glucose was measured from tail vein nicks using a glucometer (Accu-Chek, Roche, Hertfordshire, UK) at 0, 15, 30, 60, 90, and 120 minutes following an IP injection of insulin (0.75 U/kg).

3.1.11 Animals

All animal experiments and procedures were approved under the British Home Office Guidance (Animals Scientific Procedures) Act 1986 (Project Licence PIL 30/9815). Mice were housed in standard pathogen-free conditions on a 12 hour light/ 12 hour dark cycle with access to standard rodent chow and water.

3.1.12 Tissue collection

Mice were sacrificed by neck dislocation (schedule 1 method). Tissue explants were removed and snap frozen in liquid N_2, and stored at -80°C for future use. The mice were pinned onto a cork board to enable accurate dissections. Ethanol was then sprayed onto the animal to protect the tissues from contamination. Scissors were used to cut along the ventral midline and the skin was pealed back to enable a clear view of the tissues to be removed. The tissues were cut away from the animal using scissors and then trimmed of any excess artefacts.

3.1.13 Blood collection

Blood samples were obtained from the Saphenous Vein. The mouse was placed in a restraining tube, and hair was removed from the area with the use of clippers. A 25 gauge needle was used to collect the drops of blood as they appeared and collection tubes with capillary action were used to facilitate blood collection.

3.2 Introduction

GCs have become one of the mostly widely prescribed drugs in medical practice due to their potent anti-inflammatory (Hench et al 1950), antiproliferatie and antiangiogenic properties (Vilasco et al 2011). However GC excess can lead to adverse metabolic effects including; Cushing's syndrome, and type 2 diabetes (Fardet et al 2007). GCs can also be regenerated at a tissue specific level by the 11β-HSD1 enzyme (Koteleytsev et al 1997), and these reactivated GCs can play a critical role in the development of metabolic syndrome (Walker et al 2000).

The key role of 11β-HSD1 has been highlighted through the use of animal models. It has been shown that a global KO of 11β-HSD1 confers protection from glucose intolerance, hyperinsulinemia, hepatic steotosis, adiposity, and hypertension, therefore showing the potential role of 11β-HSD1 as a therapeutic target (Morton et al 2001).

Increased 11β-HSD1 activity selectively within adipose tissue results in the development of full metabolic syndrome with visceral obesity, diabetes, and hypertension (Masuzaki et al 2001) conversely transgenic mice with increased expression of 11β-HSD1 within the liver resist obesity, but have insulin resistance, dyslipidaemia, and fatty liver (Mariniello et al 2006). An important observation in these models was that they both had no change in circulating corticosterone levels, which suggests that the increase in intracellular GC regeneration is key to the observed phenotype. These studies provide further weight to the role of adipose 11β-HSD1 in the GC excess phenotype. In addition to animal studies a key study by Tomlinson et al 2002 reported a patient with Cushing's syndrome who was protected from the classic Cushing's phenotype due to a decrease in 11β-HSD1 activity. Here a case of CS was presented lacking the classical central obese and metabolic phenotype and it was hypothesised that this was due to a lack of 11β-HSD1 enzyme content. The consequential decrease in local conversion of inactive cortisone to active cortisol served to highlight to role

of 11β-HSD1 within the development of obesity, particularly within the Cushingoid phenotype.

Based on previous studies showing the link between GCs and the metabolic syndrome I hypothesise that tissue specific regeneration of GC via 11β-HSD1 is the major determinant of the phenotype of GC excess and that deletion of 11β-HSD1 specifically within adipose will protect from metabolic abnormalities. In this chapter I present data demonstrating the effect of 11β-HSD1 global KO, and also the generation of an adipose specific KO of 11β-HSD1.

3.3 Results

3.3.1 Metabolic consequences of glucocorticoid excess in 11β-HSD1 global knock-out

Studies within our lab led by Dr Stuart Morgan have investigated 11β-HSD1 (GKO) global knock-out mice. Mice were treated with corticosterone (100ug/ml) or vehicle via drinking water for 5 weeks. I initially verified that there was an effect from the glucocorticoid excess (Figure 3-2). I showed that following corticosterone (CORT) treatment there was a significant increase in serum corticosterone levels and also adrenal atrophy in both WT and GKO mice. This therefore suggests that further effects seen are independent of circulating active GC concentrations.

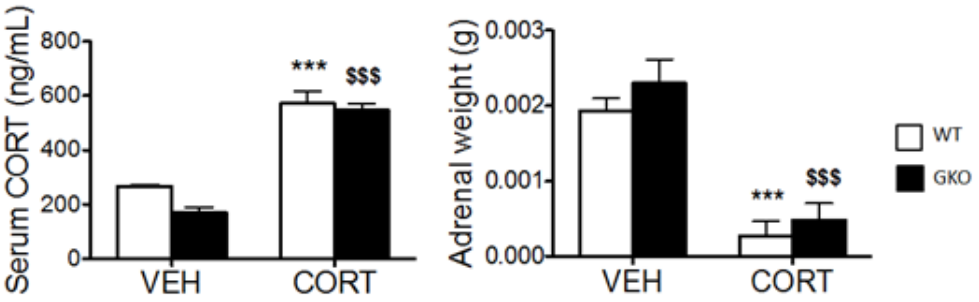

Figure 3-2 Validation of glucocorticoid excess
*Treatment with corticosterone led to a significant increase in serum corticosterone levels of both WT and GKO mice. Treatment with corticosterone also led to significant adrenal atrophy in both WT and GKO mice. Data was analysed using two way ANOVA. ***P < 0.001 vs. WT vehicle; $$$P < 0.01 vs. WT CORT. Error bars represent SEM. N=6 for all mice. Cited from Morgan et al 2014.*

By the fourth week of treatment WT mice treated with corticosterone were glucose-intolerant (Figure 3-3) hyperinsulinemic and hyperglycaemic following a 5-h fast (Figure 3-4), and had increased systolic blood pressure (Figure 3-5) when compared to those treated with vehicle. In contrast to this it was found that corticosterone-treated GKO mice remained relatively glucose-tolerant (Figure 3-2), had lower fasting insulin and glucose levels (Figure 3-3), and

showed no change in systolic blood pressure (Figure 3-4). The GKO mice were therefore protected from the adverse effects of systemic GC excess. This therefore demonstrates that global deletion of 11β-HSD1 can protect against the adverse metabolic effect of exogenous corticosterone (CORT) treatment.

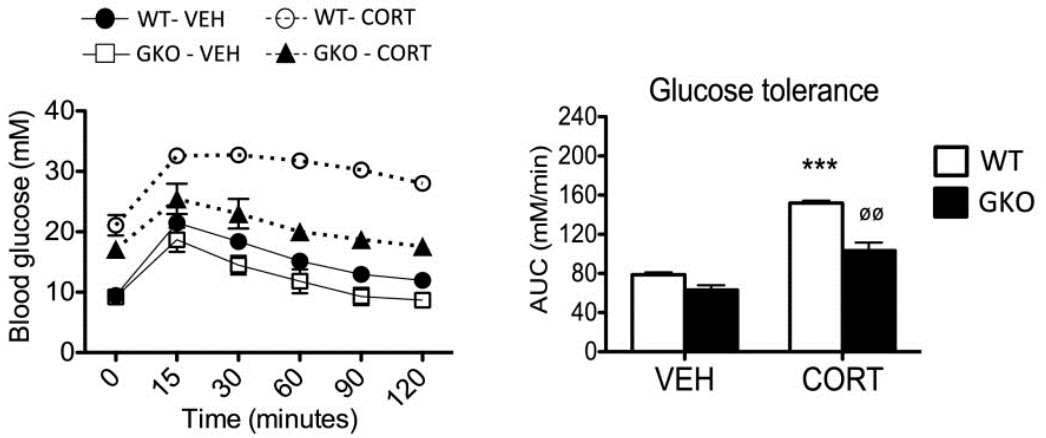

Figure 3-3 Effect of CORT treatment on glucose tolerance of WT and GKO mice
*Glucose tolerance was calculated as area under the curve, and shows improved glucose tolerance in CORT-treated GKO mice compared with CORT-treated WTs. There is also a significant decrease in glucose tolerance in WT mice following CORT treatment. Data analysed using a two way ANOVA. ***P < 0.001 vs. WT vehicle; øøP < 0.01 vs. WT CORT. Error bars represent SEM. Cited from Morgan et al 2014.*

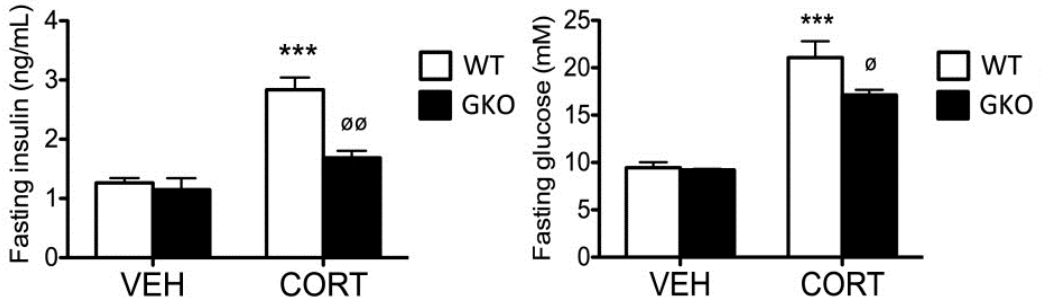

Figure 3-4 Effect of CORT treatment on insulin and glucose levels of WT and GKO mice
*Fasting insulin and fasting glucose levels are improved in CORT-treated GKO mice compared with CORT-treated WTs. There is a significant detrimental effect on fasting insulin and fasting glucose levels following CORT treatment in the WTs. Data analysed using a two way ANOVA. ***P < 0.001 vs. WT vehicle; øP < 0.05, øøP < 0.01 vs. WT CORT. Error bars represent SEM. Cited from Morgan et al 2014.*

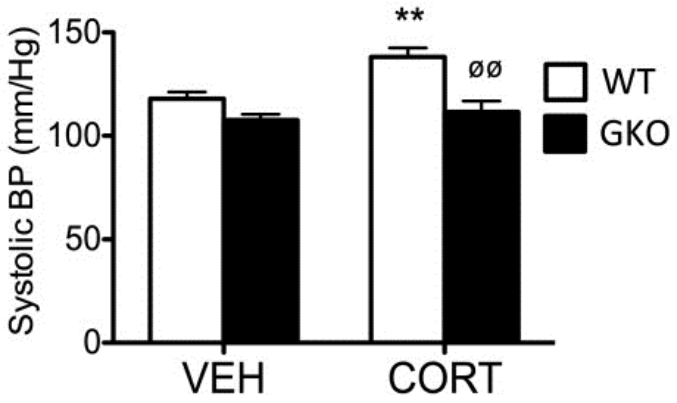

Figure 3-5 Effect of CORT treatment on systolic blood pressure in WT and GKO mice.
*Systolic blood pressure is improved in CORT-treated GKO mice compared with CORT-treated WTs. There is a significant increase in systolic blood pressure in the CORT-treated WTs compared to WT vehicle treated mice. Data analysed using a two way ANOVA. **P < 0.01 vs. WT vehicle; øøP < 0.01 vs. WT CORT. Error bars represent SEM. Cited from Morgan et al 2014.*

I also investigated the effect of GC excess on adipose tissue as GCs have been shown to increase adiposity. I found that within all fat beds tested there was a significant increase in adipose weight in CORT treated WT mice however GKO mice were protected from this increase in adipose weight (Figure 3-6). Thus indicating that GKO of 11β-HSD1 leads to protection from obesity associated with GC excess.

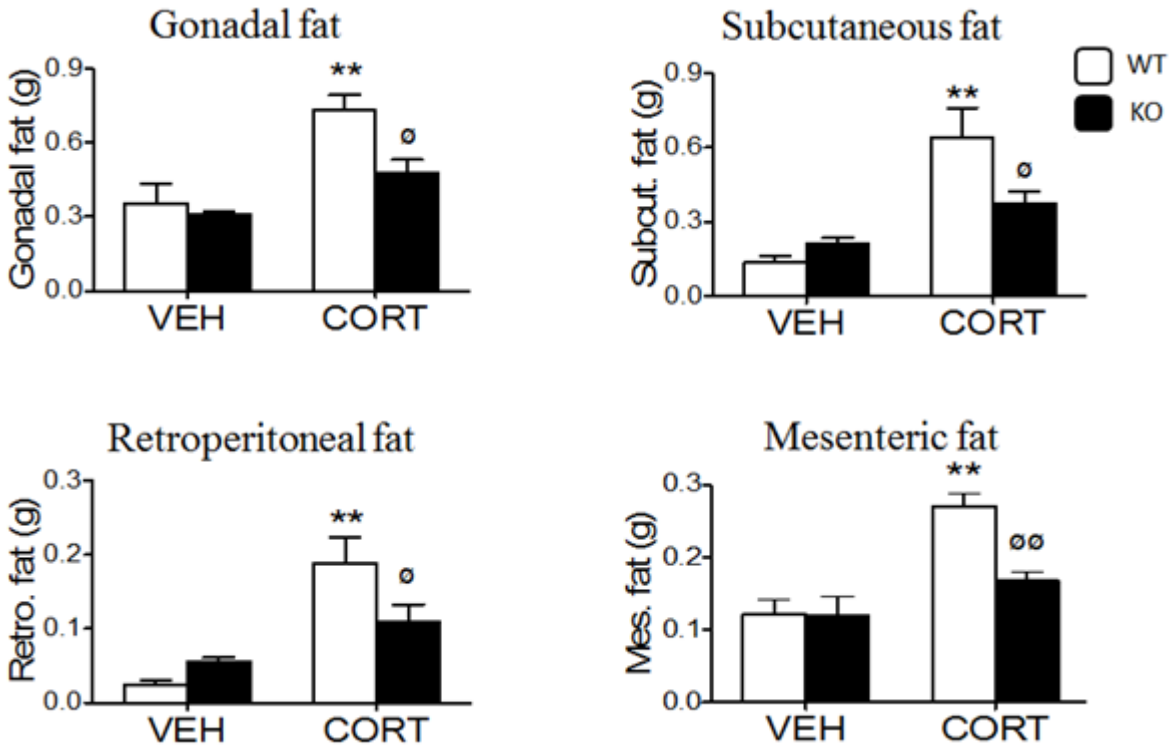

Figure 3-6 Effect of GKO of 11β-HSD1 on adipose tissue
*There is significant protection from obesity provided by global knock-out of 11β-HSD1 in all adipose depots studied. There is a significant increase in obesity in all adipose depots studied following CORT treatment in WT mice compared with vehicle treated WT mice. Data was analysed using a students T-test. **P < 0.01 vs. WT vehicle; ØØP < 0.01 vs. WT CORT ; ØP < 0.05 vs. WT CORT. Error bars represent SEM. Cited from Morgan et al 2014.*

Adipocyte size was also investigated. Adipocyte size was increased in both gonadal and subcutaneous adipose depots in both WT and GKO corticosterone treated mice. This is also demonstrated histologically for the gonadal adipose depot (Figure 3-7). This shows that the decrease in adipose depot weight shown in figure 3-5 is due to decrease in adipocyte number.

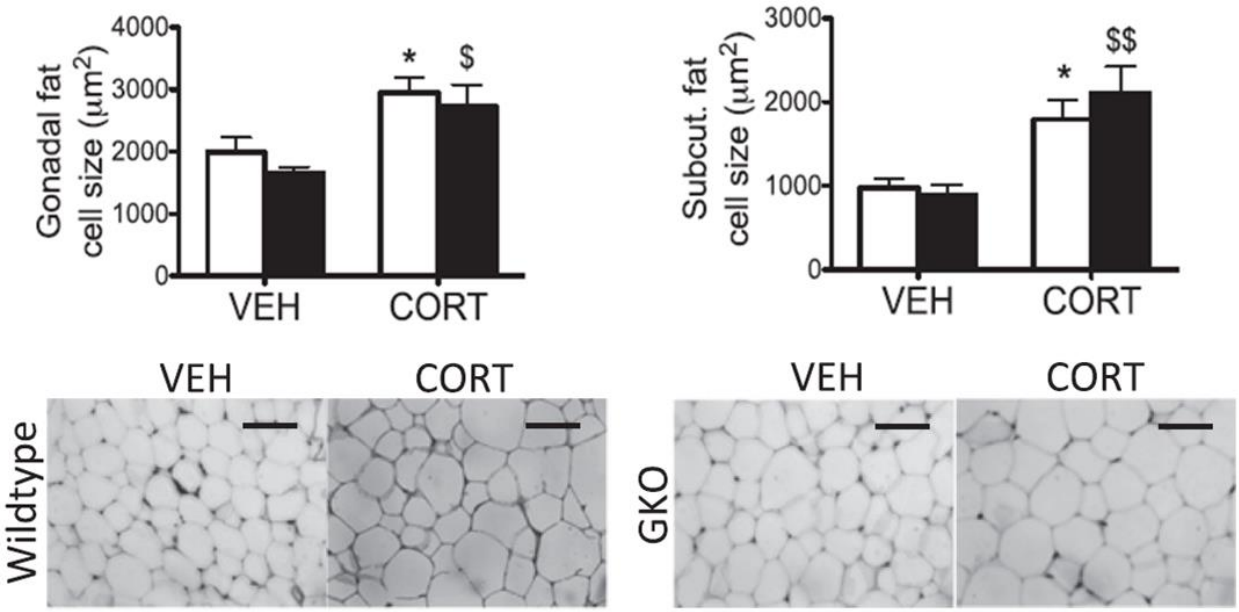

Figure 3-7 Effect of GKO of 11β-HSD1 on adipocyte size
*There is a significant increase in adipocyte size following CORT treatment in both WT and GKO mice in both gonadal and subcutaneous adipose. Data was analysed using a two way ANOVA. *P < 0.05 vs. WT vehicle; $P<0.05, $$P<0.01 vs GKO vehicle. Error bars represent SEM. Cited from Morgan et al 2014.*

Within humans non-alcoholic fatty liver disease is commonly associated with GC excess due to GCs increasing lipolysis. In keeping with this I showed that there was a significant increase in hepatic triglyceride (TAG) and serum free fatty acid content in WT mice following corticosterone treatment (Figure 3-8). In contrast GKO provided protection from hepatic TAG accumulation and increased serum free fatty acids (Figure 3-8).

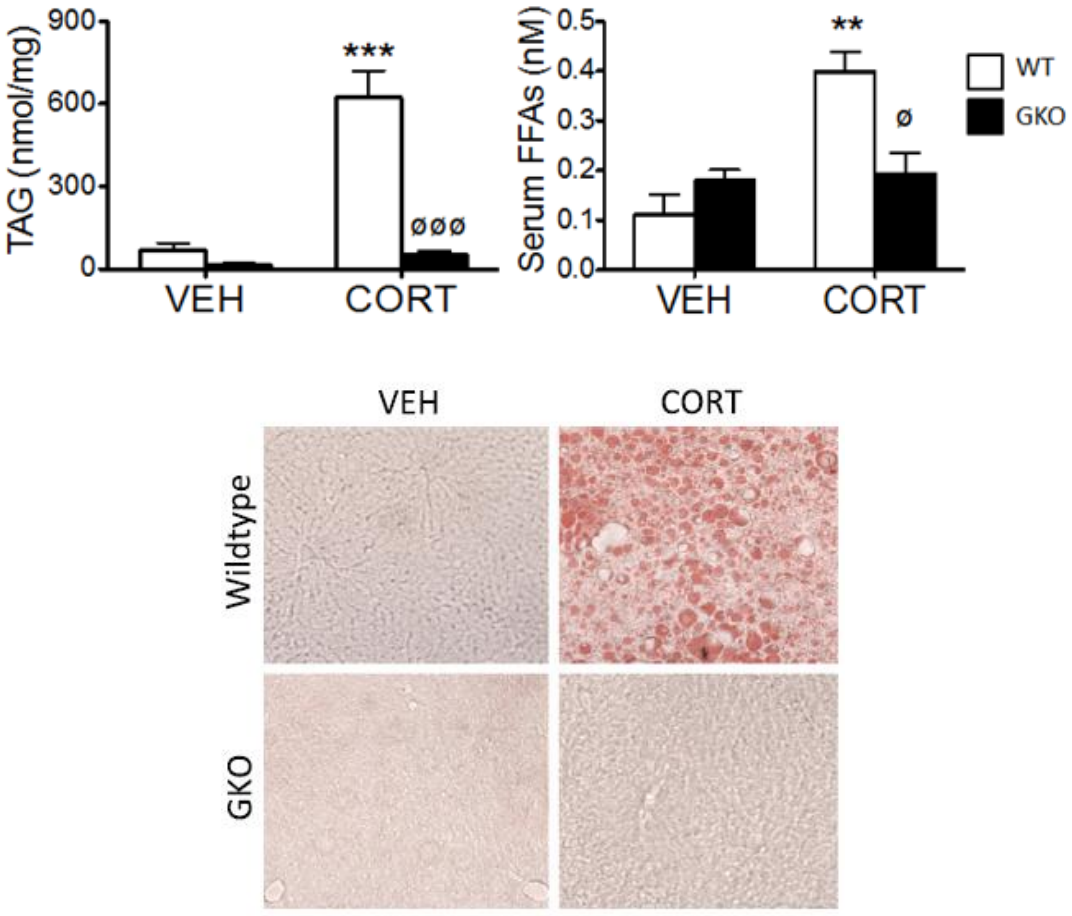

Figure 3-8 Global knock-out of 11β-HSD1 provided protection from TAG accumulation, serum free fatty acid accumulation and hepatic steatosis following CORT treatment
*GKO provided protection from an increase in TAG content following treatment with corticosterone and also provided protection from an increase in serum free fatty acid accumulation following treatment with corticosterone. In addition to this there is protection from hepatic steotosis. There is a significant increase in TAG content and serum free fatty acids following CORT treatment in WT mice. ***P < 0.001 **P < 0.01 vs. WT vehicle; ØØØP < 0.001 ØP < 0.05 vs. WT CORT. Error bars represent SEM. Cited from Morgan et al 2014.*

This data demonstrates that global deletion of 11β-HSD1 confers a beneficial phenotype on mice following excess CORT exposure. In order to elucidate the tissues responsible for this benefit I generated an adipose 11β-HSD1 specific knock-out mouse model.

3.3.2 Validation of adipose specific knock-out of 11β-HSD1

To examine the role of adipose 11β-HSD1 and its potential role in GC and insulin sensitivity I generated 11β-HSD1 adipose specific knock-out (FKO) mice. These mice were generated

using a previously determined floxed allele and adiponectin-cre line (JAX lab, B6;FVB-Tg(Adipoq-cre)1Evdr/J) as the adipose specific promoter (Figure 3-9). I used both 11β-HSD1 and cre primers to ascertain the genotypes of the mice using ear clip genotyping, which allowed us to identify target animals and corresponding litter matched controls.

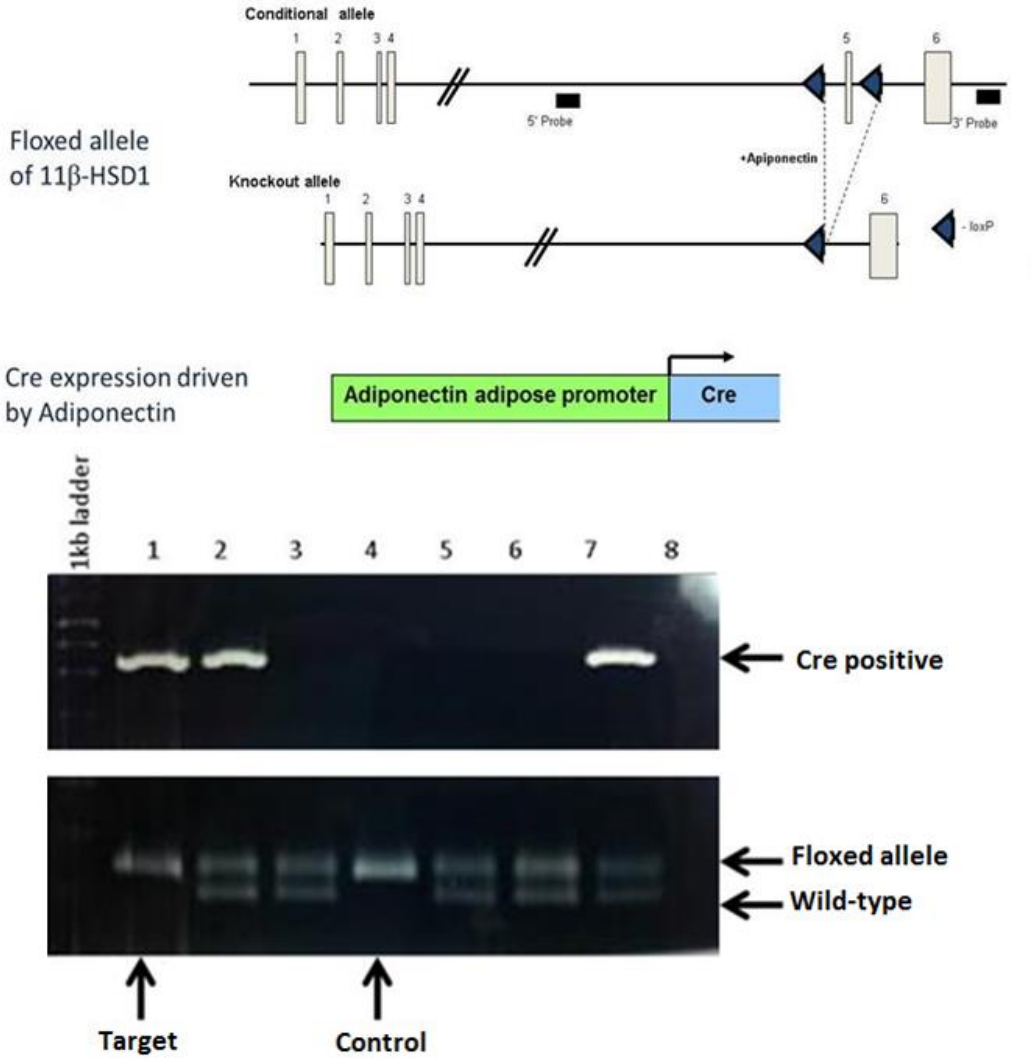

Figure 3-9 Targeting strategy and identification of adipose specific 11β-HSD1 knock-outs
I used a previously determined floxed allele of 11β-HSD1 and cre recombinase expressed from an adiponectin adipose specific promoter. Knock-out mice were identified by having the floxed allele for 11β-HSD1 and being cre positive. Cre primers were used to identify mice which had expression of cre recombinase as shown in lanes 1, 2 and 8. 11β-HSD1 primers were used to identify mice which contained the recombined KO allele, as shown in lanes 1 and 4. Mice which had both expression of cre recombinase and the recombined KO allele of 11β-HSD1 were designated as being 11β-HSD1 adipose specific KO (FKO) as shown in lane 1. Cited from Morgan et al 2014.

I assessed the genotype of the mice using ear clips from new born litters. I used cre primers to confirm the presence of cre recombinase within the mice and 11β-HSD1 primers to determine which mice contained the floxed 11β-HSD1 allele as shown in figure 3-9. The mice which contained both the cre allele and the floxed allele were designated as knock-out mice as seen in lane 1.

Once these knock-out mice were identified I confirmed that the knock-out of the 11β-HSD1 gene was specific to the adipose tissue. This was achieved by PCR amplification using 11β-HSD1 primer sets on specific tissues from the FKO mice following DNA isolation and used to confirm the presence of the recombined knock-out allele is located specifically within the adipose tissue depots (Figure 3-10). This demonstrated that the recombined KO allele for 11β-HSD1 was present only within the adipose tissue of the mice.

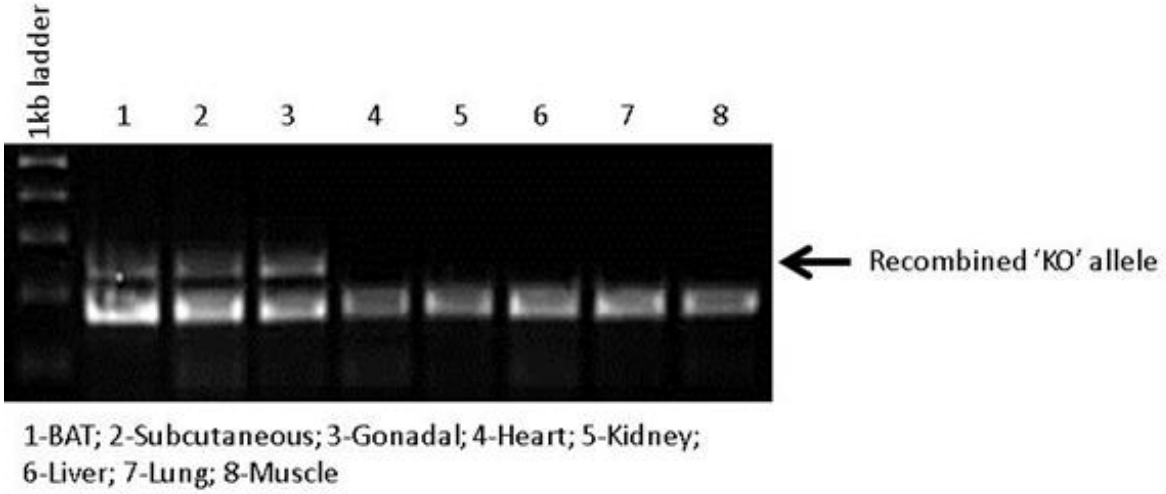

Figure 3-10 Adipose specific knock-out of 11β-HSD1 on tissue samples
Genomic DNA PCR using 11β-HSD1 primers demonstrates that the recombination is specific to adipose tissue. The recombined knock-out allele is shown to be present in the adipose tissues depots (BAT, Subcutaneous and Gonadal) of the FKO mice but absent in the other tissues of the FKO mice (Heart, Kidney, Liver, Lung, and Muscle). Cited from Morgan et al 2014.

Having confirmed the knock-out of 11β-HSD1 specifically within the adipose tissue of the mice at the genomic level I continued my validation by undertaking tissue specific mRNA

analysis on the expression of 11β-HSD1 as shown in figure 3-11. I found that there was no change in the mRNA expression level when comparing WT to FKO in the liver, lung, heart, kidney or muscle, however I showed a significant decrease in the mRNA expression of 11β-HSD1 within the adipose tissue depots (BAT, Gonadal, Subcutaneous) (P<0.0001).

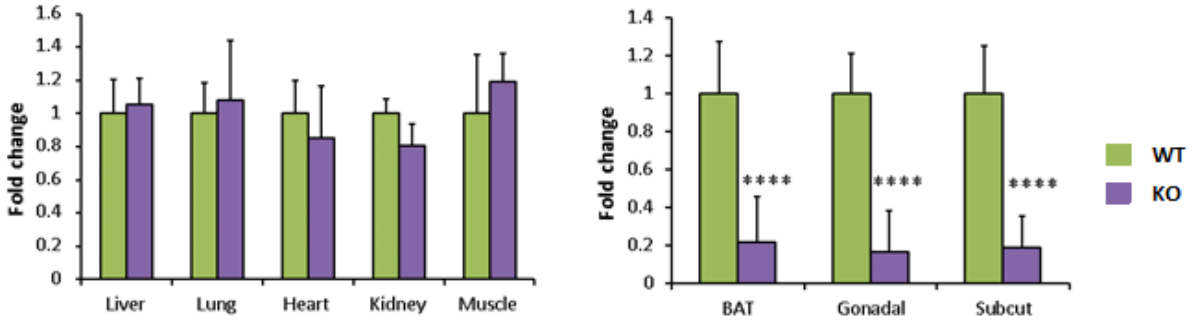

Figure 3-11 Confirmation of adipose tissue specific knock-out of 11β-HSD1 using mRNA analysis

*The fold change in 11β-HSD1 mRNA expression is significantly lower in the adipose tissues of the FKO mice compared to the WT. There is no difference in the mRNA expression of 11β-HSD1 within other tissues of the FKO mice compared to the WT. Data was analysed using student T-test. n=3. ****P<0.0001. Error bars represent SEM. Cited from Morgan S. A et al 2014.*

Having demonstrated a tissue specific knock-out of 11β-HSD1 specifically within the adipose tissue of the FKO mice at both the DNA and RNA level I continued the validation of my model by measuring the oxo-reductase activity of 11β-HSD1 within BAT, subcutaneous and liver tissue shown in figure 3-12. I found a significant decrease in 11β-HSD1 oxo-reductase activity in BAT (P<0.001) and subcutaneous adipose tissue (P<0.0001) in the FKO compared to the WT. I also found no change in the 11β-HSD1 oxo-reductase activity within the liver when comparing the WT to FKOs.

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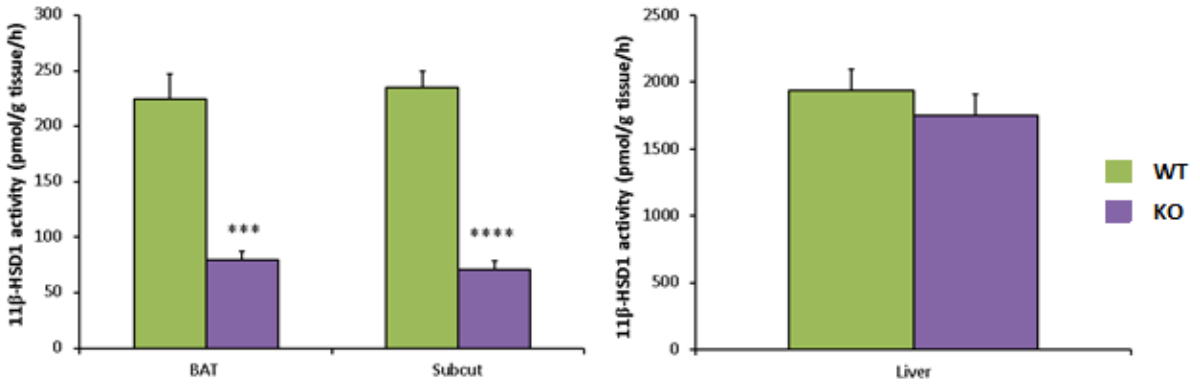

Figure 3-12 11β-HSD1 oxo-reductase activity of explant adipose tissue and liver
*The 11β-HSD1 oxo-reductase was significantly lower in the FKO mice within the BAT and subcutaneous adipose explants when compared to WT. There is no difference in the 11β-HSD1 oxo-reductase activity in the liver explants between FKO and WT. Data was analysed using the student T-test. n=3. ***P<0.001, ****P<0.0001. The error bars represent the SEM. Cited from Morgan S. A et al 2014.*

Despite having shown a significant decrease in 11β-HSD1 activity in the adipose tissue explants of the FKO mice there was higher activity than I would have expected in the knock-out mice despite there being a significant decrease. I hypothesise that this activity could be due to the presence of macrophages and/or preadipocytes present which constitute 30% of the adipose tissue and do not express the adiponectin gene and therefore do not have a knock-out of 11β-HSD1. I tested this hypothesis by separating the mature adipocyte and stromal vascular fractions and testing the 11β-HSD1 oxo-reductase activity present in the different fractions so to ascertain which area is responsible for the level of activity seen (Figure 3-13). I found that there was no change in 11β-HSD1 oxo-reductase activity in the stromal vascular fraction, however I showed a significant decrease in the oxo-reductase activity of 11β-HSD1 within BAT ($P<0.001$), subcutaneous ($P<0.0001$) and gonadal ($P<0.001$) depots within the mature adipocytes.

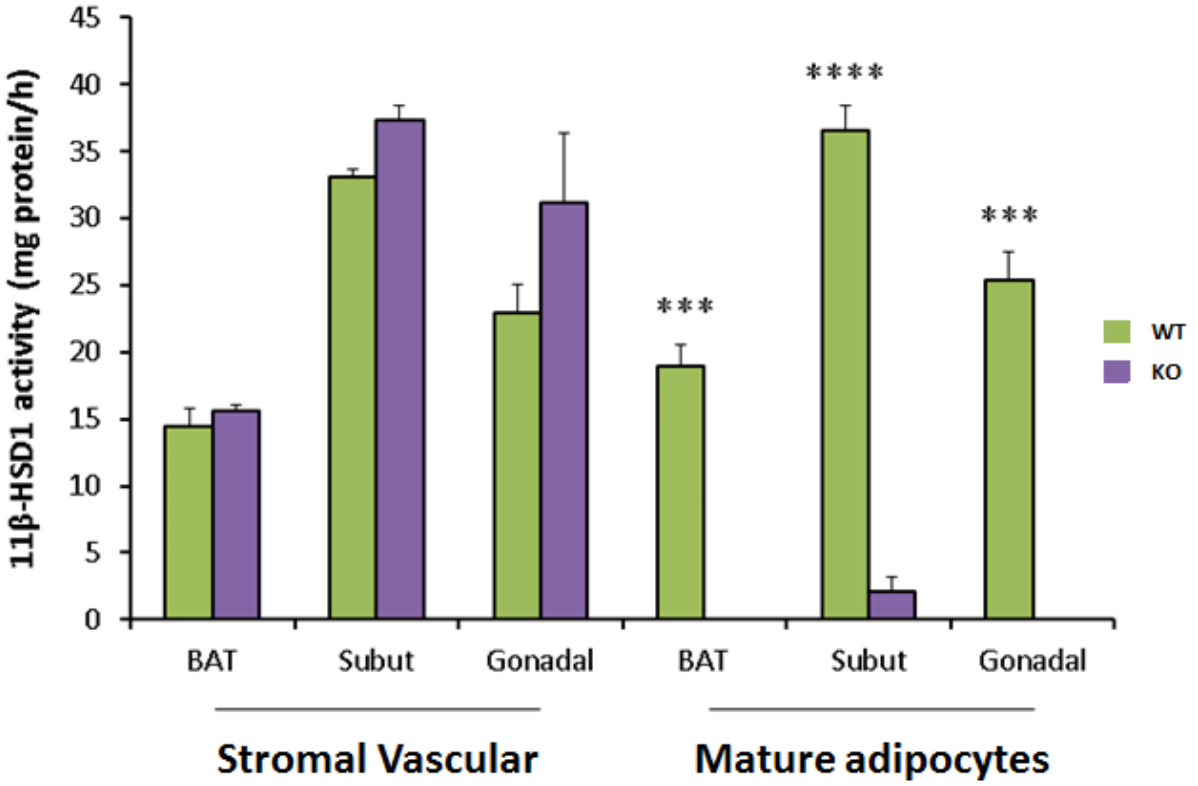

Figure 3-13 Oxo-reductase activity of mature adipocytes and stromal vascular fraction
*There is no change in 11β-HSD1 oxo-reductase activity in any of the adipose depots in the stromal vascular fraction between WT and FKO. There is a significant decrease in the 11β-HSD1 oxo-reductase activity within the BAT, subcutaneous and gonadal with the FKO compared to the WT, where the FKO demonstrated almost undetectable activity. Data was analysed using the student T-test. n=3. ***P<0.001, ****P<0.0001. The error bars represent the SEM. Cited from Morgan S. A et al 2014.*

3.3.3 Metabolic assessment of glucocorticoid supplementation in FKO mice

I utilised this FKO model generated to investigate if GCs reactivated by 11β-HSD1 within adipose tissue contribute to the adverse effects of GC excess, and if adipose specific KO of 11β-HSD1 can recapitulate the beneficial phenotype previously shown with GKO of 11β-HSD1. This aims to highlight the tissue responsible for the beneficial phenotype confirmed following global deletion of 11β-HSD1.

To investigate this I treated FKO mice with CORT (100 μg/mL), or vehicle via drinking water for 5 weeks. In contrast to GKO mice, FKO mice were not protected from

increased adiposity or a decrease in lean mass following CORT treatment. I found increases in the weight of all fat pads tested (Gonadal, mesenteric, retroperitoneal and subcutaneous) and decreases in the weight of muscle beds tested (quadriceps, tibialis anterior, and soleus). This overall led to an increase in total body weight following treatment with CORT. This data is summarised in table 3-3.

	Weight +/- SE			
	Control		FKO	
Tissues	Vehicle	CORT	Vehicle	CORT
Body weight (g)	27.74+/-0.53	26.69 +/- 0.39	27.75 +/1 0.48	29.73 +/- 0.55
Quadriceps (mg)	170+/-8	99 +/- 12*	172 +/- 11	116 +/- 6‡
Tibialis anterior (mg)	58 +/-0.0031	34 +/- 2.9*	50 +/- 1.6	32 +/- 3.7‡
Soleus (mg)	7.9 +/- 0.5	9.7 +/- 1.2	10.6 +/- 1.6	5.8 +/- 1.1
Gonadal fat (mg)	345 +/- 29	946 +/- 114*	360 +/- 21	802 +/- 79‡
Mesenteric fat (mg)	88 +/- 24	240 +/- 43	68 +/- 9	210 +/- 13
Retroperitoneal fat (mg)	86 +/- 16	319 +/- 45*	84 +/- 10	306 +/- 27‡
Subcutaneous fat (mg)	230 +/- 28	987 +/- 58†	186 +/- 22	976 +/- 77¶
Adrenal gland (mg)	1.8 +/- 0.1	0.7 +/- 0.1†	1.6 +/- 0.1	0.5 +/- 0.1¶
Liver (mg)	1.060 +/- 40	1648 +/- 189	1107 +/- 115	1243 +/- 58
Kidney (mg)	170 +/- 7	160 +/- 8	157 +/- 5	175 +/- 32

Table 3-3 Effect of CORT treatment on adiposity and lean mass
*FKO mice were not protected from increased adiposity and decreased lean mass following treatment with CORT. Data was analysed using two-way ANOVA. n = 6–7 in each group. *P < 0.01 †P < 0.001 vs. WT vehicle; §P < 0.05, ‡P < 0.01, ¶P < 0.01 vs. FKO vehicle. Cited from Morgan 2014.*

Glucose tolerance testing was conducted on WT and FKO mice, I found that there was no change in glucose intolerance (Figure 3-14) following CORT treatment between the controls and the FKOs.

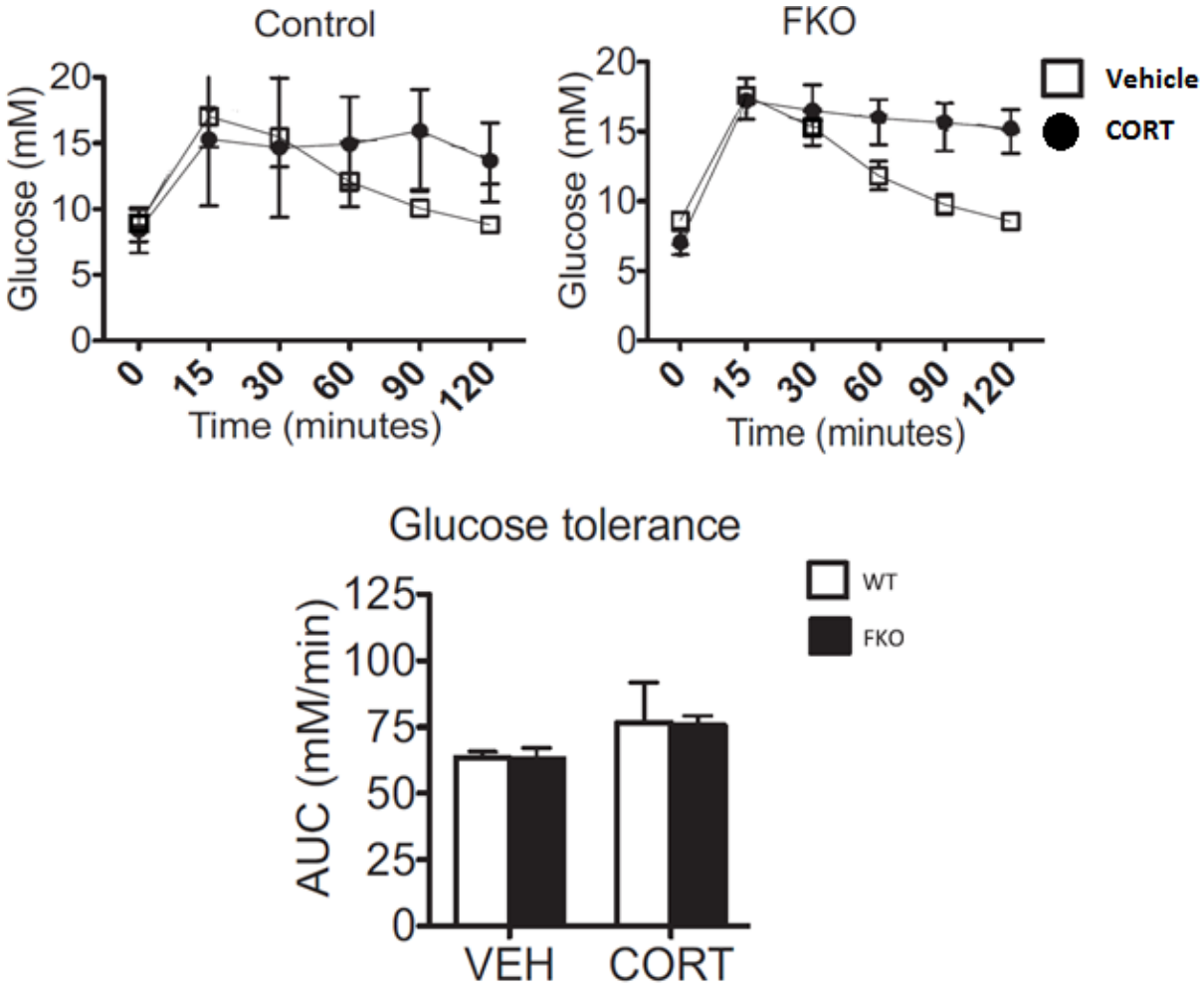

Figure 3-14 Effect of CORT treatment on glucose tolerance
FKO mice were not protected from glucose intolerance following treatment with CORT. Values are calculated as area under the curve. Data were analysed using a two-way ANOVA. n = 6–7 in each group. Cited from Morgan et al 2014.

Fasting insulin levels were not changed in WT and FKO mice (Figure 3-15) following CORT treatment, the FKOs were not protected from hyperinsulinemia where they showed a significant increase (<0.01) in fasting insulin levels following CORT treatment.

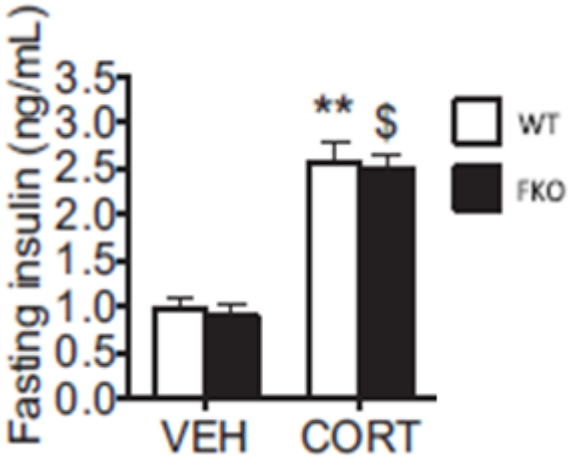

Figure 3-15 Effect of CORT treatment on insulin tolerance
*FKO mice were not protected from hyperinsulinemia when treated with CORT. There was a significant increase in the fasting insulin levels following CORT treatment in the WTs compared to vehicle treated, and there was a significant increase in the fasting insulin levels following CORT treatment in the FKOs compared to vehicle treated. Data were analysed using two-way ANOVA. n = 6–7 in each group. **P < 0.01, vs. WT vehicle; $P < 0.05, $$P < 0.01, vs. FKO vehicle. Error bars represent SEM. Cited from Morgan S. A et al 2014.*

FKO mice showed protection from CORT induced hepatic steotosis and increased expression of key lipolytic mediators. This was shown using oil red O staining on frozen liver sections and hepatic TAG quantification. However FKO mice were not protected from CORT induced increased expression of the hepatic fatty acid transporter CD36 (figure 3-16).

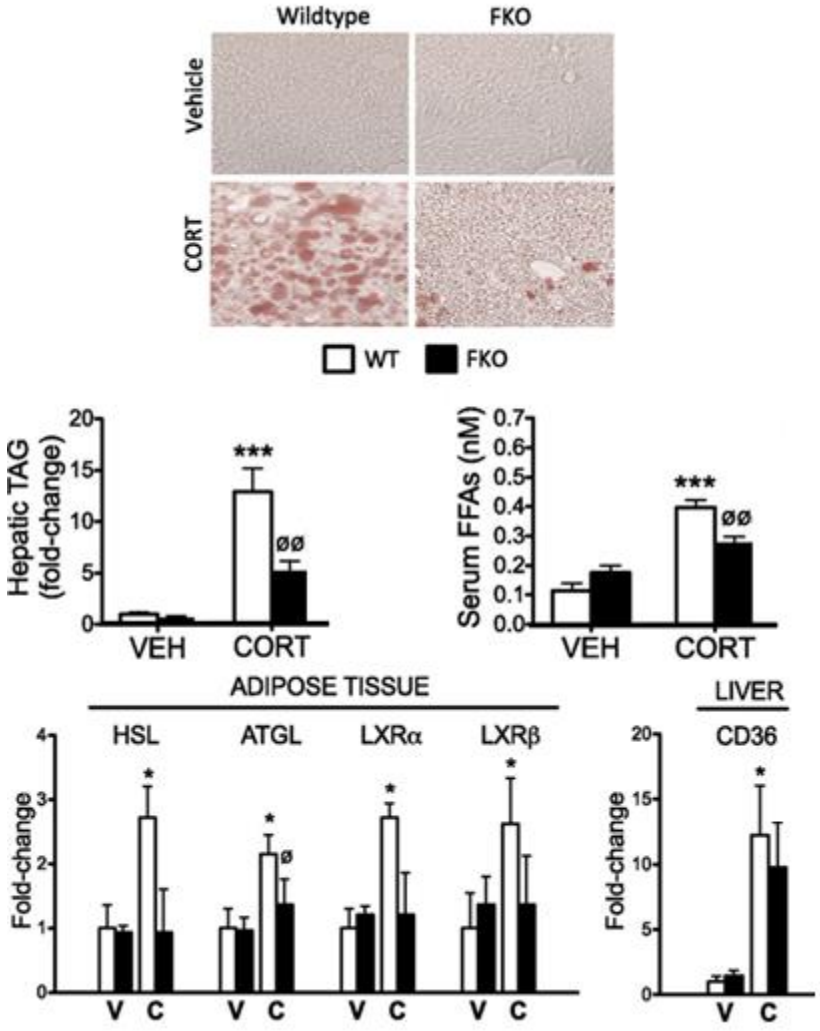

Figure 3-16 WAT-specific 11β-HSD1 KO mice, and not liver-specific 11β-HSD1 KO mice, are protected from the development of CORT-induced hepatic steatosis.
*Oil red O staining showed that FKO mice were protected from CORT induced hepatic steotosis compared with CORT treated controls (wildtype). Hepatic TAG quantification also showed that FKO mice were protected from CORT induced hepatic steotosis compared with CORT treated controls (wildtype). In addition FKO mice, were protected from increased serum free fatty acids and increased mRNA expression of key lipolytic mediators in adipose tissue following CORT treatment. However FKO mice were not protected from increased expression of the hepatic free fatty acid transporter CD36 following CORT treatment. Data were analysed using two-way ANOVA (n = 6–7 in each group). *P < 0.05, ***P < 0.001 vs. WT vehicle; ØP < 0.05, ØØP < 0.01 vs. WT CORT. C, CORT; V, vehicle. Error bars represent SEM. Cited from Morgan et al 2014.*

Taken together, these data suggest intracellular GC excess in adipose tissue is influential in the regulation of lipid metabolism.

3.4 Discussion

Since their discovery as potent anti-inflammatory agents (Hench et al 1950) GCs have become one of the most widely prescribed drugs in medical practice. GCs however have many adverse effects and long term GC therapy can lead to suppression of the HPA axis (Krasner et al 1999), insulin resistance (Lansang et al 2011) and diabetes (Lansang et al 2011). This effect of GCs is amplified by 11β-HSD1 which regulates intracellular GC metabolism at the pre-receptor level (Rabbitt et al 2002), via its oxo-reductase activity increasing intracellular GC levels by converting circulating inert GC to active GC (Tomlinson et al 2001) (Napolitano et al 1998). These negative effects of GC excess therefore are an active area of research in an attempt to establish a mechanism to control their adverse effects.

The data shows that GKO of 11β-HSD1 protects from the adverse effects of GC excess, and the mice showed less of an increase in adipose mass following GC treatment. This has demonstrated that reactivation of GCs by 11β-HSD1 is the major determinant of the phenotype seen with GC excess as opposed to circulating GC levels. This leads to the possibility of 11βHSD1 acting as a potential target for the therapeutic treatment of patients with Cushing's syndrome, and to limit the adverse effects seen following the prescription of GCs.

In order to further examine the role of 11β-HSD1 within the body, I generated an 11β-HSD1 adipose specific knock-out mouse model (FKO), in order to try to elucidate the tissue responsible for the protective affects seen following global knock-out of 11β-HSD1.

FKO mice were generated by utilising cre-lox technology. I found a significant decrease in expression of 11β-HSD1 within the adipose of the FKO mice, and normal levels of 11β-HSD1 mRNA expression within the other tissue depots of the FKO mice when compared to WT. I further confirmed the adipose specific knock-out of 11β-HSD1 by

performing an 11β-HSD1 oxo-reductase activity assay. This showed a significant decrease in 11β-HSD1 activity within the adipose depots and normal 11β-HSD1 activity within the liver of the FKO mice compared to the WTs. Despite this significant decrease in expression I did however find a higher level of 11β-HSD1 activity than would be expected in a knock-out model. This could have been due to the presence of other factors within adipose tissue, for example immune and endothelial cells (Dani et al 2012). In order to ascertain what was responsible for this activity I performed 11β-HSD1 oxo-reductase activity assays on the stromal vascular fraction and also mature adipocytes. I found that within the stromal vascular fraction there was a change in the 11β-HSD1 activity between the WT and FKO mice. However within the mature adipocytes I found that there was a significant decrease in 11β-HSD1 expression in the FKO compared to the WT, and that the level of activity seen was the level to be expected from a knock-out model. These experiments allowed me to confirm that I had successfully generated an adipose specific 11β-HSD1 knock out model. This means that the model will be devoid of local GC regeneration within mature adipose tissue cells.

It has been shown that GKO mice are protected from CS when mice are exposed to excess GC. These mice showed improved glucose tolerance, insulin sensitivity, improved systolic blood pressure, liver, muscle and skin. I utilised my generated 11β-HSD1 adipose specific 11β-HSD1 KO model (FKO) to ascertain the contribution of adipose 11β-HSD1 to this protective phenotype. I showed that these mice were not protected from adiposity, decreased lean body mass, glucose intolerance, hyperimsulinemia, increased expression of CD36 or reduced grip strength following CORT treatment

Due to not being protected from an increase in the expression of the fatty acid transporter CD36, the FKO mice hepatic fatty acid uptake is unaffected by FKO of 11β-HSD1 within adipose tissue. In addition the failure of the mice to be protected from adiposity

decreased lean body mass, glucose intolerance, hyperimsulinemia, or reduced grip strength following CORT treatment suggests the role played by 11β-HSD1 within other tissues, such as skeletal muscle.

However these mice were protected from hepatic TAG accumulation, increased serum free fatty acids, and hepatic steotosis, and increased adipose expression of hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL), liver X receptor a (LXRa), and liver X receptor b (LXRb). In contrast to this LKO mice were not protected from these adverse effects of GC treatment, which suggests that increased intracellular GC availability within adipose tissue as opposed to the liver is central to the beneficial effects seen in the GKO.

Triglycerides located within adipose tissue are the main form of energy storage within animals (Herada et al 2003). These energy stores are realised in times of need and this process is advantages to survival. However the balance between storage and release needs to be controlled as dysregulation of these processes may result in metabolic disorders, such as obesity and insulin resistance (Herada et al 2003). The classical enzyme, HSL, hydrolyses TG, diglycerides (DG), and monoglycerides (MG), with the highest specific activity against DG (Belfrage et al. 1978).

HSL is primarily involved in the mobilisation of stored fats and is stimulated by catecholamines and ACTH and inhibited by insulin and has traditionally been considered the key lipolytic enzyme in adipocytes (Holm et al 2003). Its hormonal activation occurs via cAMP dependent PKA which phosphorylates HSL (Yeaman et al 1990). It hydrolyses the first fatty acid from the triglyceride molecule resulting in the release of a fatty acid and diglyceride and plays a pivotal role in providing the major source of energy for most tissues, and HSL KO mice have been shown to be resistant to obesity (Kraemer et al 2002; Herada. K. 2003). ATGL has triglyceride-specific lipase activity, and catalyzes the initial step in

triglyceride hydrolysis (Kershaw et al 2006; Zimmermann et al 2004). ATGL specifically removes the first fatty acid from the TG molecule generating FFA and DG. LXRs are regulators of cholesterol, fatty acid and glucose homeostasis and are involved in the stimulation of lipogenesis. LXR KO mice have been shown to have decreased adiposity with decreased lipid size (Gerin et al 2005). LXRa and LXRb have been identified as key regulators of lipid homeostasis in multiple cell types (Repa et al 2000).

Therefore the control of their expression shown in the FKO mice in my study demonstrated a beneficial effect of 11β-HSD1 KO within adipose tissue, as an increase in HSL, ATGL. LXRa or LXRb expression would lead to an increase in the breakdown of stored triglycerides, and a consequential increase in free fatty acids.

Our GKO and FKO models demonstrate how knock-out of the 11β-HSD1 gene can go some way to ameliorate the negative effects of excess. Although the FKO mice did not show the striking protection seen with the GKO mice, they still demonstrated protection from some of the adverse effects of GC excess. This theory that KO of 11β-HSD1 will be beneficial is based on 11β-HSD1s role in the regeneration of active GC from 11-DHC. In addition to this when there is an increase in CORT there is an increase in the production of 11β-HSD1, leading to an increased potential for the production of CORT. Also an increase in CORT leads to an increase in the regeneration of 11-DHC by 11β-HSD2. This activated CORT can enter the cell by binding to the GR within the cytoplasm. Therefore knock-out of 11β-HSD1 can have far reaching benefits on the overall effect of CORT on the body. This relationship is demonstrated in figure 3-17.

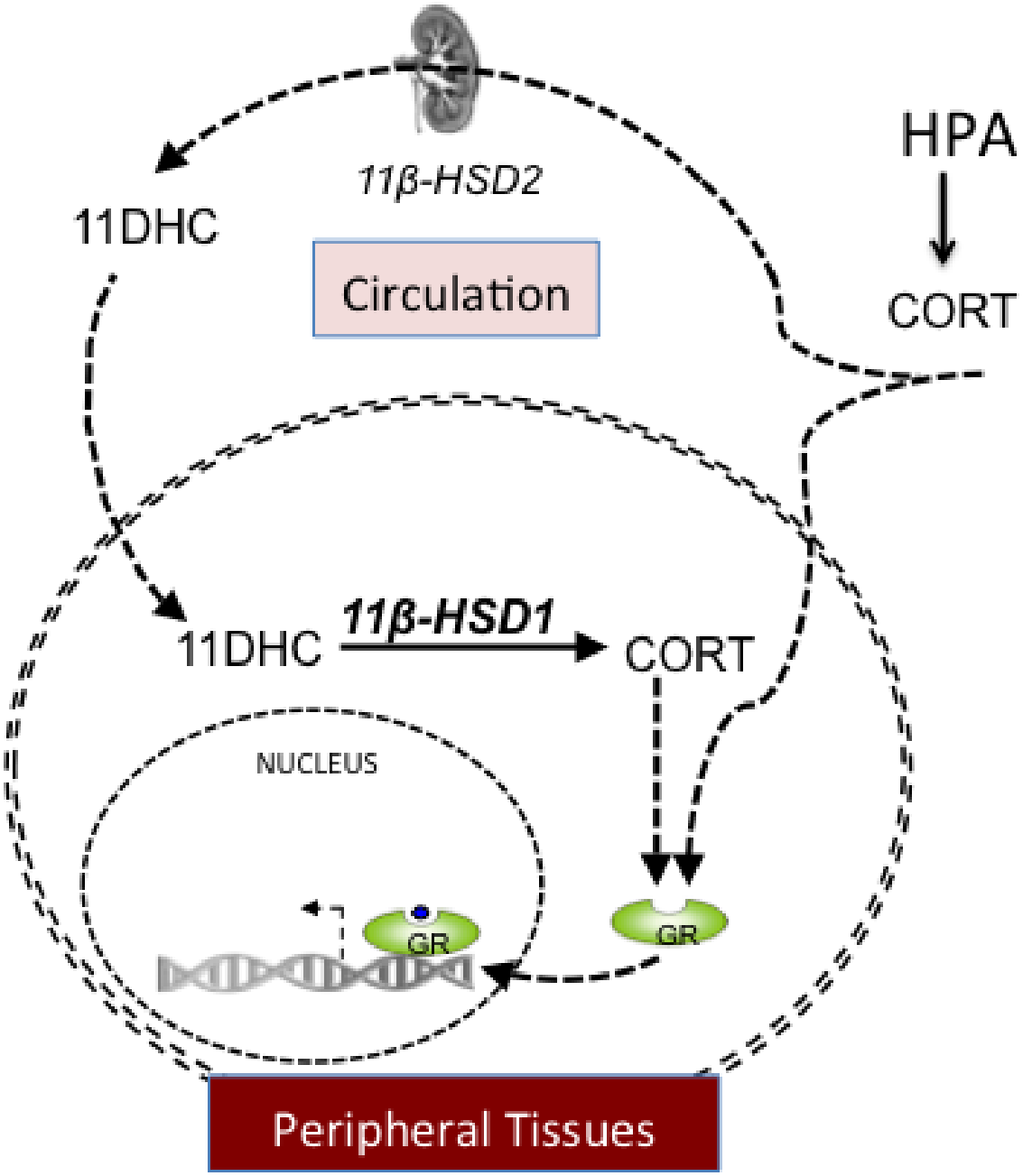

Figure 3-17 The relationship between 11β-HSD1, 11-DHC and CORT
11β-HSD1 converts 11-DHC into CORT. An increase in the concentration of cort leads to an increase in the production of 11-DHC by 11β-HSD2 and also an increase in the production of the 11β-HSD1 enzyme. Cited from Morgan et al 2014.

Due to the increasing prevalence of obesity on a global scale and its consequential detrimental effect on health and the economy where is represents a major risk factor for the development of insulin resistance, hyperlipidaemia, type 2 diabetes, and cardiovascular disorders,

mechanisms to combat its rise are the centre of much interest. Due to GCs role within the development of obesity there is a potential for 11β-HSD1 as a potential target (Bjorntrop et al 1999).

Despite the negative side effects of GC excess their action as anti-inflammatory agents is still required. Therefore therapy would be required to maintain the anti-inflammatory effect yet eliminate the undesirable metabolic side effects.

The data presented suggests that 11β-HSD1 may offer a novel target to limit the side affects seen with exogenous GC administration. I have shown that GKO of 11β-HSD1 ameliorates the side effects seen with an excess of GC administration and that the adipose tissue 11β-HSD1 is partially responsible for the effects seen. This therefore highlights adipose 11β-HSD1 as a potential tissue for targeting.

4. Chapter 4 - 11β-hydroxysteroid dehydrogenase type 1 and brown adipose tissue phenotype

4.1 Methods

4.1.1 Mitochondrial copy number analysis

4.1.1.1 Method

Mitochondrial copy number was assessed using an mtDNA/nDNA ratio on extracted DNA. Real-time quantitative PCR was performed using ABI Prism 7,500 Sequence Detection System (Applied Biosystems, Warrington, UK). A NovaQUANT™ Human Mitochondrial to Nuclear DNA Ratio Kit was used to compare the levels of nuclear to mitochondrial DNA (mtDNA). Each well individually tested for one of the four target genes. Nuclear gene 1 was compared to mitochondrial gene 1 and nuclear gene 2 was compared to mitochondrial gene 2. Ratios between the two genes were calculated and average for each sample to represent the mtDNA copy number per cell. The reactions contained: 10 µl of DNA template (2 ng) diluted using the appropriate amount of nuclease free water, and 10 µl of 2X RT2 Fast SYBR Green Mastermix. The plates were sealed using a clear adhesive film (Applied biosystems, Warrington, UK). The results were given as Ct values and the ΔCt values were calculated (Ct of gene of interest-Ct of 18S) and given as a fold change ($2^{-\Delta\Delta Ct}$).

4.1.1.2 Probe and primer sequences and details

A NovaQUANT™ Human Mitochondrial to Nuclear DNA Ratio Kit was used, and was supplied by Novagen, this kit composes qPCR plates and PCR primer pairs. The kit has been verified to not amplify mitochondrial pseudogenes and to effectively amplify target sequences.

4.2 Introduction

Brown adipose tissue (BAT) has emerged as a target for potential exploitation in the treatment of obesity and its related metabolic complications. BAT is predominantly localised in the intrascapular and paraspinal areas of both humans and rodents, however the concentration of BAT shows a decrease with increasing age (Almind et al 2007).

BAT is the main site for adaptive thermogenesis and responds to fluctuations in environmental factors, for example cold and diet, by increasing energy expenditure. Following exposure to cold BAT utilises lipids and carbohydrates to generate heat by using UCP1 to uncouple electron transport from oxidative phosphorylation, thus playing an active role in energy expenditure and fatty acid oxidation (Cannon et al 2008). Being located in the mitochondrial inner membrane UCP1 acts as a proton transporter to abolish the proton gradient, the driving force of ATP synthesis, allowing the free energy conserved in the proton gradient to be dissipated as heat (Watanabe et al 2008).

With its role in energy expenditure BAT has a central role in the regulation of adipose stores within the body, and as such has potential in battling both obesity and diabetes due to its striking metabolic capacity, further evidence to support this is that mice deficient in BAT develop obesity, insulin resistance and other metabolic abnormalities (Mirbolooki et al 2011; Enerback et al 2010). BAT has recently been shown to be present within adult humans using 18F-FDG PET-CT scans (Cypress et al 2009). This therefore identifies BAT as possible therapeutic target within humans.

Many factors have a negative impact on BAT; two main players are glucocorticoids and ageing. With an increase in GC exposure and an increase in ageing there is a decrease in BAT function.

Excess GCs appear to suppress BAT thermogenesis in both mice and rats, this is hypothesised to be due to a central action suppressing BAT thermogenesis (Walker et al 1992; Soumano et al 2000). Due to their high rate of administration the side effects of GCs are highly prevalent, and therefore there is a need for the development of a mechanism of treatment for GC excess. The effect of GC is further increased via the 11β-HSD1 enzyme, which converts inactive cortisone to active cortisol. Exposure of adipose cells to GCs significantly increases 11β-HSD1 expression and activity, further contributing to GC excess (Bujalska et al 1997).

Increasing age has been shown to have negative effects on BAT and may contribute to thermal dysregulation and energy imbalance. Increasing age shows a strongly negative correlation with BAT mass and BAT's metabolic effect, further there is a higher incidence of BAT activation following cold exposure in a young rather than an aged cohort (Pfannenberg et al; Saito et al 2009). This however has been contradicted by Van Marken Lichenbelt et al 2009, who showed that there was no correlation between BAT activity and age. Further ageing also affects the architecture and distribution of adipose tissue where there is a positive correlation between age and lipid size.

I hypothesised that with increased exposure to GCs and with increasing age there would be a detrimental effect on the functioning of BAT. I also hypothesised that with a knock-out of 11β-HSD1 and the consequential decrease in tissue regeneration of GC there would be a beneficial effect on the functioning of BAT and a possible delay or prevention of the negative affects on BAT associated with increasing age.

To test this hypothesis I analysed BAT samples from young (10 weeks) and aged (100 weeks) wild-type or global 11β-HSD1 knock-out (GKO) mice. Further separate cohorts have been treated with exogenous GC (corticosterone). This allowed me to test the effects of

ageing, GC excess and possible beneficial effects of 11β-HSD1 global knock-out on BAT. Further to this I generated primary cultures of BAT from young (10 weeks) mice, and assessed the effects of GC administration on overall markers of BAT function.

4.3 Results

4.3.1 Effect of ageing on brown adipose tissue in vivo

I wanted to investigate the effect of ageing on BAT. To do this I obtained tissue samples from young (10 weeks) and aged (100 weeks) cohorts, of both wild-type (WT) black 6 and 11β-HSD1 global knock-out (GKO) mice. I initially analysed the base line effect of ageing on these tissues by comparing WT young and aged tissues. Potential changes in gene expression, mitochondrial copy number analysis, protein blot analysis, histological analysis and tissue weight were assessed.

To begin I analysed the tissue weight data (Figure 4-1) to see if ageing resulted in a change in BAT mass. I found that there was a significant increase in the tissue weight/body weight ratio with ageing ($p<0.001$). This was a combination of an increase in body weight and an increase in BAT weight within the aged cohort (Figure 4-1).

Tissue (g)	Young	Aged
Body weight	23.79 +/- 0.381	36.59 +/- 0.54
Brown adipose tissue	0.069 +/- 0.003	0.161 +/- 0.38

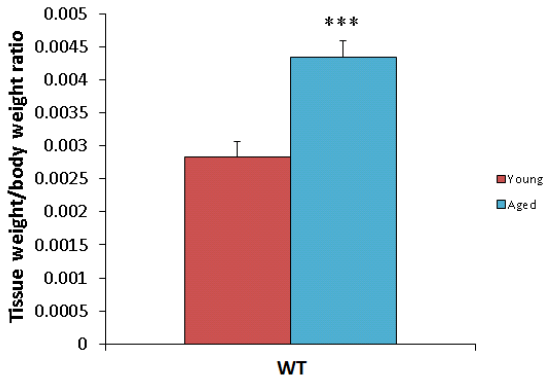

Figure 4-1 Effect of ageing on brown adipose tissue weight in wild-type mice
*Ageing leads to a significant increase in BAT tissue weight/body weight ratio within WT mice. Aged mice showed a significantly higher tissue weight/body weight ration than the young mice. Data was analysed using student T-test. ***P<0.001. n=11 (young) n=10 (aged). Error bars represent SEM.*

Having noted an increase in tissue weight with age I investigated potential mechanisms which may be responsible for this change and also if the increase in BAT tissue weight led to a potential improvement in BAT function. I initially analysed potential age related changes in 11β-HSD1 expression, as the 11β-HSD1 enzyme has previously been shown to be a mediator of BAT function. I found that there was a significant increase in 11β-HSD1 expression in the aged mice compared to the young ($P<0.0001$) as shown in figure 4-2. Having seen this striking increase in 11β-HSD1 expression with age, and the consequential potential for an increase in local GC reactivation I wanted to see if there was also an increase in glucocorticoid receptor expression (nr3c1). I again measured mRNA expression via RT-PCR. I found that there were no changes in nr3c1 expression (figure 4-2) with age and therefore infer that there is no change in glucocorticoid receptor content.

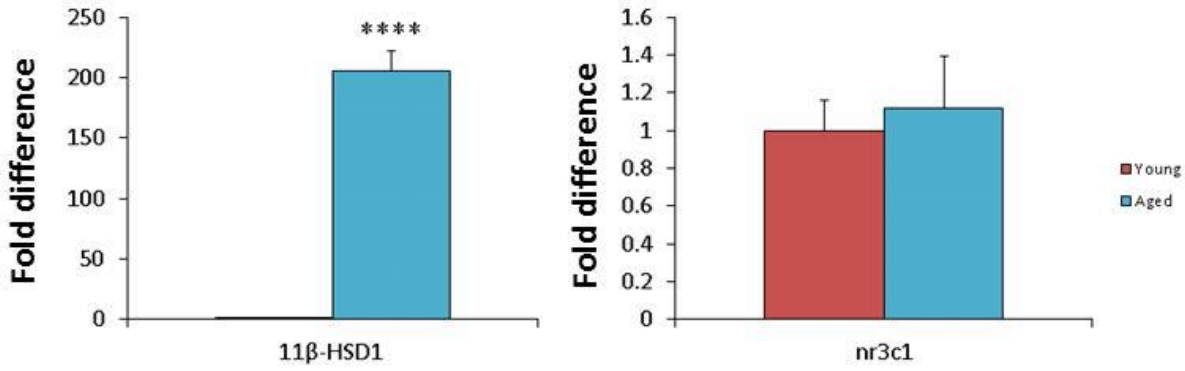

Figure 4-2 Effect of ageing on 11β-HSD1 and nr3c1 mRNA expression in brown adipose tissue

*Ageing leads to a significant increase in 11β-HSD1 mRNA expression in WT mice. Aged mice showed significantly higher 11β-HSD1 mRNA expression than the young mice. Error bars represent SEM. Ageing leads to no change in the mRNA expression of nr3c1. Aged and young mice showed the same level of nr3c1 expression. Data was analysed using student T-test. ***. P<0.001. ****P<0.0001. n=11 (young) n=10 (aged). Error bars represent SEM.*

Due to BAT being central to non-shivering thermogenesis and this process involving the utilisation of mitochondria, I continued my investigations by analysing potential effects of

ageing on markers of mitochondrial function in an attempt to ascertain its effects on the potential for non-shivering thermogenesis. I began by performing RT-PCR analysis on mitochondrial transcription factors which are related to potential improvements in mitochondrial function. I analysed changes in the expression of PGC-1α, PRDM16 and PPARγ with age (Figure 4-3) and found a significant ($p<0.05$) increase in the expression of PRDM16 and PPARγ with age.

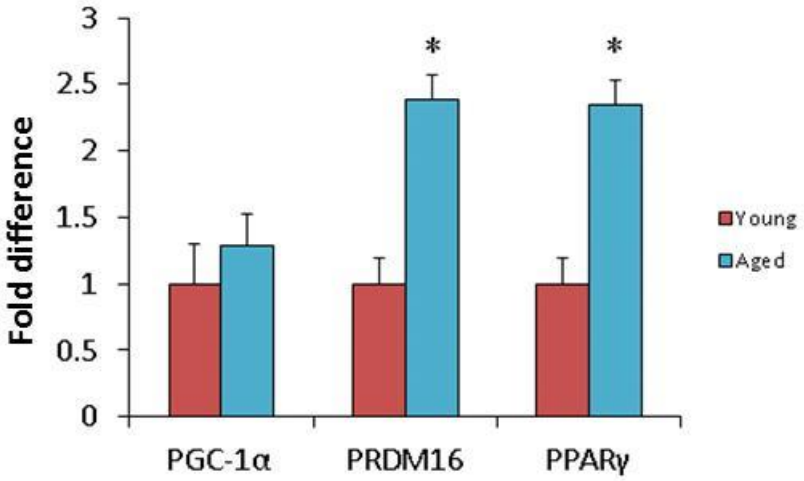

Figure 4-3 Effect of ageing on PGC-1α, PRDM16 and PPARγ mRNA expression in brown adipose tissue
*Ageing leads to a significant increase in the mRNA expression of PRDM16 and PPARγ. Aged mice showed a significantly higher mRNA expression of PRDM16 and PPARγ than the young mice. There was no significant change in the mRNA levels of PGC-1α between young and aged mice. Data was analysed using student T-test. * P<0.05. n=11 (young) n=10 (aged). Error bars represent SEM.*

Having noted increases in mitochondrial transcription factors I proceeded to see if there were age related changes in the expression of genes located within the electron transport chain and non-shivering thermogenesis. I analysed the expression of UCP1 (Figure 4-4), Cox7a1, Cox8b, and CoxIV (Figure 4-5). Further to this I analysed changes in protein concentration of UCP1 via western blot analysis (Figure 4-4). I found a significant increase in the mRNA

(p<0.001) and protein (p<0.05) of UCP1, and also a significant increase in both Cox7a1 and Cox8b mRNA expression (p<0.05) with age.

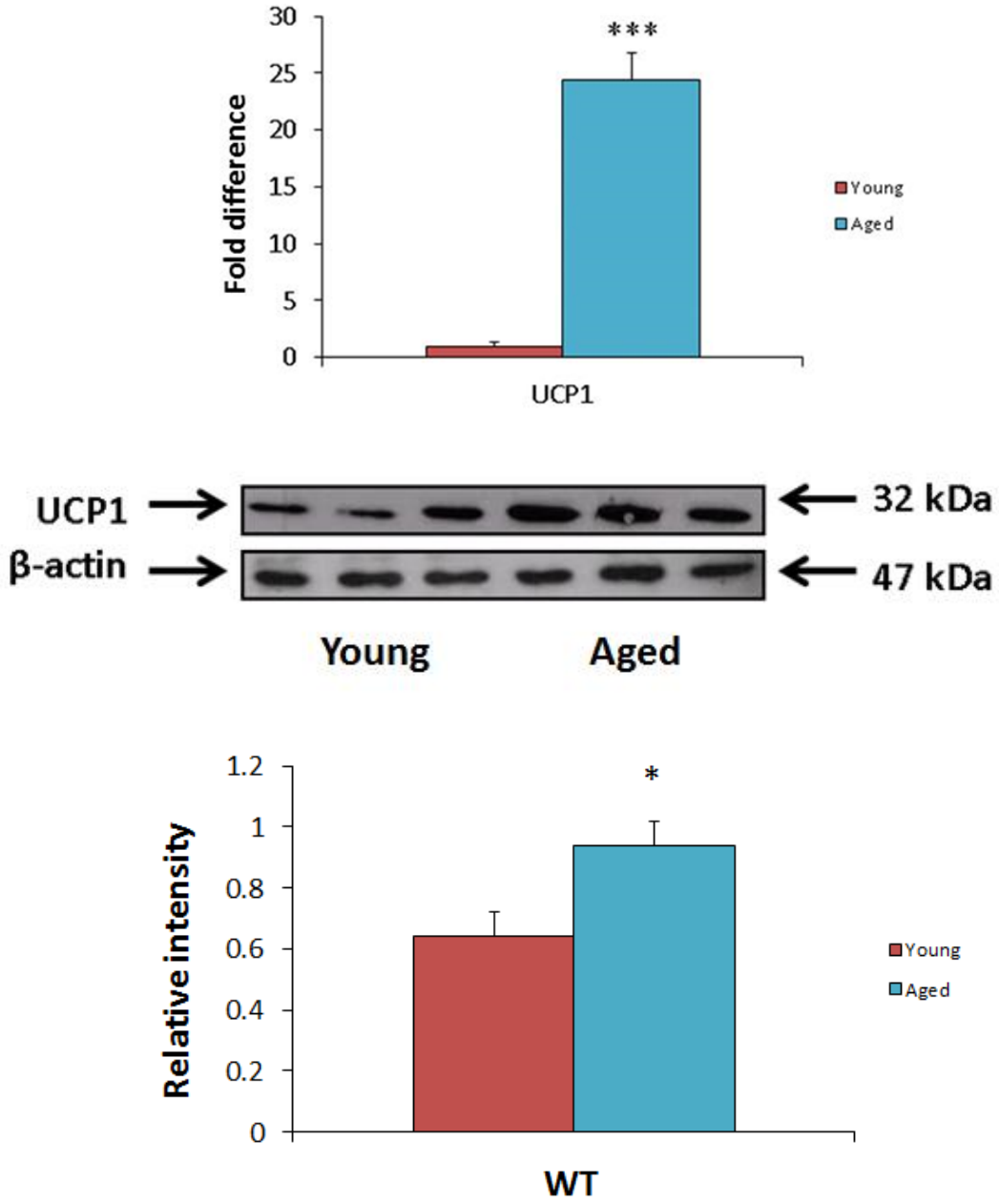

Figure 4-4 Effect of ageing on UCP1 mRNA and protein expression in brown adipose tissue
*Ageing leads to a significant increase in the mRNA expression of UCP1. Aged mice showed a significantly higher mRNA and protein expression of UCP1 than the young mice. Data was analysed using student T-test. *** P<0.001. n=11 (young) n=10 (aged). Data was analysed using densitometry. * P<0.05. n=3. Error bars represent SEM.*

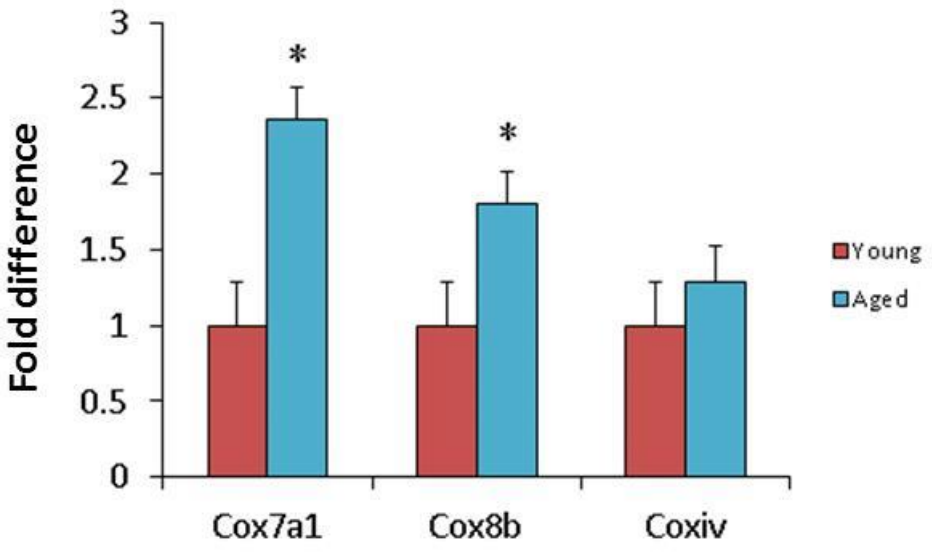

Figure 4-5 Effect of ageing on Cox7a1, Cox8b and CoxIV mRNA expression in brown adipose tissue

*Ageing leads to a significant increase in the mRNA expression of Cox7a1 and Cox8b. Aged mice showed a significantly higher mRNA expression of Cox7a1 and Cox8b than the young mice. There was no significant difference in the mRNA expression of CoxIV between the young and aged mice. Data was analysed using student T-test. * P<0.05. n=11 (young) n=10 (aged). Error bars represent SEM.*

Having seen that there are increases in the concentration of BAT markers with age I then went on to see if there were age related changes in the expression of genes involved in the enhancement of mitochondrial biogenesis. I analysed the expression of NRF1 and TFAM (Figure 4-6). I found significant increases in the expression of TFAM ($p<0.05$) and NRF1 ($p<0.01$) in the aged cohort compared to the young cohort.

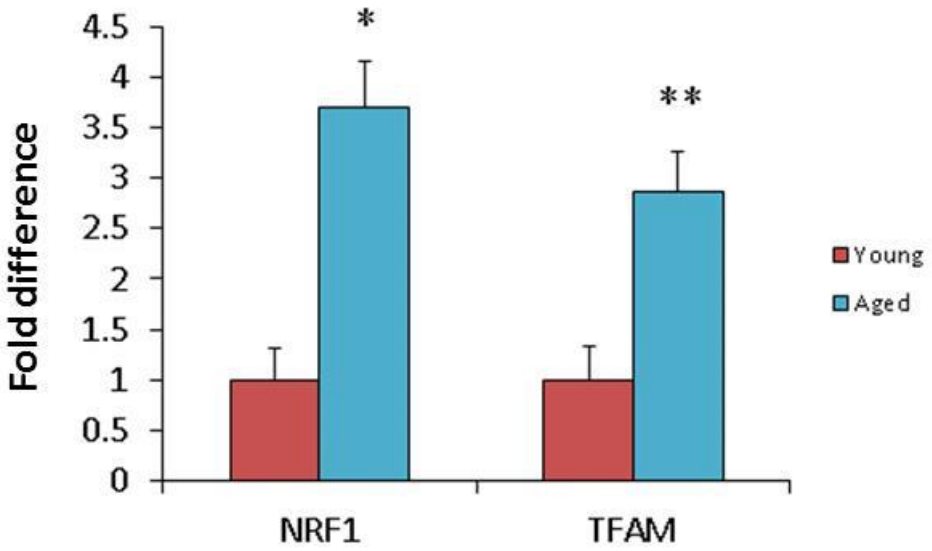

Figure 4-6 Effect of ageing on expression of TFAM and NRF1 in brown adipose tissue
*Ageing leads to a significant increase in the mRNA expression of NRF1 and TFAM. Aged mice showed a significantly higher mRNA expression of NRF1 and TFAM than the young mice. Data was analysed using student T-test. * P<0.05, **P<0.01. n=11 (young) n=10 (aged). Error bars represent SEM.*

Having noted increases in genes involved in mitochondrial function and biogenesis I investigated possible effects on mitochondrial copy number. I assessed changes in mitochondrial copy number and also changes in the protein concentration of mitochondrial complexes (Figure 4-7). I found a significant increase in mitochondrial copy number with age ($p<0.05$) and also a significant increase in the protein expression of complex 2 ($p<0.05$) with age.

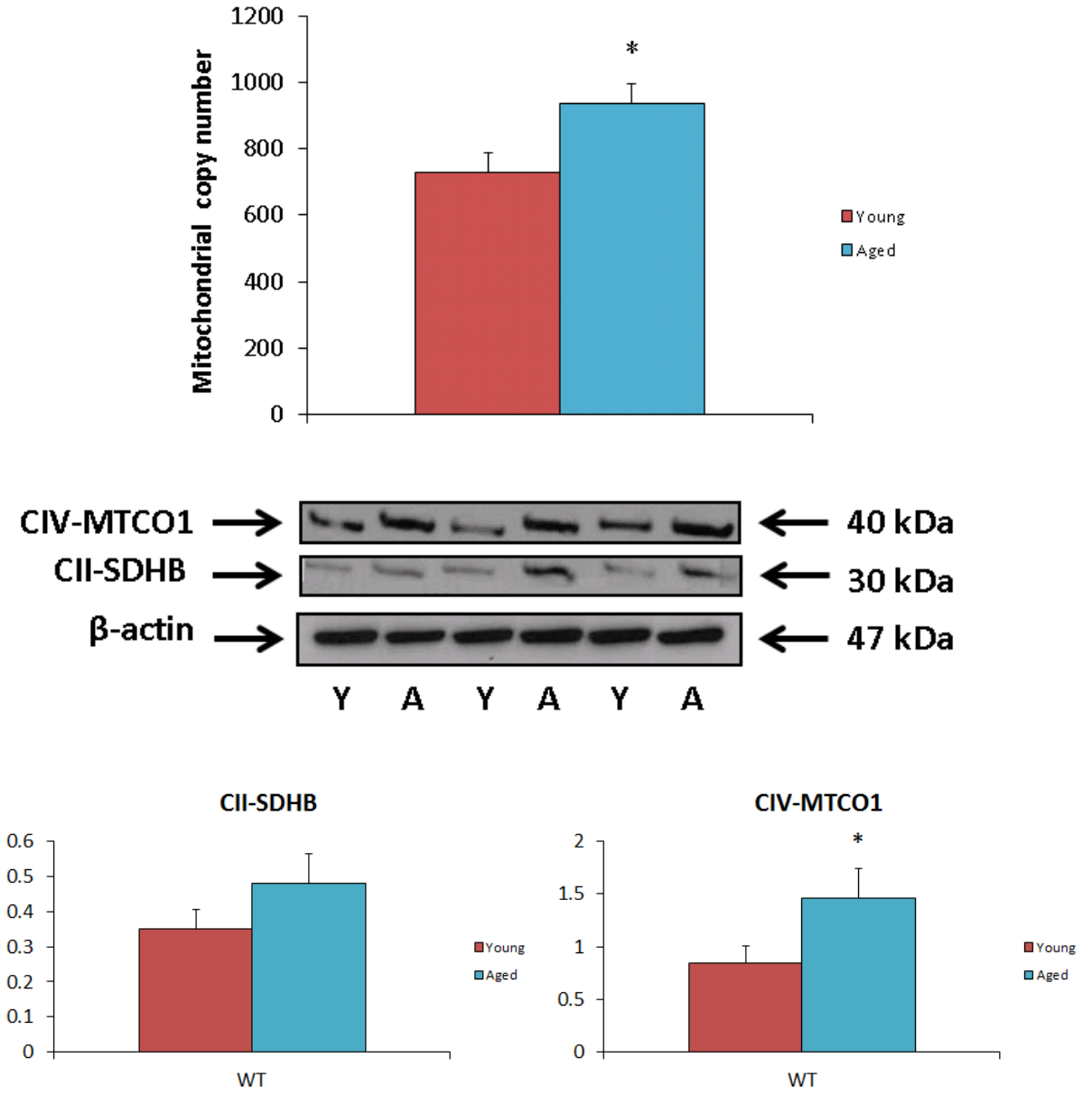

Figure 4-7 Effect of ageing on mitochondrial copy number and mitochondrial complex protein expression in brown adipose tissue

*Ageing leads to a significant increase in mitochondrial copy number and protein expression of CIV-MTCO1. There was no significant change in the protein expression of CII-SDHB between the young (Y) and aged (A) mice. Data was analysed using student T-test. * P<0.05. n=6. Error bars represent SEM. Data was analysed using densitometry. * P<0.05. n=3.*

4.3.2 Effect of exposure to exogenous glucocorticoid on brown adipose tissue in vivo

Previous studies have demonstrated the negative effects of GCs on BAT function. I wished to test the effect of exogenous GC treatment on BAT in an attempt to mimic the effect of

prescribed GCs. To test this I obtained archived tissue samples from young (10 weeks) mice, of WT genotype that had been exposed to either corticosterone or vehicle. I performed RT-PCR to measure potential changes in gene expression with and without the administration of exogenous GCs. I also measured mitochondrial copy number, and measured changes in tissue weight.

I began by analysing the effect of administration of exogenous GCs on BAT tissue weight as ratio to total body weight. I found that there was significant decrease ($p<0.01$) in the BAT weight/body weight ratio in the mice treated with exogenous GC compared to those treated with vehicle (Figure 4-8).

Tissue (g)	Vehicle	CORT
Body weight	23.79 +/- 0.381	24.43 +/- 0.42
Brown adipose tissue	0.069 +/- 0.003	0.029 +/-0.002

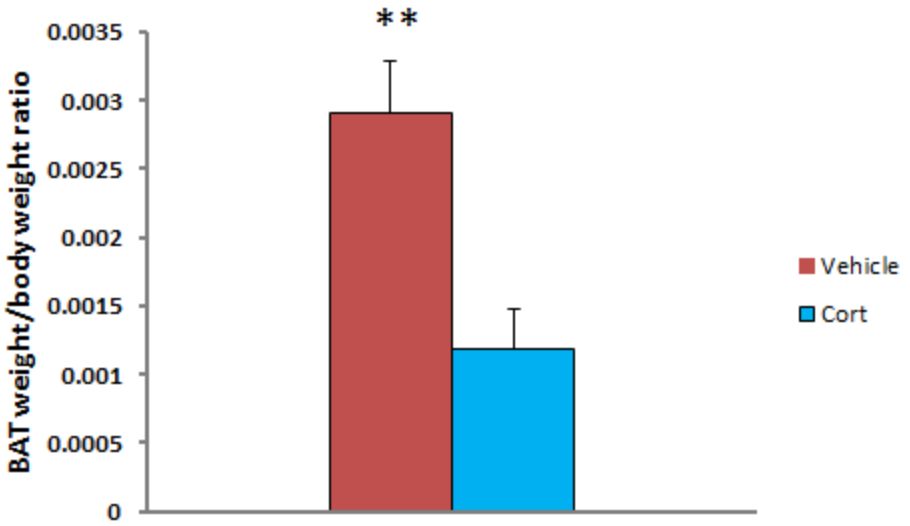

Figure 4-8 Effect of exposure to exogenous glucocorticoid on BAT mass in brown adipose tissue

*The exposure to exogenous GC, CORT leads to a significant increase in the body weight/BAT weight ratio. Mice treated with exogenous CORT had a higher body weight/BAT weight ratio than those treated with vehicle. Data was analysed using student T-test. * P<0.05, **P<0.01. n=7 (vehicle) n=6 (CORT). Error bars represent SEM.*

Having seen these significant changes in the body weight/BAT weight ratio I investigated the possible reasons behind this. I firstly looked to see if there were changes in 11β-HSD1 expression following GC administration (Figure 4-9). I found that there was a significant increase in the expression of 11β-HSD1 in the mice treated with corticosterone ($p<0.01$) compared with those treated with vehicle. Having seen these significant effects on 11β-HSD1 expression following exogenous GC administration I then investigated the effect of endogenous GC administration on glucocorticoid receptor (nr3c1) mRNA expression (Figure 4.9). I found no changes in nr3c1 mRNA expression in mice treated with corticosterone when compared to those treated with vehicle.

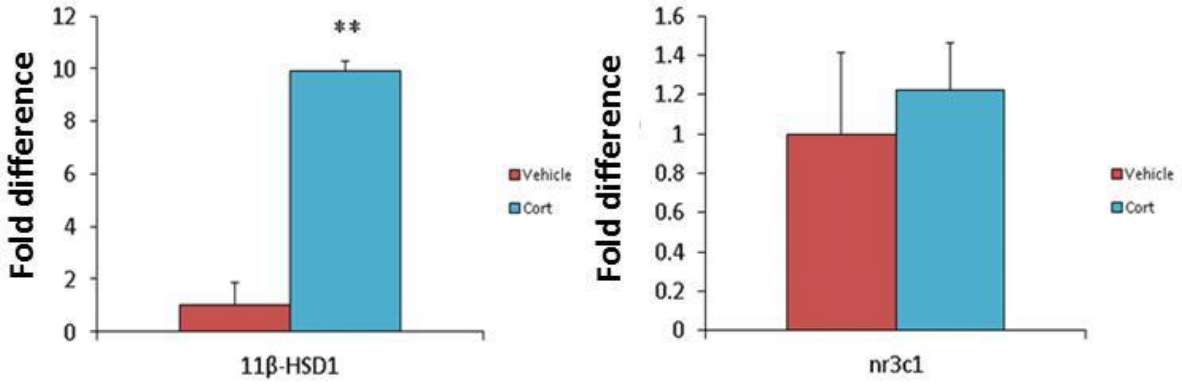

Figure 4-9 Effect of exogenous glucocorticoid administration on 11β-HSD1 and nr3c1 expression in brown adipose tissue
*The exposure to exogenous GC, CORT leads to a significant increase in mRNA expression of 11β-HSD1 and no change in the mRNA expression of nr2c1. Mice treated with exogenous CORT had a higher mRNA expression level of 11β-HSD1 than those treated with vehicle. Data was analysed using student T-test. **P<0.01. n=7 (vehicle) n=6 (CORT). Error bars represent SEM.*

I went on to investigate the effect of GC administration on the expression of BAT transcription factors. I analysed changes in the expression of PGC-1α, PRDM16 and PPARγ (Figure 4-10). I found no changes in the mRNA expression of any of the transcription factors following exposure to exogenous GCs. I then went on to see if there were GC administration

related changes in the expression of BAT markers. I analysed the expression of UCP1, Cox7a1, Cox8b, and CoxIV (Figure 4-10). I found a significant decrease in the mRNA expression of UCP1 ($p<0.05$) following treatment with corticosterone.

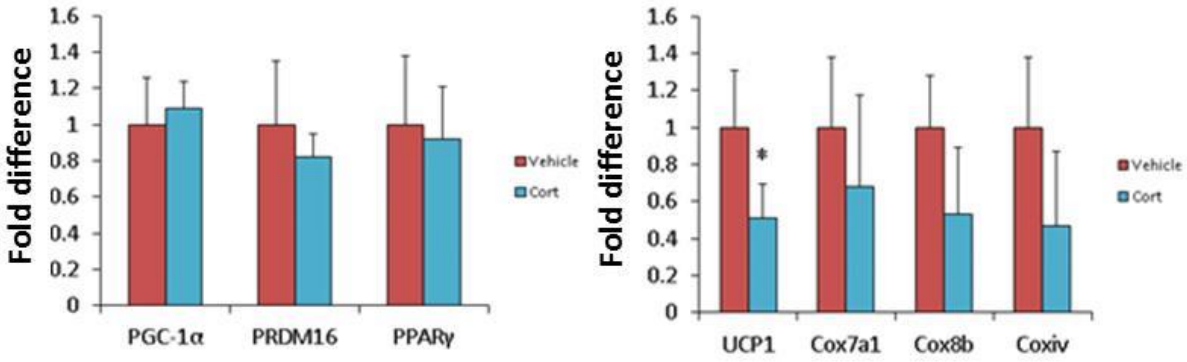

Figure 4-10 Effect of exogenous glucocorticoid administration on PGC-1α, PRDM16, PPARγ, UCP1, Cox7a1, Cox8b and CoxIV expression in brown adipose tissue
*The exposure to exogenous GC, CORT lead to a significant decrease in the expression of UCP1. Mice treated with exogenous CORT had a significantly lower mRNA expression of UCP1 than those treated with vehicle. There was no significant change in the mRNA expression of PGC-1α, PRDM16, PPARγ, Cox7a1, Cox8b or CoxIV following exposure to CORT. Data was analysed using student T-test. *P<0.05, **P<0.01. n=7 (vehicle) n=6 (CORT). Error bars represent SEM.*

Having noted changes in the expression of UCP1 following GC treatment I then went on to see if there were GC administration related changes in the expression genes related to mitochondrial biogenesis. I analysed the expression of NRF1 and TFAM and found no changes in their expression following GC treatment (Figure 4.11).

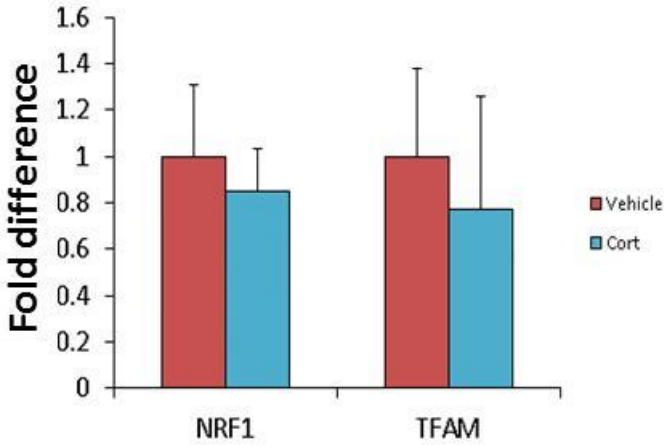

Figure 4-11 Effect of exposure to exogenous glucocorticoid on NRF1 and TFAM mRNA expression in brown adipose tissue
The exposure to exogenous GC, CORT leads to no change in mRNA expression of NRF1 and TRAM. Mice treated with exogenous CORT had the same mRNA expression level of NRF1 and TFAM as those treated with vehicle. Data was analysed using student T-test. n=7 (vehicle) n=6 (CORT). Error bars represent SEM.

Having noted a decrease in the mRNA expression of UCP1 following the administration of exogenous GC I investigated possible effects on mitochondrial DNA copy number (Figure 4-12). I found no change in the mitochondrial copy number following exposure to corticosterone.

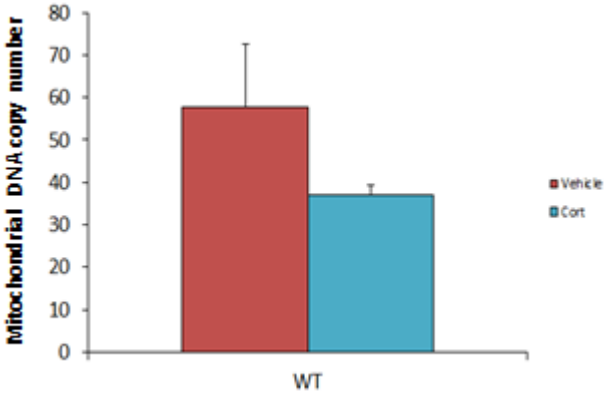

Figure 4-12 Effect of exogenous glucocorticoid administration on mitochondrial DNA copy number in brown adipose tissue
The exposure to exogenous GC, CORT leads to no change in mitochondrial copy number. Mice treated with exogenous CORT had no significant change in mitochondrial copy number as those treated with vehicle. Data was analysed using student T-test. n=3. Error bars represent SEM.

4.3.3 Effect of 11β-HSD1 knock-out on animals treated with exogenous glucocorticoids

Having seen significant changes in mRNA expression of mitochondrial related genes with GKO of 11β-HSD1 and also significant changes in the mRNA expression of mitochondrial related genes with exogenous GC treatment I wished to test whether GKO of 11β-HSD1 would protect BAT function from the negative effects of exogenous GC treatment. To test this I obtained tissue from WT and GKO genotypes and also those treated with corticosterone. The tissue samples were from young (10 week) mice.

I investigated the effect of GC administration on the expression of BAT transcription factors. I analysed changes in the expression of PGC-1α, PRDM16 and PPARγ with GKO in mice treated with corticosterone (Figure 4-13). I found a significant increase in the mRNA expression of PRDM16 ($P<0.01$) with GKO in mice treated with corticosterone.

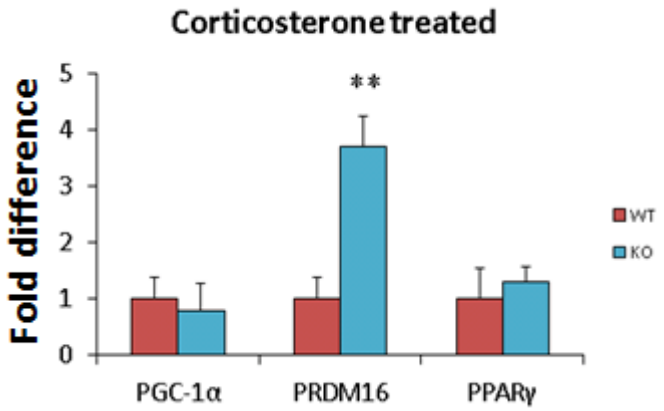

Figure 4-13 Effect of GKO on PGC-1α, PRDM16 and PPARγ expression in samples treated with exogenous glucocorticoid in brown adipose tissue
*11β-HSD1 knock-out leads to a significant increase in PRDM16 expression following treatment with corticosterone. There was no significant change in the mRNA expression of PGC-1α or PPARγ with genotype following treatment with corticosterone. Data was analysed using student T-test. *P<0.05, ** P<0.01. n=7 (vehicle) n=6 (cort). Error bars represent SEM.*

I then went on to see if there were GC administration related changes in the expression of BAT markers. I analysed the expression of UCP1, Cox7a1, Cox8b, and CoxIV (Figure 4-14). I found a significant increase in the mRNA expression of UCP1 ($P<0.05$), Cox8b ($P<0.01$), and CoxIV ($P<0.05$) with GKO following treatment with corticosterone. Further to this I analysed changes in UCP1 protein expression with GKO following treatment with corticosterone (Figure 4-14). I found a significant increase in UCP1 expression with GKO in the samples treated with corticosterone ($P<0.05$).

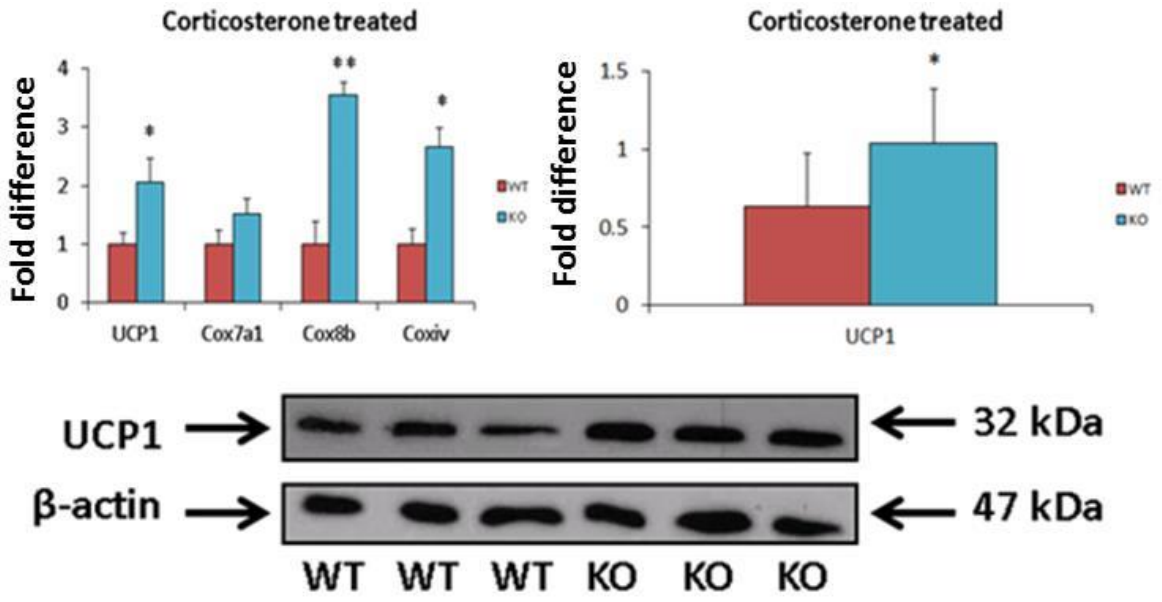

Figure 4-14 Effect of 11β-HSD1 GKO on mRNA expression of UCP1, Cox7a1, Cox8b, and CoxIV in mice exposed to exogenous glucocorticoid in brown adipose tissue
*11β-HSD1 knock-out led to a significant increase in UCP1, Cox8b, and CoxIV mRNA expression and a significant increase in UCP1 protein expression following treatment with corticosterone. There was no significant change in the mRNA expression of Cox7a1 with genotype following exposure to corticosterone. Data was analysed using student T-test. *P<0.05, ** P<0.01, ***P<0.001. n=7 (vehicle) n=6 (cort).Data was analysed using densitometry. *P<0.05, ** P<0.01. n=3. Error bars represent SEM.*

I then went on to see if there were GC administration related changes in the expression of transcription factors. I analysed the expression of NRF1 and TFAM (Figure 4-15). I found no

change in the mRNA expression of either NRF1 or TFAM in the GKOs following treatment with corticosterone.

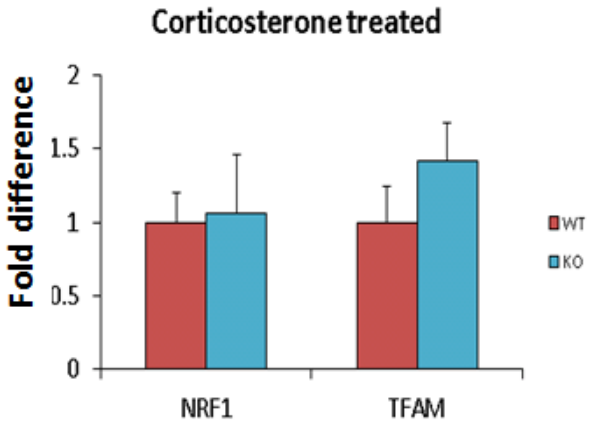

Figure 4-15 Effect of 11β-HSD1 GKO on mRNA expression of NRF1 and TFAM in mice exposed to exogenous glucocorticoid in brown adipose tissue
There was no significant change in the mRNA expression of NRF1 or TFAM with genotype following treatment with corticosterone. Data was analysed using student T-test. n=7 (vehicle) n=6 (cort). Error bars represent SEM.

4.3.4 Effect of glucocorticoid excess on brown adipose tissue in vitro

Having seen effects following the administration of GCs I wished to investigate this effect further. To achieve this I generated primary tissue cultures from intra-scapular BAT from wild-type mice aged between 9 and 10 weeks. I analysed the effect of exposure of these primary cultures to GC treatment for a four hour period where dexamethasone was added for 6 hours. I tested the effect of exogenous GC treatment on cells maintained at 37°C. I performed; RT-PCR to measure potential changes in gene expression, and 11β-HSD1 activity assays to test potential changes in the activity of 11β-HSD1 following GC administration.

I initially investigated the effect of the addition of exogenous GC on 11β-HSD1 expression (figure 4.16). I found that there was a significant increase in the expression of 11β-HSD1 following the administration of GC ($p<0.05$). However no significant changes in 11β-HSD1 activity.

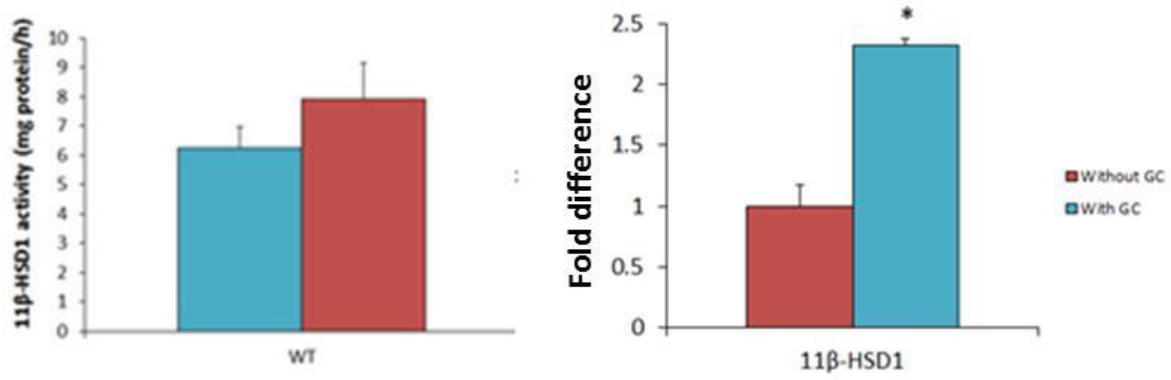

Figure 4-16 Effect of GC on 11β-HSD1 expression and activity in brown adipose tissue
*The exposure to exogenous GC led to an increase in mRNA expression of 11β-HSD1. Mice treated with exogenous GC had a significant increase in mRNA expression of 11β-HSD1 compared to untreated cells. There was no change in 11β-HSD1 activity following GC treatment. Data was analysed using student T-test. *P<0.05, **P<0.01 n=3. Error bars represent SEM.*

I went on to investigate the effect of exposure to GC on the expression of BAT transcription factors. I analysed changes in the expression of PGC-1α, PRDM16 and PPARγ (Figure 4-17). I then went on to see if there were GC related changes in the expression of BAT markers. I analysed the expression of UCP1, Cox7a1, and Cox8b with and without GC treatment (Figure 4-17). I found a significant decrease in the expression of Cox8b (P<0.05) when treated with exogenous GC.

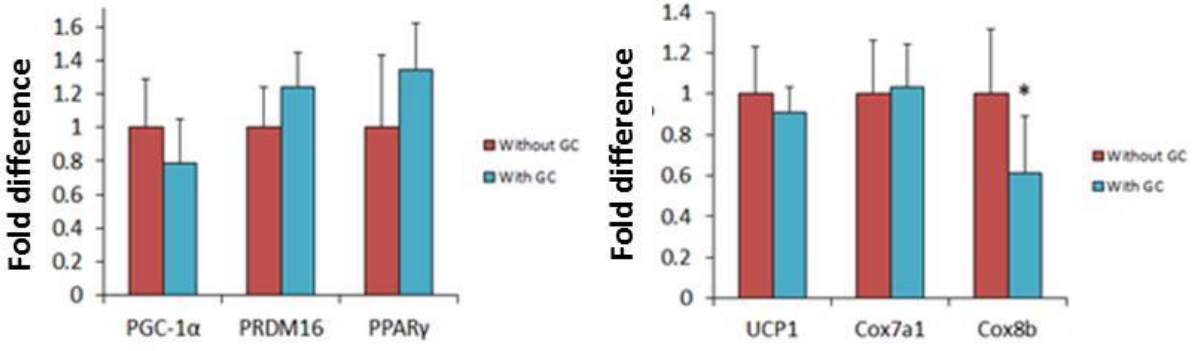

Figure 4-17 Effect of exogenous GC treatment on the expression of PGC-1α, PRDM16, PPARγ, UCP1, Cox7a1, and Cox8b in brown adipose tissue
*The exposure to exogenous GC led to a significant decrease in mRNA expression of Cox8b. Data was analysed using student T-test. *P<0.05. n=3. Error bars represent SEM.*

Having seen significant changes in genes located within the mitochondrial I went on to see if there were GC related changes in the expression of mitochondrial transcription factors. I analysed the expression of NRF1 and TFAM (Figure 4-18). I found a significant decrease in the mRNA expression of NRF ($p<0.05$) and TFAM ($p<0.01$).

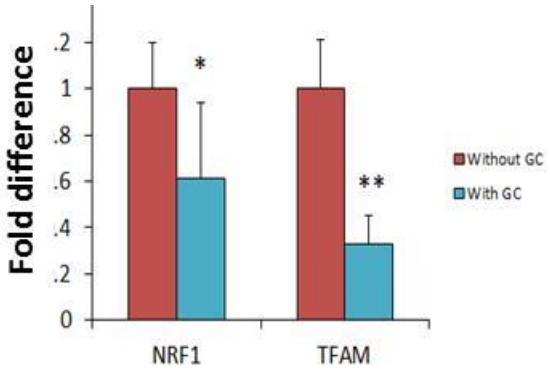

Figure 4-18 Effect of exogenous GC treatment on the expression of NRF1 and TFAM in brown adipose tissue
*The exposure to exogenous GC led to a decrease in mRNA expression of NRF1 and TFAM when compared to untreated cells. Data was analysed using student T-test. *P<0.05, **P<0.01. n=3. Error bars represent SEM.*

4.3.5 Effect of 11β-HSD1 knock-out on brown adipose tissue in vivo

Previous work has highlighted the beneficial effects of 11β-HSD1 knock-out on BAT function and also conversely how an increase in 11β-HSD1 can be of detriment to BAT function. This led me to analyse the potential beneficial effects of global knock-out of 11β-HSD1 (GKO) on BAT without exposure to exogenous GCs for a short and a prolonged period. I used tissue samples from young (10 weeks) and aged (100 weeks) cohorts, of wild-type and 11β-HSD1 knock-out genotypes.

I began by seeing if there was a change in BAT weight/body weight ratio with GKO in both the young and aged cohorts (Figure 4.19). I found a significant increase in BAT weight/body weight ratio in the aged cohort ($p<0.05$), however I found no change in BAT weight/body weight ratio in the young cohort.

	Young		Aged	
Tissue (g)	**WT**	**GKO**	**WT**	**GKO**
Body weight	23.79 +/- 0.381	22.14 +/- 0.302	30.18 +/- 0.395	28.54 +/- 0.418
Brown adipose tissue	0.069 +/- 0.003	0.084 +/- 0.002	0.127 +/- 0.005	0.160 +/- 0.008

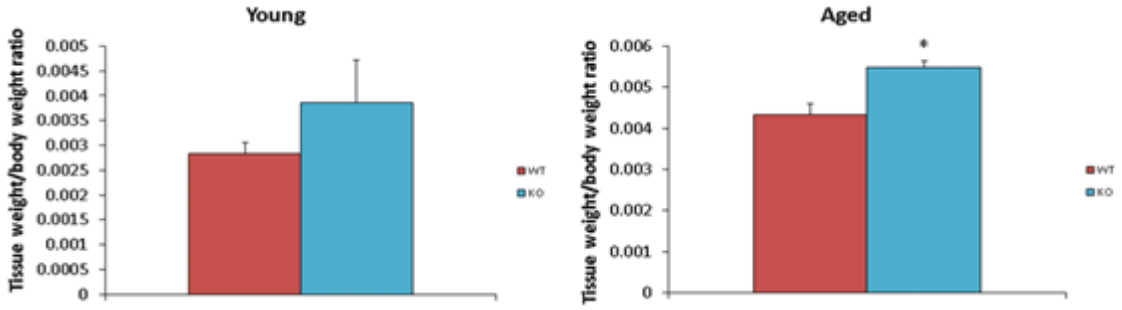

Figure 4-19 Effect of 11β-HSD1 knock-out on brown adipose tissue weight in brown adipose tissue
*11β-HSD1 knock-out leads to a significant increase in BAT weight/body weight ratio in aged mice. Aged mice showed a significantly higher BAT weight/body weight ratio with 11β-HSD1 KO compared to WT. There was no significant change in BAT weight/body weight ratio in young mice between WT and GKO. Data was analysed using student T-test. * P<0.05. n =5 for WT young, n=9 for GKO young. N = 6 for WT aged, n =6 for GKO aged. Error bars represent SEM.*

Due to BAT being central to non-shivering thermogenesis and this involves the utilisation of mitochondria; I continued my investigations by analysing potential effects of 11β-HSD1 GKO on markers of mitochondrial function. I began by performing RT-PCR analysis on mitochondrial transcription factors which are related to potential improvements in mitochondrial function. I analysed changes in the expression of PGC-1α, PRDM16 and PPARγ with age (Figure 4.20). I found a significant increase in the mRNA expression of PGC-1α ($p<0.05$), PRDM16 ($p<0.05$), and PPARγ ($p<0.05$) with 11β-HSD1 GKO in the young cohort, and a significant increase in the mRNA expression of PGC-1α ($p<0.001$), PRDM16 ($p<0.05$) and PPARγ ($p<0.01$) with 11β-HSD1 GKO in the aged cohort.

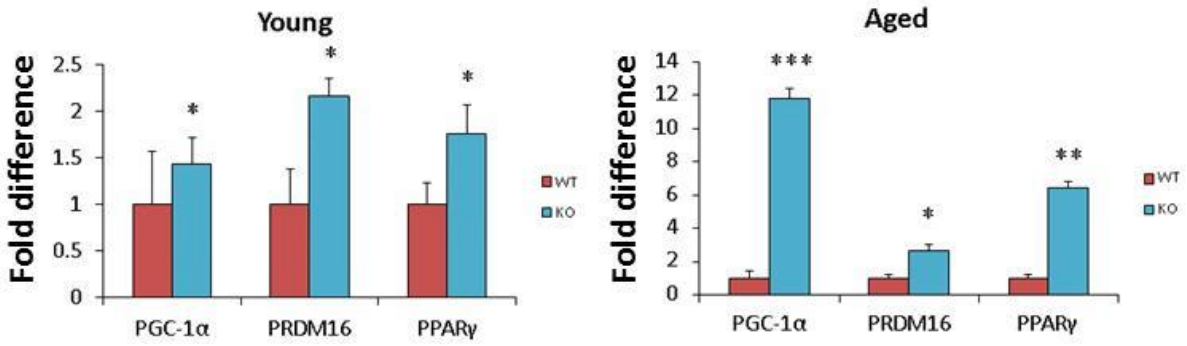

Figure 4-20 Changes in mRNA of PGC-1α, PRDM16, and PPARγ between WT and GKO in young and aged cohorts in brown adipose tissue
*11β-HSD1 knock-out leads to a significant increase in PGC-1α, PRDM16 and PPARγ expression in both young and aged mice. Young and aged mice showed a significantly increased PGC-1α, PRDM16, and PPARγ expression with 11β-HSD1 GKO compared to WT. Data was analysed using student T-test. * P<0.05, **P<0.01, ***P<0.001. n =5 for WT young, n=9 for GKO young. N = 6 for WT aged, n =6 for GKO aged. Error bars represent SEM.*

Due to the transcription factors being regulators of the expression of mitochondrial genes I then went on to see if there were changes in the expression of an array of mitochondrial structural genes. I analysed the expression of UCP1, Cox7a1, Cox8b, and CoxIV (Figure 5.21). I found no change in expression in the young cohort. Within the aged cohort I found significant increases in the mRNA expression of Cox7a1 ($p<0.01$), Cox8b ($P<0.01$), and CoxIV ($P<0.05$). Further to this I analysed changes in protein concentration of UCP1 via western blot analysis (Figure 4.21). I found no change in the protein expression of UCP1 in the young cohort, however I found a significant increase in the expression of UCP1 within the aged cohort ($P<0.05$).

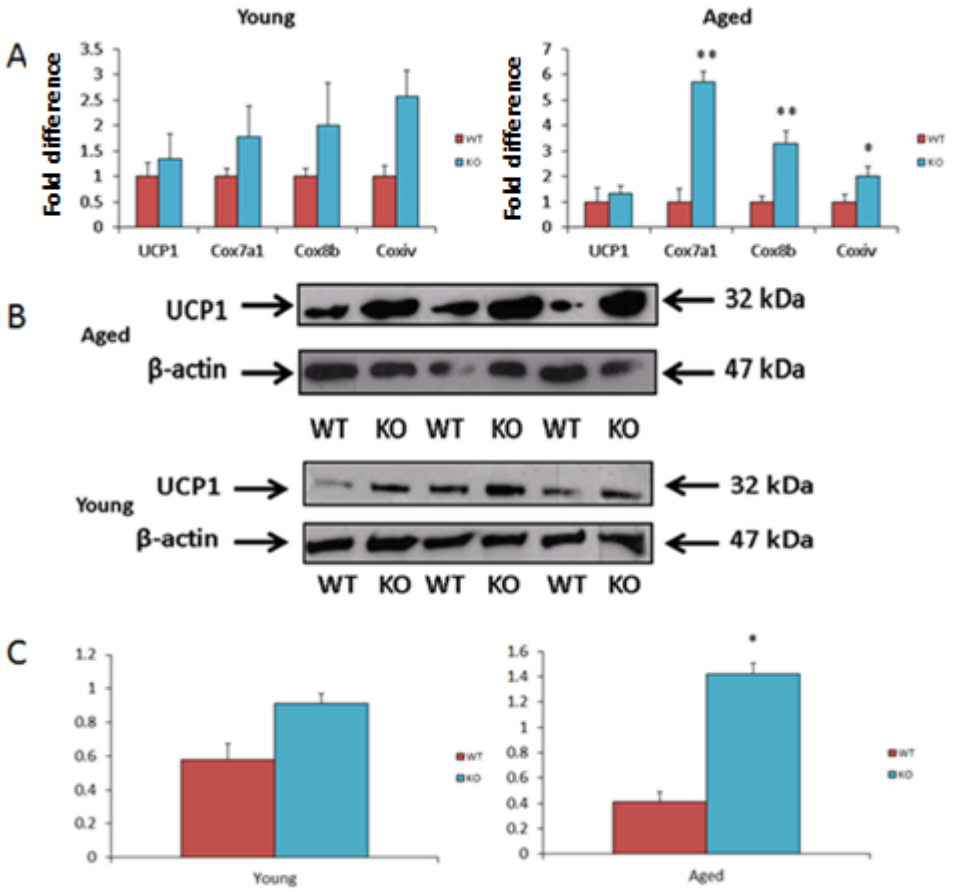

Figure 4-21 Changes in mRNA of UCP1, Cox7a1, Cox8b, and CoxIV and UCP1 protein between WT and GKO in young and aged cohorts in brown adipose tissue
*11β-HSD1 knock-out led to a significant increase in Cox7a1, Cox8b, and CoxIV mRNA expression and UCP1 protein expression in aged mice. Aged mice showed a significantly increased Cox7a1, Cox8b and CoxIV mRNA expression with 11β-HSD1 GKO compared to WT but no significant change in UCP1 expression. Aged mice showed a significant increase in UCP1 protein expression with 11β-HSD1 GKO compared to WT. Young mice showed no significant change in mRNA UCP1, Cox7a1, Cox8b or CoxIV expression or UCP1 protein expression between genotypes. Data was analysed using student T-test. * P<0.05, **P<0.01. n =5 for WT young, n=9 for GKO young. N = 6 for WT aged, n =6 for GKO aged. Data was analysed using densitometry. * P<0.05. n =3. Error bars represent SEM.*

Having seen increases in the expression of mitochondrial genes I investigated possible effects of GKO on the expression of genes involved in regulating mitochondrial biogenesis. I analysed the expression of NRF1 and TFAM (Figure 4.22). I found that there was a significant increase in both NRF1 ($p<0.05$) and TFAM ($p<0.05$) with GKO in the young

cohort. I found that in the aged cohort there was a significant increase in NRF1 ($p<0.05$), but no change in TFAM.

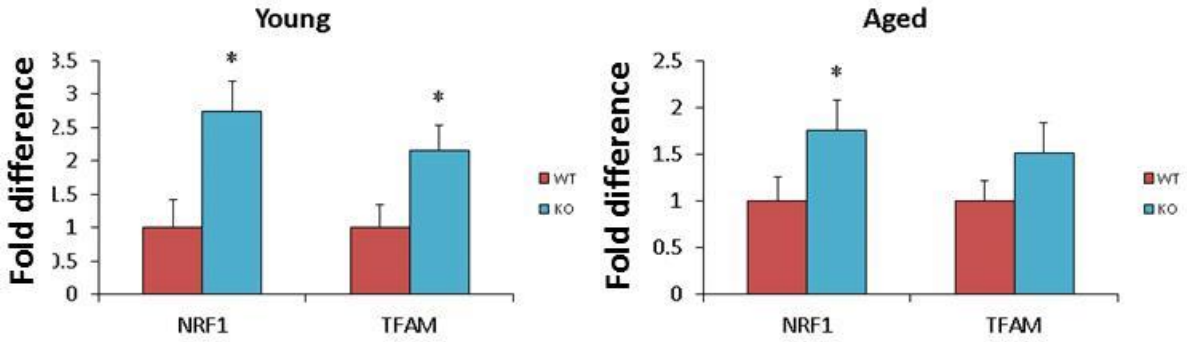

Figure 4-22 Changes in mRNA of NRF1 and TFAM comparing WT to GKO in young and aged cohorts in brown adipose tissue
*11β-HSD1 knock-out leads to a significant increase in NRF1 and TFAM mRNA expression in young mice, and a significant increase in NRF1 mRNA expression in aged mice. Young mice showed a significantly increased NRF1 and TFAM mRNA expression with 11β-HSD1 GKO compared to WT. Aged mice showed a significant increase in NRF1 mRNA expression with 11β-HSD1 GKO compared to WT. aged mice showed no significant change in TFAM expression in the GKO compared to WT. Data was analysed using student T-test. * P<0.05. n =5 for WT young, n=9 for GKO young. N = 6 for WT aged, n =6 for GKO aged. Error bars represent SEM.*

Having noted increases in genes involved mitochondrial function I investigated possible effects on mitochondrial copy number. I assessed changes in mitochondrial copy number, and performed western blot analysis on mitochondrial complexes (Figure 4-23). I found no changes in mitochondrial copy number between WT and GKO in the young cohort, however I found a significant increase in mitochondrial copy number in the GKOs compared to the wild-types in the aged cohort ($p<0.05$). In addition to this I found a significant increase in protein expression of mitochondrial complex 2 ($p<0.05$) with GKO in the aged tissue samples.

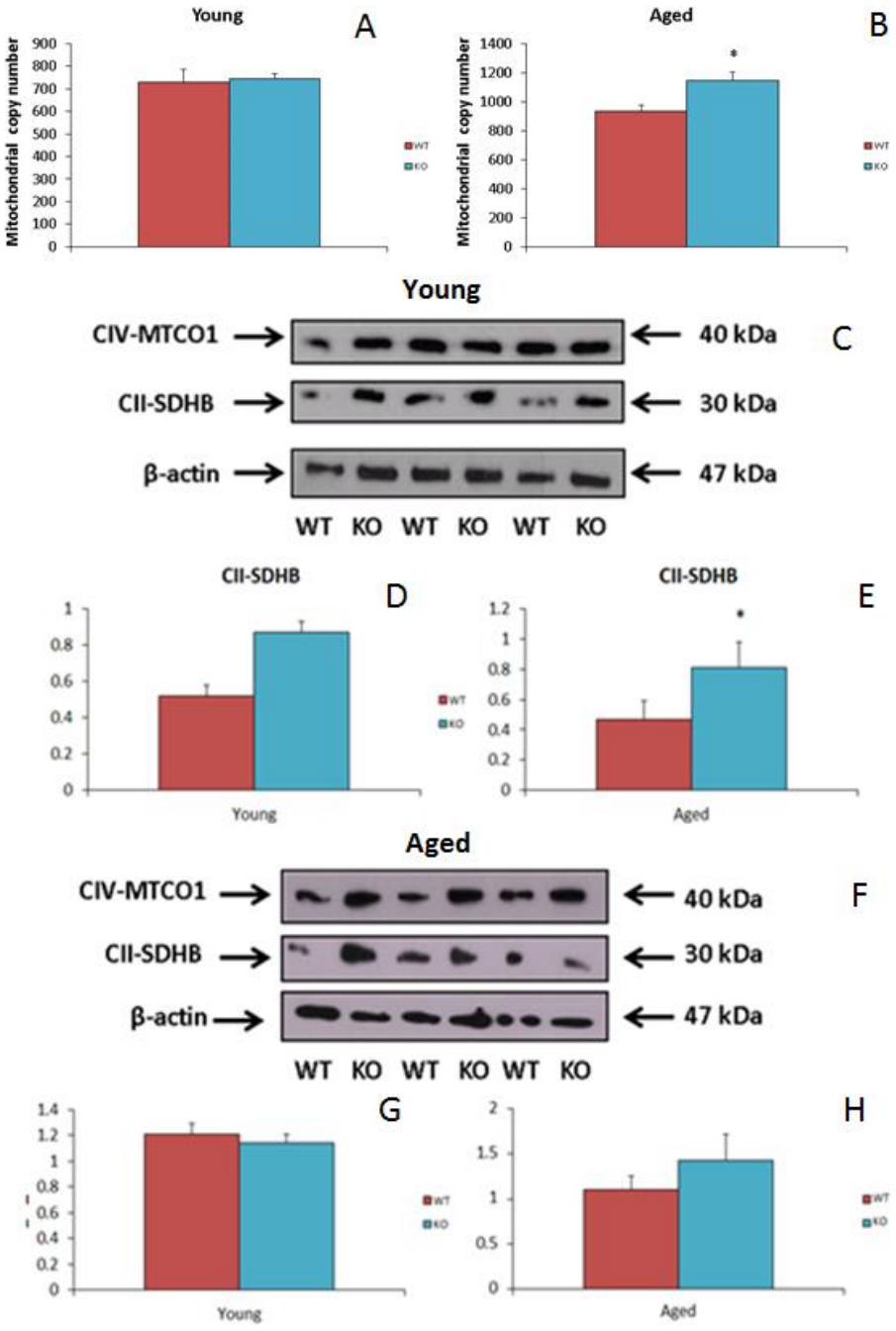

Figure 4-23 Changes in mitochondrial copy number and protein concentration of mitochondrial complexes comparing WT to GKO in young and aged cohorts in brown adipose tissue

*11β-HSD1 knock-out led to significant increase in mitochondrial copy number in aged mice (B) but no change in young mice (A). 11β-HSD1 knock-out also led to a significant increase in the protein expression of C11-SDHB in aged mice (E, F) but no change in the protein expression of CIV-MTCO1 (F, H). In addition there was no change in protein expression of C11-SDHB or CIV-MTCO1 in the young mice with 11β-HSD1 knock-out (C, D, G). Data was analysed using student T-test. * P<0.05. n =6. Data was analysed using densitometry. * P<0.05. n =3. Error bars represent SEM.*

4.3.6 Effect of 11β-HSD1 knock out on primary cultures of brown adipocytes

Having seen changes in gene and protein expression following global 11β-HSD1 knock-out I wished to take these investigations further by investigating the effect of global knock-out of 11β-HSD1 on BAT primary culture. I obtained primary brown adipose tissue cultures from intrascapular BAT from both 11β-HSD1 global knock-out and wild-type mice genotypes of mice aged 10 weeks.

I investigated the effect of 11β-HSD1 GKO on the expression of PGC-1α, PRDM16 and PPARγ (Figure 4-24). I found a significant increase in the expression of PGC-1α ($p<0.05$) and PPARγ ($p<0.05$). I then proceeded to test the effect of 11β-HSD1 knock-out on the mRNA expression of UCP1, Cox7a1, and Cox8b (Figure 4.24). I found a significant increase in the expression of UCP1 ($p<0.05$), Cox7a1 ($p<0.05$), and Cox8b ($p<0.05$) in the 11β-HSD1 knock-out mice.

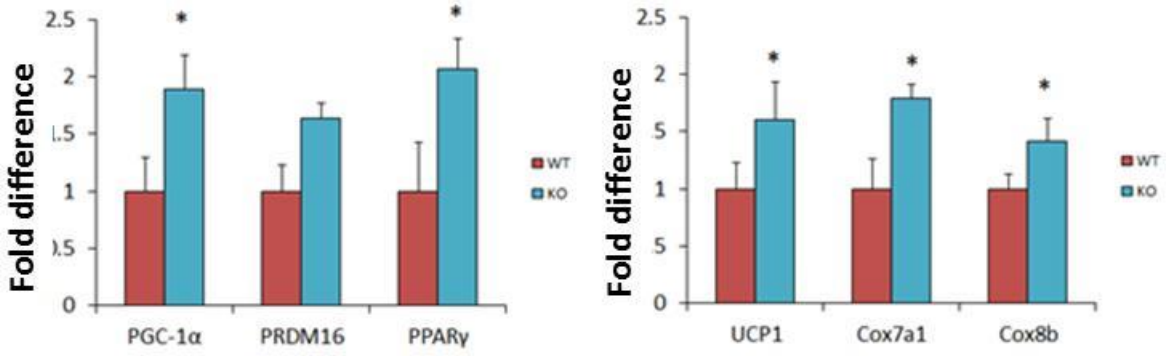

Figure 4-24 Effect of 11β-HSD1 knock-out on the expression of PGC-1α, PRDM16, PPARγ, UCP1, Cox7a1 and Cox8b in brown adipose tissue
*11β-HSD1 knock-out led to a significant increase in the mRNA expression of UCP1, Cox7a1, Cox8b PGC-1α and PPARγ in primary cultures. There was no significant change in the mRNA expression of PPARγ or Cox7a1. Data was analysed using student T-test. * P<0.05. n =3. Error bars represent SEM.*

I went on to investigate the effect of knock-out of 11β-HSD1 on the expression of mitochondrial regulators (figure 4.25). I found a significant increase in the expression of NRF1 ($p<0.05$) but no change in the expression of TFAM.

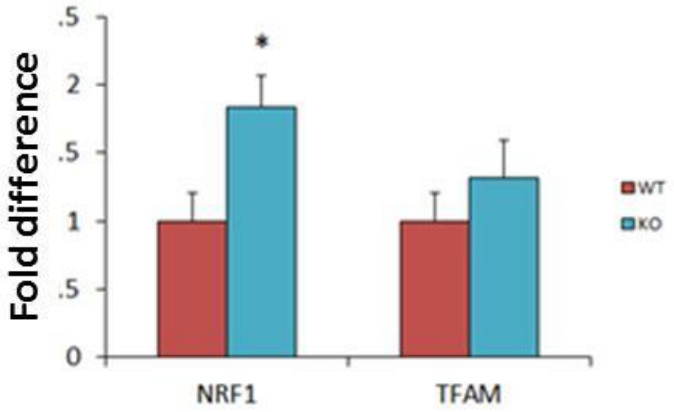

Figure 4-25 Effect of 11β-HSD1 knock-out on the expression of NRF1 and TFAM in brown adipose tissue
*11β-HSD1 knock-out leads to a significant increase in NRF1 mRNA expression in primary cultures. There was no change in the expression of TFAM. Data was analysed using student T-test. * P<0.05. n =3. Error bars represent SEM.*

4.4 Discussion

Ageing has been shown to have a negative effect on BAT in humans, where there is a strong negative correlation with age and BAT mass which may contribute to thermal dysregulation and energy imbalance (Cypress et al 2009). In addition, the metabolic effect of BAT shows a decrease with increasing age (Pfannenberg et al 2010). It is thought that the decrease in BAT mass in humans seen with age is due to a decreased need for thermogenesis. I investigated the effect of age on BAT via young (10 weeks) and aged (100 weeks) WT tissue samples in mice.

I found a significant increase in BAT weight/body weight ratio with age; which contradicts with previous literature which showed a decrease in BAT weight with age (Pfannenberg et al 2010). This increase in quantity of BAT with age could lead to a higher output from the BAT, leading to positive metabolic effects. However looking purely at tissue weight does not assess the quality of the adipose tissue, which may have a larger mass due to an increase in lipid quantity, and the function of the BAT may have diminished as a consequence of age (Lecoulte et al 2011).

To further analyse the BAT samples I performed mRNA analysis on an array of genes. I found that there was a significant increase in 11β-HSD1 mRNA expression in the aged mice. This consequently leads to a higher capacity for local reactivation of GCs in the aged mice via the oxo-reductase activity of the 11β-HSD1 enzyme, converting inactive 11-dehydrocorticosterone to corticosterone (Seckl et al 2001). This potentially higher concentration of local active GC could have detrimental effects on the BAT of the aged mice due to the negative effects of GC on BAT activity and function, where they suppress BAT thermogenesis (Walker et al 1992) (Soumano et al 2000).

It has previously been shown that 11β-HSD1 expression has a negative effect on BAT and therefore I hypothesised that the aged mice would have a decrease in BAT activity and

function due their increased exposure to higher intra-cellular levels of GC. I found significant increases in the mRNA expression of PRDM16 and PPARγ in the aged mice. This is contradictory to what I expected as age has been shown to be a negative regulator of BAT function and due to these genes being positively correlated with an increase in BAT function I would expect a decrease in their expression with age.

I hypothesise that the increases in PRDM16 and PPARγ that I showed is due to the non-thermoneutral temperatures that the mice have been housed at. All animals used within these experiments were housed within non-thermoneutral conditions within the animal house (24°C), which is a lower temperature than thermoneutrality (30°C). Normal animal house conditions have been shown to lead to chronic thermal stress to mice (Golozoubova et al 2004). As a consequence of this the aged mice have been exposed to a lifetime at non-thermalneutral conditions and therefore have activation of non-shivering thermogenesis, and an increase in cold-inducible genes. PRDM16 and PPARγ are both cold inducible (Murholm. M. 2009). I infer that this lifelong exposure to non-thermoneutrality overrides the previously seen and expected negative effects of ageing. In addition these increases in PRDM16 and PPARγ with age could explain my observed increase in BAT mass with age as both PRDM16 and PPARγ contribute to an increase in BAT differentiation and BAT mass (Murholm. M. 2009; Tzu-Ann. C. 1996). In addition older mice are likely to be less active and therefore have a greater require for BAT activity to maintain the correct temperature.

I further investigated the effect of age by examining its effect on traditional markers of BAT. I found significant increases in the expression of UCP1, Cox7a1, and Cox8b at the mRNA level, and also a significant increase in UCP1 expression at the protein level. I hypothesise that these increases in expression are a consequence of the non-thermoneutral conditions that the mice were housed in, as described earlier. Further these markers are

downstream targets of PPARγ and PRDM16 and therefore due to the increases previously seen in PPARγ and PRDM16 it would be thought that there would be consequential increases in these genes too which is what was seen in my data (Seale et al 2007). In addition to this PPARγ has been shown to cooperate with PGC-1α to induce the expression of UCP1 and other typical brown adipose markers (Pisani et al 2011; Watanabe et al 2008).

UCP1 is involved in regulating the energy balance, which was first proposed by Rothwell and Stock in 1979, and therefore an increase in its expression could have beneficial effects on obesity (Rothwell et al 1979). It has previously been shown that with decreasing temperature there is an increase in UCP1, and this supports the hypothesis that due to the ageing mice having been exposed to a lifetime of below thermoneutral temperatures there is an adaptive response which leads to an increase in UCP1 expression (Van Marken Lichtenbelt et al 2009).

I continued my studies by analysing differences with ageing on the expression of NRF1 and transcriptional factor A (TFAM) which are involved in mitochondrial DNA transcription and regulation including respiratory subunits and factors involved in the replication and transcription of mitochondrial DNA (Chau et al 1992; Scarpulla et al 1997). I found significant increases in both NRF1 and TFAM in the aged mice, I again hypothesise that this is due to the non-thermoneutral conditions that the mice were housed at, due to the expression of NRF1 being rapidly induced by cold-exposure (Puigserver et al 1998).

NRF1 has been shown to interact with PGC-1α to be involved in the transcriptional control of mitochondrial biogenesis during adaptive thermogenesis; therefore its increase in expression may correlate with an increase in mitochondria and therefore an increase in the potential for non-shivering thermogenesis, which could have beneficial metabolic effects (Wu et al 1999).

I found that there was a significant increase in mitochondria copy number in the aged mice compared to the young, and that there was a significant increase in complex 4 expression with age. This increase in mitochondrial copy number was expected due to the previous data I have generated showing increases in mitochondrial genes and also TFAM and NRF1 which are involved in mitochondrial biogenesis and have previously been shown to be positively correlated with mitochondrial copy number (Ekstrand et al 2004).

My findings are contradictory to studies which have shown that with increasing age there is a decrease in BAT function, which has been shown in both humans and rodents (Pfannenberg et al 2010). I hypothesise that this is due to the non-thermoneutral temperatures that the mice were housed at, and that a lifetime exposure to non-thermal temperatures is responsible to the increase in BAT markers that I found. This effect of standard animal house conditions (18-22°C) and the chronic thermal stress that it produces has been previously highlighted by Golozoubova et al 2004, who showed that these mice increase their food intake by 50-60%. In addition recent studies within humans have shown that cold exposure leads to an increase in BAT (Lichtenbelt et al 2015). I therefore conclude that the constant thermal stress incurred from animal house conditions severely effects the animals BAT development and function. Overall my ageing studies are supportive of 11β-HSD1 having a regulatory role in BAT metabolism, these affects are directionally consistent with young and old, however it is the aged cohort which reach statistical significance more often. This may be a consequence of the effect of 11β-HSD1 KO having a greater period of time to exert its effects within the aged cohort. This is due to 11β-HSD1's role in the reactivation of active GC and with prolonged decrease in reactivated GC being present there is a more striking effect seen.

I extended my study to look into the effects of exogenous GC administration on BAT. GCs have previously been shown to play a crucial role in adipocyte biology, where they are

central to the differentiation of pre-adipocytes to mature adipocytes (Hauner. H. 1987). Further previous studies have shown that GCs suppress BAT thermogenesis, and that UCP1 expression negatively correlates with GC concentration (Soumano. K. 2000).

I found that there was a significant increase in the BAT weight/body weight ratio in the mice treated with GC. This data suggests that with an increase in GC availability there is an increase in BAT mass. I further analysed the effect of exogenous GC treatment on BAT by measuring its effect on 11β-HSD1 expression. I found a significant increase in 11β-HSD1 expression in tissues from mice treated with corticosterone. This is in agreement with previous data which shows that GCs are stimulatory to the expression of 11β-HSD1 (Kaur et al 2007). As a consequence of this increase in 11β-HSD1 expression in the GC treated tissues, there will be a potential for an increase in the intra-cellular regeneration of active GC, further adding to the increase in GC concentration in these mice.

Having noted these changes I assessed the effect of endogenous GC treatment on the expression of an array of BAT markers. I found no changes in the expression of nr3c1, PGC-1α, PRDM16, PPARγ, Cox7a1, Cox8b, CoxIV, NRF1, or TFAM following treatment with corticosterone. However I did find significant decreases in the expression of UCP1. This shows that following treatment with exogenous GC there is a detrimental effect on UCP1 expression within BAT which would lead to a decreased capacity for the tissue to undergo non-shivering thermogenesis, due to UCP1 being central to this process in its role in uncoupling respiration. Due to this there would be a decrease in BAT function within these mice leading to possible obesity and metabolic consequences.

To further investigate the effect of GC on BAT function I analysed the effect of GC administration on terminally differentiated BAT culture. I found that with the addition of GC there was a significant increase in the mRNA expression of 11β-HSD1. This is in keeping

with my in vivo findings. In addition to this I found a significant decrease in the expression Cox8b, NRF1 and TFAM following GC treatment. This was not shown in vivo, however this data did show a trend towards a decrease in expression.

Overall these in vivo and in vitro experiments showed that following exposure to GC, there is a significant increase in the expression of 11β-HSD1, which would lead to the potential for further increases in active GC regeneration and further to this a decrease in the expression of BAT genes is seen which can be used to infer that GCs have a negative effect on BAT function.

Inactive GCs can be reactivated at the pre-receptor level by the 11β-HSD1 enzyme, leading to an increase in the local availability of active GC (Seckl et al 2001). When 11β-HSD1 is over expressed within adipose tissue there is development of full metabolic syndrome with visceral obesity, diabetes, dyslipidemia and hypertension (Masuzaki. H. 2001). Conversely, mice that are deficient in 11β-HSD1 are insulin sensitised and resist the adverse effects of a high-fat diet, showing a cardio-protective phenotype. I therefore hypothesise that a GKO of 11β-HSD1 will be beneficial to BAT function; I tested the effects of 11β-HSD1 in vivo and in vitro.

I found that within the young cohort there was no change in BAT weight/body weight ratio; however in the aged cohort I found a significant increase in BAT weight/body weight ratio in the GKO mice. This may be a combined genotype and ageing effect. Despite not reaching significance there is a trend towards an increase in BAT weight/body weight ratio in the young mice and this increase reaches significance in the aged mice, this therefore demonstrates that with a long-term decrease in reactivation of GCs via 11β-HSD1 there is a beneficial effect on BAT mass and with age this effect is able to reach significance.

I found significant increases in the expression of PGC-1α, PRDM16 and PPARγ in both the young and aged mice. In addition I found a significant increase in PGC-1α and PPARγ in the GKO primary culture. This is in agreement with my hypothesis that a decrease in local GC concentration achieved by the GKO of 11β-HSD1 would lead to an increase in BAT gene expression.

Having seen the increase in expression of PRDM16 I would also expect the increase in PGC-1α to be partly as a consequence of this due to PRDM16 inducing its expression. PRDM16 also acts as a co regulator to activate PGC-1α and together they drive the molecular phenotype of brown fat cells (Seale et al 2007). Further PGC-1α is a positive regulator of mitochondrial biogenesis and respiration, adaptive thermogenesis, gluconeogenesis as well as many other metabolic processes, therefore an increase in its expression is positive for BAT function and may lead to a more desirable phenotype (Handschin and Spiegelman, et al 2006) (St-Pierre et al., 2003). In addition an increase in PRDM16 and PPARγ within the GKO aged mice could explain why there is an increase in BAT mass with the GKO, as both PRDM16 and PPARγ contribute to increase in BAT differentiation and BAT mass (Murholm. M. 2009; Tzu-Ann et al 1996). Further an increase in expression of PRDM16 has been shown to offer protection from obesity due to an increase in energy expenditure, an increase in glucose tolerance, and more insulin sensitivity (Seale et al 2014; Montague et al 1998).

I further investigated the effect of GKO by examining its effect on traditional markers of BAT. I found significant increases in the expression of CoxIV, Cox7a1, and Cox8b, at the mRNA level in the aged mice, and also a significant increase in UCP1 expression at the protein level within the aged cohort. In addition to this I found a significant increase in the expression of UCP1, Cox7a1 and Cox8b with GKO in the BAT primary culture experiments. I hypothesise that these increases in expression are due to the beneficial effects of decreasing

local GC availability in the GKO. In addition previous studies have shown that PGC-1α expression levels correlate with that of mitochondrial genes, notably those encoding various subunits of the electron transport chain, adaptive thermogenesis, and genes involved in oxidative phosphorylation (Leone et al 2005). Also PGC-1α can coactivate the PPARγ2/RXRa heterodimer to stimulate the UCP1 promoter (Tiraby et al 2003).

I continued my studies by analysing differences with GKO on the expression of NRF1 and TFAM which are involved in mitochondrial biogenesis. I hypothesise that there would be an increase in their expression with the GKO, firstly due to the GKO of 11β-HSD1 lowering the local GC level and also due to the increases in the genes I have already demonstrated. PGC-1α expression has been shown to increase the expression of the nuclear respiratory factors (NRFs), and also that PGC-1α increases the expression and acts as a coactivator for NRF1 to regulate the expression of mitochondrial transcription factor A, (TFAM), which is primarily responsible for the transcription and replication of mitochondrial genes from the mitochondrial genome (Wu et al., 1999). I found significant increases in both NRF1 and TFAM with GKO in the young mice, and NRF1 with GKO in the aged mice. In addition to this I found a significant increase in the expression of NRF1 in the GKO with the primary culture experiments. I again hypothesise that this is due to the decrease in GC regeneration in the GKO genotype. Both NRF1 and TFAM are involved in mitochondrial biogenesis and it can be assumed that an increase in these genes will lead to an increase in mitochondrial number and a possible increase in BAT thermogenic function.

I found that there was a significant increase in mitochondria copy number with GKO in the aged mice, and significant increases in mitochondrial complexes 2 and 4 protein expression. This increase in mitochondrial copy number was expected due to the previous data I have generated showing increases in mitochondrial genes and also NRF1 which is

involved in mitochondrial biogenesis and have previously been shown to be positively correlated with mitochondrial copy number. I would have expected an increase in mitochondrial copy number with GKO in the young mice too; however this was not the case. This could be due to these mice being younger (10 weeks) and therefore the effect of the GKO has only had a limited amount of time to fully exert its effects on mitochondrial copy number within these mice.

Overall I showed that with GKO of 11β-HSD1 there was an increase in markers of overall BAT function and also an increase in BAT mass. This is in keeping with previous studies that have shown that GCs are detrimental to BAT function and also that GKO of BAT leads to beneficial effects. I hypothesise that the increase in markers of BAT function in the GKO mice is due to the consequential decrease in local GC reactivation by the 11β-HSD1 enzyme. This therefore points to a possible therapeutic role for the targeted disruption of the activity of 11β-HSD1 in ameliorating obesity and its metabolic complications.

Having shown a beneficial effect of GKO of 11β-HSD1 on BAT function in young and aged mice, and also the detrimental effect of the addition of exogenous GC on BAT I decided to further investigate this by analysing the effect of GKO of 11β-HSD1 on mice treated with exogenous GC. This would allow for the mimicking of the effect of people treated with exogenous GC as a therapy, and investigate whether a disruption of the 11β-HSD1 enzyme would be beneficial for patients being treated with exogenous GC.

I found a significant increase in the mRNA expression of PRDM16, UCP1, Cox8b and CoxIV in GKO mice treated with corticosterone. The increase in expression of UCP1 in the GKOs will allow for a higher level of non-shivering thermogenesis to take place, and lead to a consequential decrease in obesity and metabolic complications, therefore giving the mice the potential for a healthier phenotype. The increase in expression of Cox8b and CoxIV show an

increase in members of the mitochondrial electron transport chain. Therefore there is an increase in the capacity for non-shivering thermogenesis, and it can be inferred from this that there is an increase in BAT function in GKO mice.

I however found no change in the mRNA expression of PGC1α, PPARγ, Cox7a1 NRF1 or TFAM or in the mitochondrial copy number with GKO following treatment with corticosterone. This could be due to the length of treatment with exogenous GC was not sufficient enough to cause a significant response.

It is well established that excess GC and ageing have negative effects on BAT, however I have demonstrated that by knocking out 11β-HSD1 and therefore removing the local regeneration of active GC I can negate the negative effects of ageing and GCs on BAT. As shown in the figure below ageing leads to an increase in 11β-HSD1 expression and the consequential increase in active GC regeneration. This increase in active GC leads to negative effects on mitochondrial biogenesis, where there is a decrease in UCP1 expression, a decrease in mitochondrial gene expression, and a decrease in mitochondrial biogenesis and size.

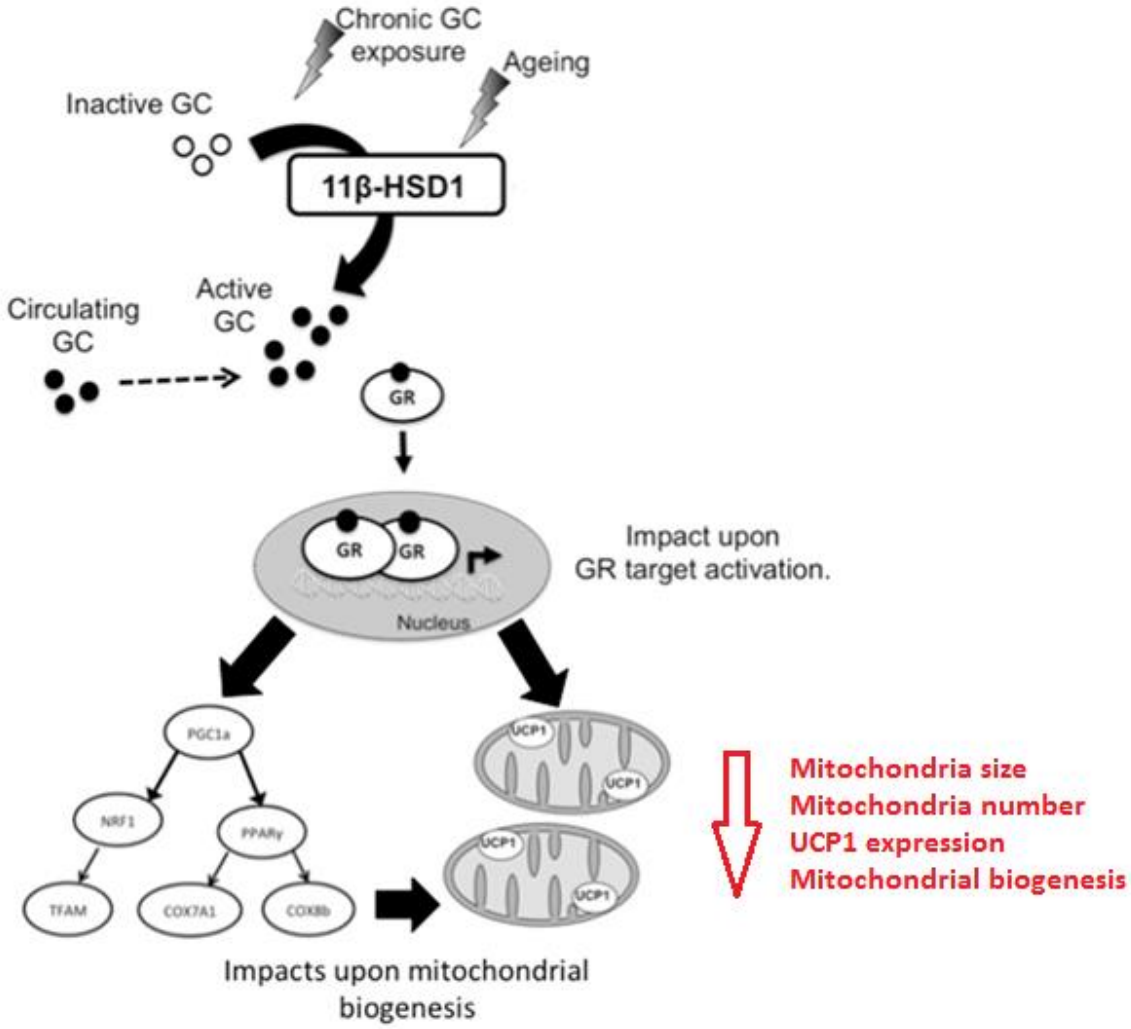

Figure 4-26 Effect of age and chronic glucocorticoid exposure on BAT
Ageing leads to an increase in 11β-HSD1 activity and a consequential increase in the potential for local reactivation of GC. An increase in active GC has negative effects on BAT where it impacts upon mitochondrial biogenesis.

However I have demonstrated that knocking out 11β-HSD1 prevents this and my data even shows that with age there was a potential improvement in mitochondrial function, however this may be exacerbated by the chronic thermal stress the mice were housed in. Overall I have shown that 11β-HSD1 represents an exciting possible target for the prevention of GC and age induced negative effects on BAT function. Furthermore this opens up the potential of utilising BAT as a means to combat obesity. This is of particular importance for translation to humans. An increase in BAT mass has been linked with decreases in obesity and with the global

epidemic of obesity in humans on the rise it offers an exciting prospect to potential treatment of obesity within the human population.

5. Chapter 5 - 11β-hydroxysteroid dehydrogenase type 1 and white adipose tissue browning

5.1 Introduction

11β-HSD1 has been implicated in the pathogenesis of several human disorders including, obesity, insulin resistance and hyperglycaemia (Kannisto et al 2004; Baudrand et al 2010). 11β-HSD1 is responsible for the intracellular regeneration of active GC, and up regulation of this activity within adipose appears to be a key contributor to central obesity and metabolic dysfunction (Morton et al 2008; Paulmyer-Lacroix et al 2002; Rask et al 2002).

In addition to this the expression of 11β-HSD1 varies between different adipose tissues, there is higher 11β-HSD1 mRNA content in visceral compared to subcutaneous adipose depots (Paulmyer-Lacroix et al 2002; Bujalska at al 1997). This may lead to the accumulation of the more metabolically disadvantageous visceral adipose tissue, leading to a central obese phenotype (Paulmyer-Lacroix et al 2002). The effects of adipose-specific 11β-HSD1 over-expression are seen more strongly within visceral depots due to higher GR concentrations (Morton et al 2004; Rebuffe-Scrive et al 1990).

Mice deficient in 11β-HSD1 are insulin sensitised and resist the adverse metabolic effects of a high-fat diet, showing a cardio-protective phenotype, this has been demonstrated in various transgenic models. 11β-HSD1 knock-out mice show resistance to diet-induced visceral obesity and diabetes through improved insulin sensitivity (Wamil et al 2011) (Ataab et al 2010) (Morton et al 2004). This is due to a reduction in the synthesis and secretion of triglycerides and increasing fatty acid oxidation. In addition 11β-HSD1 GKO mice placed on a HFD accumulate fat at a higher rate in peripheral areas which are metabolically more desirable sites as visceral depots which are metabolically less safe (Morton et al 2004) (Wamil et al 2011). Overall a decrease in the level of adipose 11β-HSD1 aids in the

production of a protective metabolic phenotype, adding weight to its role as a therapeutic target.

White adipose tissue (WAT) in mice is located predominantly within three areas; subcutaneous, mesenteric and gonadal. It functions to store energy in the form of triglycerides when there is energy excess, and releases fatty acids when there is an energy deficit, thus regulating whole body energy homeostasis.

Studies have demonstrated that white adipocytes contain two types of cells; pure white adipocytes and brite cells. Brite cells have a more brown-like phenotype and have the ability to express UCP1, however do not otherwise possess the full molecular characteristics of brown adipocytes (Petrovic et al 2009; Guerra et al 1998; Seale et al 2008). These brite cells are induced following a given stimulus for example cold exposure, or β-adrenergic receptor agonists (Petrovic et al 2009). This has been demonstrated in mice and most recently within humans (Kolodny. G. M. et al. 2014).

Cold-acclimatised pre adipocytes in WAT have slightly more pronounced brown characteristics often with large mitochondria (Barbatelli et al 2010). This demonstrates that brite cells have the capacity to switch between an energy storage and energy dissipation phenotype (Wu et al 2012). Further Young et al 1984 showed similar changes in brite cells following cold acclimatisation, and also that in mice maintained at standard animal house conditions (23°C) there was the existence of brown fat cells in traditional white fat cell pads due to chronic stimulation by cold exposure (Young 1984; Seale et al 2014). Within the subcutaneous adipose tissue there was significant expression of PRDM16, UCP1, Cidea, Cox8b and Elovl3, showing that under chronic cold exposure there is induction of a brown fat like gene programme within WAT (Seale et al 2014). This ability of cells within classical

WAT depots to express UCP1 has been recognised for 30 years as first demonstrated by Young et al in 1984.

The white/brown plasticity of adipose tissues might have considerable medical implications, since the brown-like phenotype seems to correlate with a reduced propensity for developing obesity and diabetes in mice (Murano et al 2009; Bachman et al 2002). Further an increase in expression of PGC-1α in WAT may lead to an increase in UCP1 expression and an increase in oxidation of fatty acids (Tiraby et al 2003). Further Fisher et al 2012 have shown that there is an increase in brite cells within WAT in 11β-HSD1 GKO mice which demonstrate a resistance to obesity induced by diet and demonstrate an improvement in glucose metabolism.

Given the data generated in Chapter 4 of this thesis I wanted to assess the impact of 11β-HSD1 on the ability of WAT to assume a brown/ brite like phenotype, and hypothesise that increased exposure to GCs and the activity of 11β-HSD1 with age there will be an inhibitory effect. As is seen in BAT in chapter 4. On this basis I hypothesise that loss of 11β-HSD1 and the consequential decrease in tissue regeneration of GC there will be beneficial effects on WAT cells where they assume a brown phenotype and delay or prevent age associated negative effects.

5.2 Results

5.2.1 Effect of ageing on white adipose tissue in vivo

I wanted to investigate the effect of ageing on WAT. To do this I obtained tissue samples from young (10 weeks) and aged (100 weeks) cohorts, of both wild-type (WT) and 11β-HSD1 global knock-out (GKO) male black 6 mice. I initially analysed the base line effect of ageing on these tissues by comparing WT young and aged tissues.

Initially I analysed potential age related changes in 11β-HSD1 expression, as the 11β-HSD1 enzyme has previously been shown to be a mediator of WAT function, where it led to an increase in WAT mass. I found that there was no change in 11β-HSD1 expression within gonadal adipose tissue with age (Figure 5-1).

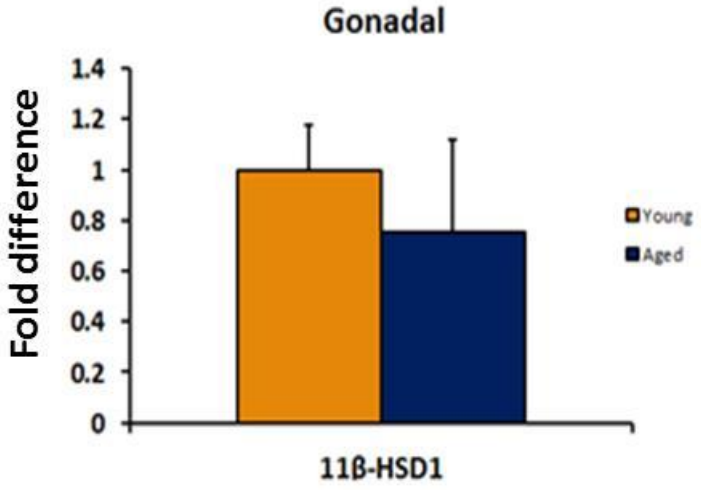

Figure 5-1 Effect of ageing on 11β-HSD1 mRNA expression in gonadal white adipose tissue
Ageing lead to no change in the mRNA expression of 11β-HSD1in gonadal adipose. Data was analysed using student T-test. n=11 (young) n=10 (aged). Error bars represent SEM.

I wished to investigate if ageing had an effect on browning/ brite marker within WAT. I analysed changes in the expression of PGC-1α, PRDM16 and PPARγ with age. I found no significant changes in their expression levels in gonadal adipose tissue (Figure 5-2). Despite this I also examined potential age related changes in the expression of genes involved in the

mitochondrial electron transport chain. I analysed the expression of UCP1, Cox7a1, Cox8b. I found a significant increase in the mRNA expression of UCP1 ($p<0.01$) with age (Figure 6-2) in gonadal adipose tissue.

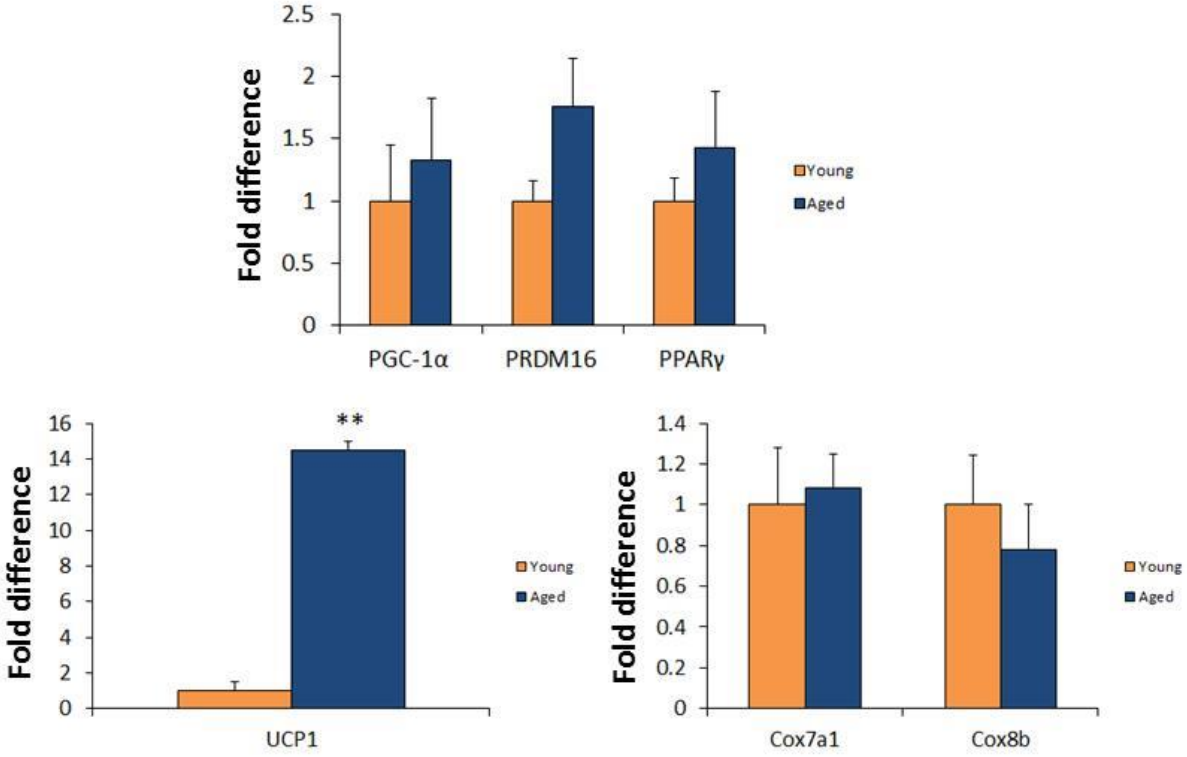

Figure 5-2 Effect of ageing on PGC-1α, PRDM16, PPARγ, UCP1, Cox7a1 and Cox8b mRNA expression in gonadal white adipose tissue
*Ageing lead to no change in the mRNA expression of PGC-1α, PRDM16, PPARγ, Cox7a1, or Cox8b. Ageing lead to a significant increase in the mRNA expression of UCP1.Data was analysed using student T-test. n=11 (young) n=10 (aged). **p<0.01. Error bars represent SEM.*

5.2.1 Effect of temperature on white adipose tissue in vitro

I hypothesised that the increase in expression of UCP1 seen within the aged cohort was a consequence of the temperature in which the mice were housed in which is below the level of thermoneutrality for mice. In order to test this I generated primary tissue cultures of WAT from wild-type mice all aged between 9 and 10 weeks. I analysed the effect of exposure of these primary cultures to decreased (25°C) temperatures for a four hour period. I performed

RT-PCR and 11β-HSD1 activity assays to measure potential changes in gene expression between cells maintained at 37°C and those at 25°C for 4 hours.

I initially investigated potential effects of colder temperatures on the expression of 11β-HSD1 within primary WAT culture. I found no change in the mRNA expression of 11β-HSD1 with a decrease in temperature, and also no change in 11β-HSD1 activity (Figure 5-3).

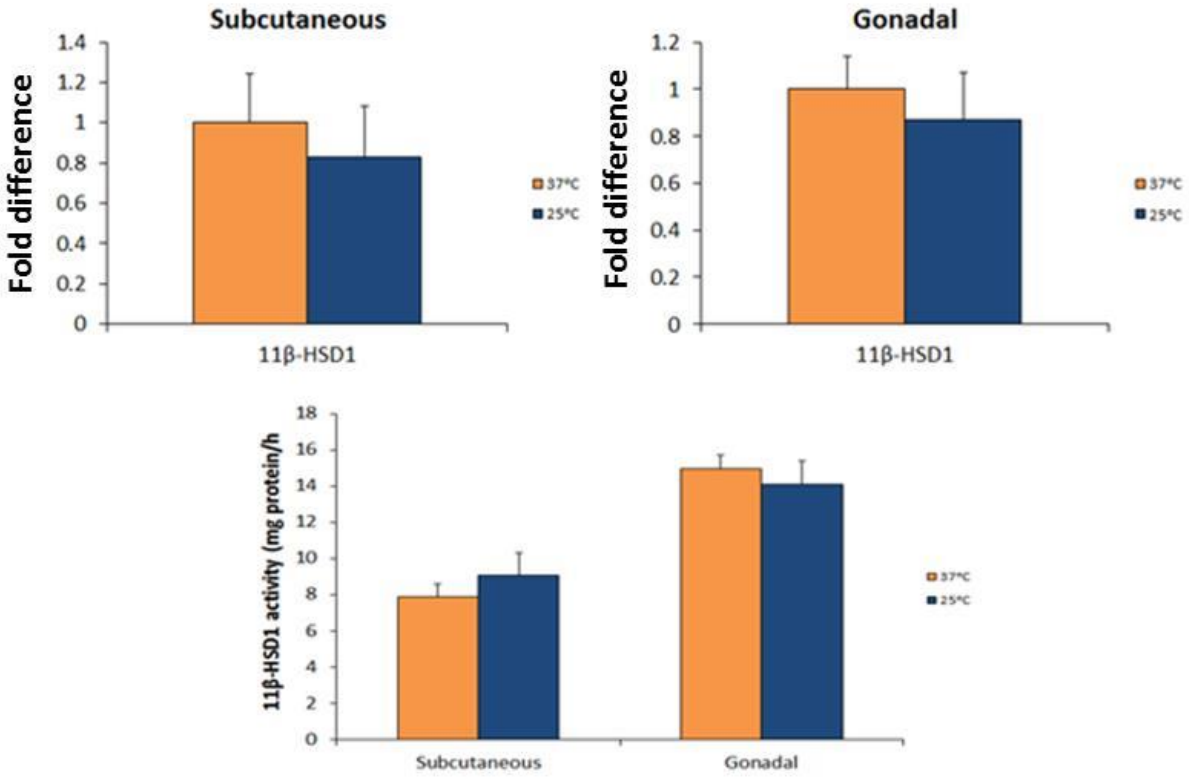

Figure 5-3 Effect of temperature change on 11β-HSD1 expression and activity in primary WAT culture in gonadal white adipose tissue
A decrease in temperature led to no change in the mRNA expression or activity of 11β-HSD1. Primary cultures of both subcutaneous and gonadal adipose tissue maintained at 25°C had the same mRNA level and activity as primary cultures maintained at 37°C. Data was analysed using student T-test. n=3. Error bars represent SEM.

I went on to investigate the effect of exposure to non-thermoneutral temperature on the expression of PGC-1α, PRDM16 and PPARγ with changing temperatures. I found a significant increase in the mRNA expression of PGC-1α ($p<0.001$), PRDM16 ($p<0.05$), and PPARγ ($p<0.01$) at 25°C in subcutaneous adipose primary culture. I also found a significant

increase in the mRNA expression of PGC-1α ($p<0.05$), and PRDM16 ($p<0.01$) at 25°C in gonadal adipose primary culture (Figure 5-4).

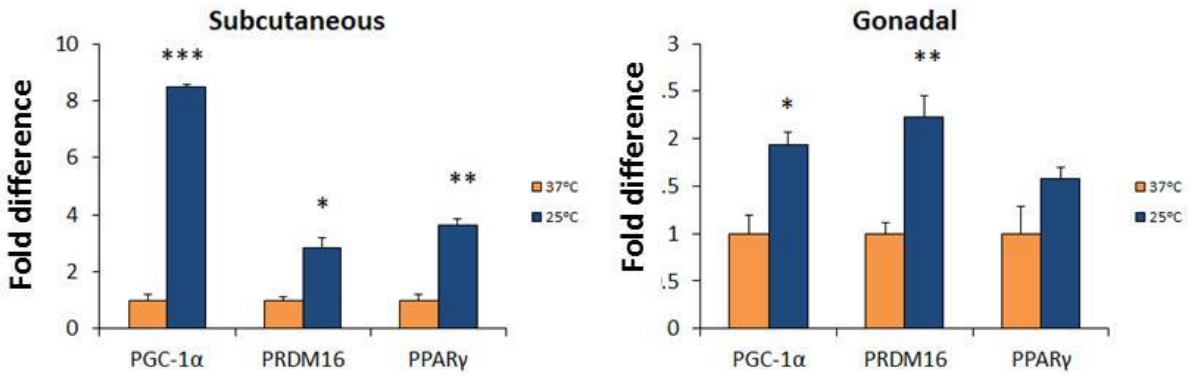

Figure 5-4 Effect of temperature on the expression of PGC-1α, PRDM16, and PPARγ expression in gonadal white adipose tissue
*A decrease in temperature led to a significant increase in the mRNA of PGC-1α, PRDM16 and PPARγ in subcutaneous primary adipose tissue culture, it also led to a significant increase in the mRNA expression of PGC-1α and PRDM16 in gonadal primary adipose tissue culture. There was no significant change in the mRNA expression of PPARγ following a decrease in temperature in the gonadal primary adipose tissue culture. Data was analysed using student T-test. n=3. *p<0.05, **p<0.01. Error bars represent SEM.*

I then went on to investigate if there were temperature related changes in the expression of brite related genes. I analysed the expression of UCP1, Cox7a1, and Cox8b. I found a significant increase in the mRNA expression of UCP1 ($p<0.0001$), Cox7a1 ($p<0.05$), and Cox8b ($p<0.001$) within the subcutaneous primary culture and also a significant increase in the mRNA expression of UCP1 ($p<0.05$), Cox7a1 ($p<0.05$) and Cox8b ($p<0.01$) in the gonadal adipose tissue (Figure 5-5).

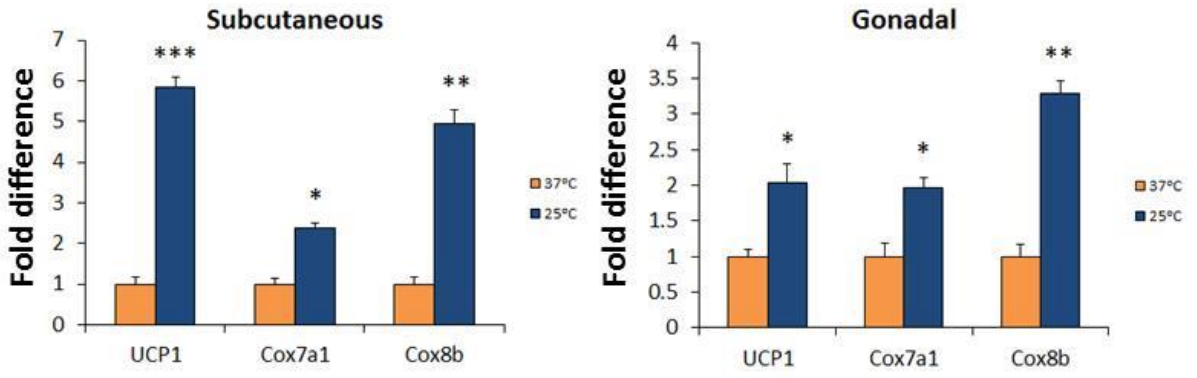

Figure 5-5 Effect of temperature change on the expression of UCP1, Cox7a1, and Cox8b in gonadal white adipose tissue
*A decrease in temperature led to a significant increase in the mRNA of UCP1, Cox7a1 and Cox8b in both subcutaneous and gonadal primary adipose tissue culture. Data was analysed using student T-test. n=3. *p<0.05, **p<0.01, ***p<0.001. Error bars represent SEM.*

5.2.2 Effect of glucocorticoid excess on white adipose tissue in vitro

To examine the role of excess GC on WAT function I generated primary tissue cultures of mature adipocytes derived from subcutaneous and gonadal WAT from wild-type mice. I analysed the effect of exposure of these primary cultures to either GC treatment or vehicle and examined the expression of key markers and performed 11β-HSD1 activity assays,.

I found that there was no change in the mRNA expression of 11β-HSD1 following GC addition in subcutaneous adipose primary cultures (Figure 5-6), however I found a significant increase in the mRNA expression of 11β-HSD1 within the gonadal adipose primary culture ($p<0.05$) (Figure 5-6). I further went on to see if there were changes in 11β-HSD1 activity with exposure to GC. I found no changes in 11β-HSD1 activity following administration of GC in either subcutaneous or gonadal primary cultures (Figure 5-6).

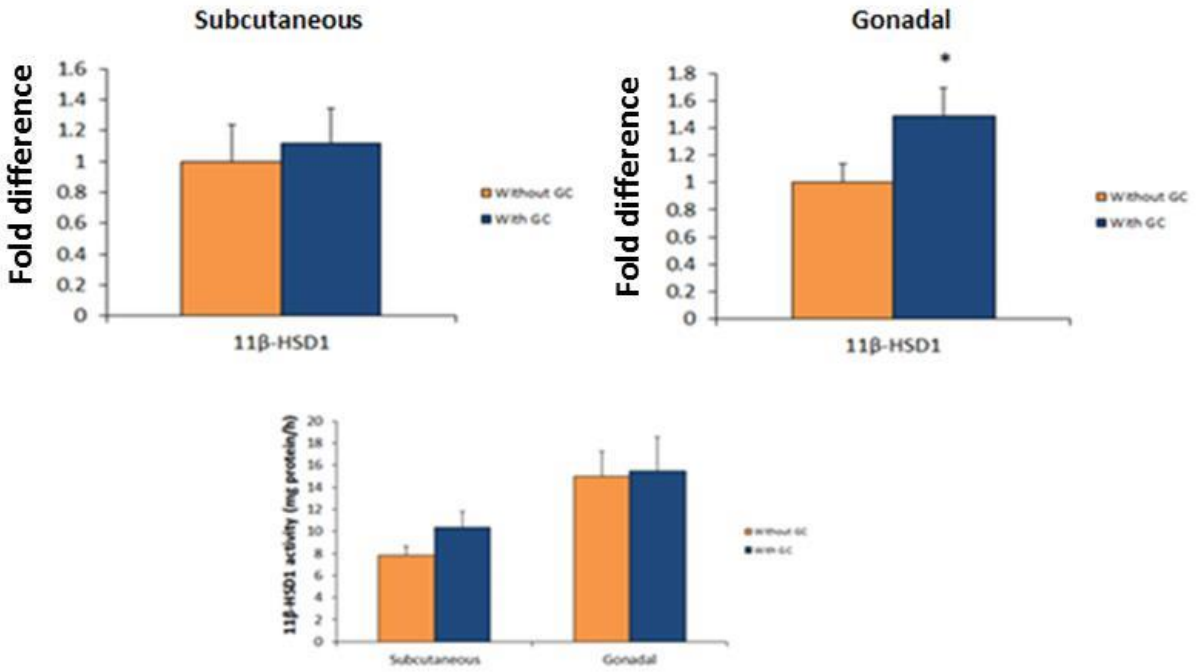

Figure 5-6 Effect of GC on 11β-HSD1 expression and activity in subcutaneous and gonadal adipose in gonadal white adipose tissue
Exposure to excess GC led to no change in the mRNA expression of 11β-HSD1 in subcutaneous primary adipose tissue culture. There was no change in 11β-HSD1 activity following administration of exogenous GC in either subcutaneous or gonadal primary adipose tissue culture. Data was analysed using the students T-test. N=3. Error bars are representative of SEM.

I went on to investigate the effect of exposure to GC on the expression of transcription factors. I analysed changes in the expression of PGC-1α, PRDM16 and PPARγ. I found a significant decrease in the mRNA expression of PRDM16 ($p<0.05$), and PPARγ ($p<0.05$) in the subcutaneous primary adipose culture (Figure 5-7). I also found a significant decrease in the mRNA expression of PRDM16 ($p<0.05$) in the gonadal primary adipose culture (Figure 5-7).

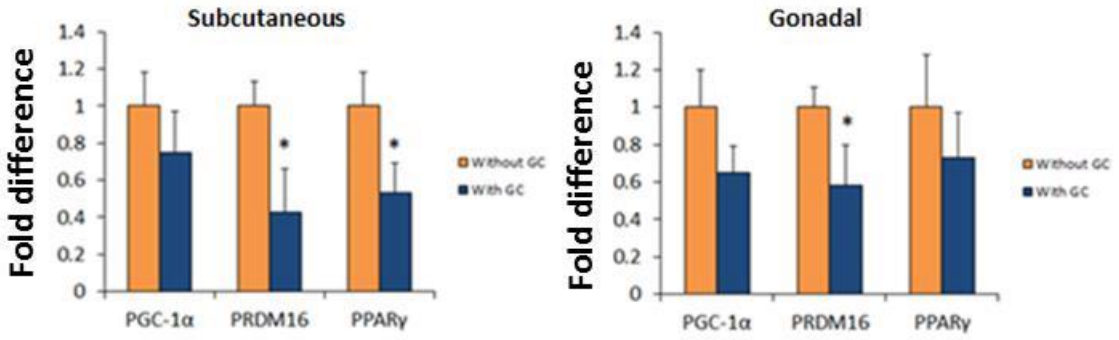

Figure 5-7 Effect of endogenous GC treatment on the expression of PGC-1α, PRDM16, and PPARγ in subcutaneous and gonadal white adipose tissue
*Exposure to excess GC led to significant decrease in the mRNA expression of PRDM16 and PPARγ in subcutaneous primary adipose tissue culture and PRDM16 in gonadal primary adipose tissue culture. Data was analysed using student T-test. n=3. *p<0.05. Error bars represent SEM.*

I then went on to see if there were GC related changes in the expression of UCP1, Cox7a1, and Cox8b. I found that there was a significant decrease in the mRNA expression of UCP1 ($p<0.05$), Cox7a1 ($p<0.05$) and Coxb8 ($p<0.05$) following the addition of GC in the subcutaneous adipose primary cultures (Figure 5-8). I also found that there was a significant decrease in the mRNA expression of UCP1 ($p<0.01$), Cox7a1 ($p<0.05$) and Coxb8 ($p<0.05$) following the addition of GC in the gonadal adipose primary cultures (Figure 5-8).

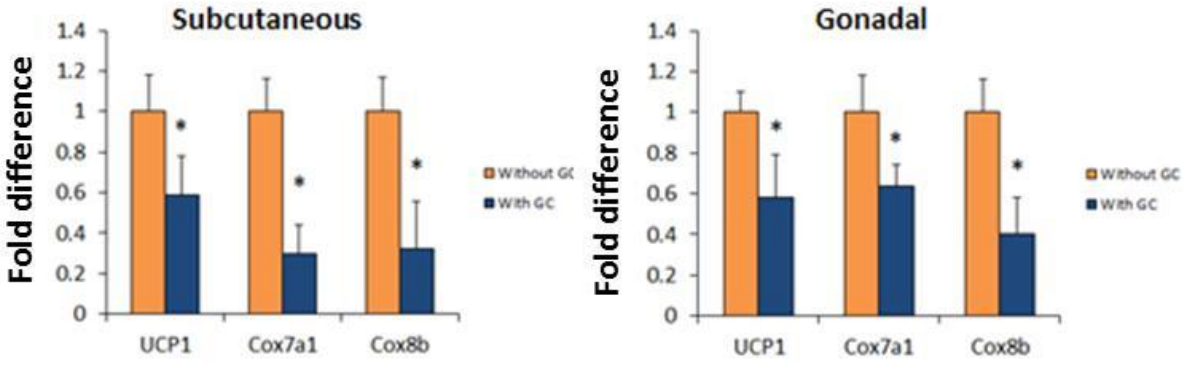

Figure 5-8 Effect of exogenous GC treatment on the expression of UCP1, Cox7a1 and Cox8b in subcutaneous and gonadal adipose tissue in white adipose tissue
*Exposure to excess GC led to a significant decrease in the mRNA expression of UCP1, Cox7a1 and Cox8b in subcutaneous and gonadal primary adipose tissue culture. Data was analysed using student T-test. n=3. *p<0.05, **p<0.01. Error bars represent SEM.*

5.2.3 Effect of 11β-HSD1 knock-out on white adipose tissue in vivo

Previous work has highlighted the beneficial effects of 11β-HSD1 knock-out on WAT function and also conversely how an increase in 11β-HSD1 can be of detriment to WAT function. In addition my data demonstrated the negative effect of GC administration on WAT, therefore I hypothesised that GKO of 11βHSD1 would be beneficial to WAT function. This led us to analyse the potential beneficial effects of global knock-out of 11β-HSD1 on WAT browning/brite markers in subcutaneous tissue samples from young (10 weeks) and aged (100 weeks) cohorts, of wild-type and 11β-HSD1 knock-out (GKO) genotypes.

To do this I measured the expression of markers of browning. I began by performing RT-PCR analysis on mitochondrial transcription factors which are related to potential improvements in mitochondrial function. I analysed changes in the expression of PGC-1α, PRDM16 and PPARγ with age (Figure 5-9). I found no significant increase in the mRNA expression of PGC1α PRDM16 and PPARγ in the GKO mice in the young cohort, however I found a significant ($p<0.05$) increase in the mRNA expression of PRDM16 and PPARγ in the aged cohort following global GKO of 11β-HSD1 (Figure 5-9).

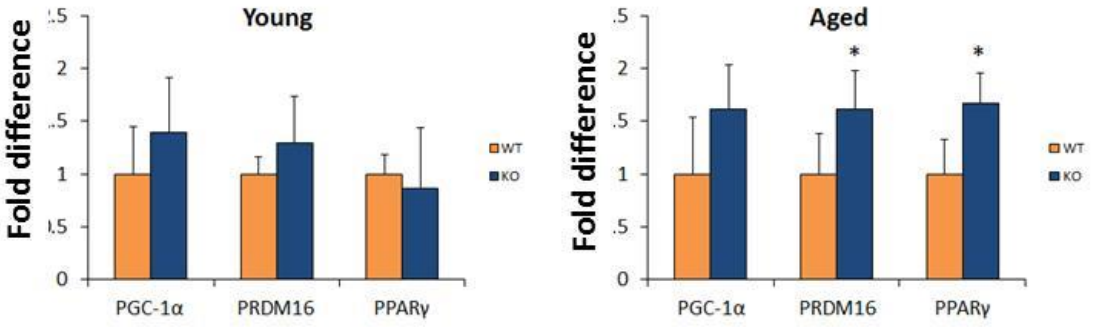

Figure 5-9 Changes in mRNA of PGC-1α, PRDM16, and PPARγ between WT and GKO in young and aged cohorts in gonadal white adipose tissue
*11β-HSD1 knock-out led to a significant increase in PRDM16 and PPARγ mRNA expression in aged mice, but no significant change in the mRNA expression of PGC-1α within the aged mice. There was no significant change in the mRNA expression of PGC-1α, PRDM16 or PPARγ with GKO in the young mice. Data was analysed using student T-test. * P<0.05. n =5 for WT young, n=9 for GKO young. N = 6 for WT aged, n =6 for GKO aged. Error bars represent SEM.*

Due to the transcription factors being regulators of the expression of mitochondrial genes I then went on to see if there were changes in the expression of an array of mitochondrial structural genes. I analysed the expression of UCP1, Cox7a1, and Cox8b (figure 5-10). I found a significant increase in the mRNA expression of Cox8b ($p<0.01$) in the GKO of the young cohort, and a significant increase in the mRNA expression of Cox7a1 ($p<0.01$) and Cox8b ($p<0.05$) in the GKO of the aged cohort (Figure 5-10).

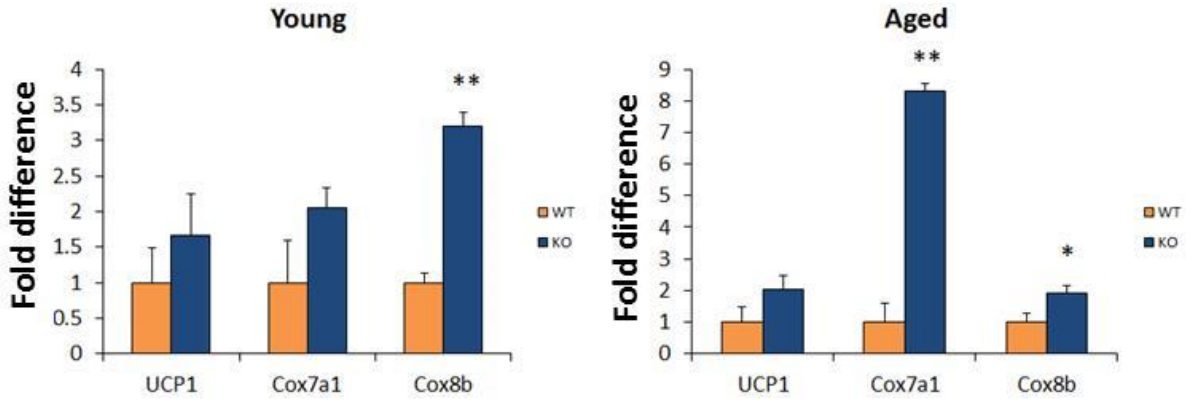

Figure 5-10 Changes in mRNA of UCP1, Cox7a1, Cox8b, and CoxIV between WT and GKO in young and aged cohorts in gonadal white adipose tissue
*11β-HSD1 knock-out leads to a significant increase in Cox8b mRNA expression in young mice, and a significant increase in Cox7a1 and Cox8b mRNA expression in aged mice. There was no significant change in the mRNA expression of UCP1, or Cox7a1 with GKO in the young mice, and there was no significant change in the mRNA expression of UCP1 with KI in the aged mice. Data was analysed using student T-test. * P<0.05, *p<0.01. n =5 for WT young, n=9 for GKO young. N = 6 for WT aged, n =6 for GKO aged. Error bars represent SEM.*

5.2.4 Effect of 11β-HSD1 knock-out on white adipose tissue in vitro

To further examine if 11β-HSD1 mediates the effect of the observed GC suppression of WAT browning markers I investigated primary white adipose tissue cultures from subcutaneous and gonadal WAT from both global knock-out 11β-HSD1 and wild-type mice.

I investigated the effect of GKO on the expression of PGC-1α, PRDM16 and PPARγ. I found a significant increase in the mRNA expression of PGC-1α ($p<0.01$), PPARγ ($p<0.05$) in the primary subcutaneous culture (Figure 5-11). I also found a significant increase in the

mRNA expression of PGC-1α ($p<0.05$), PRDM16 ($p<0.05$), and PPARγ ($p<0.05$) in the primary gonadal culture (Figure 5-11).

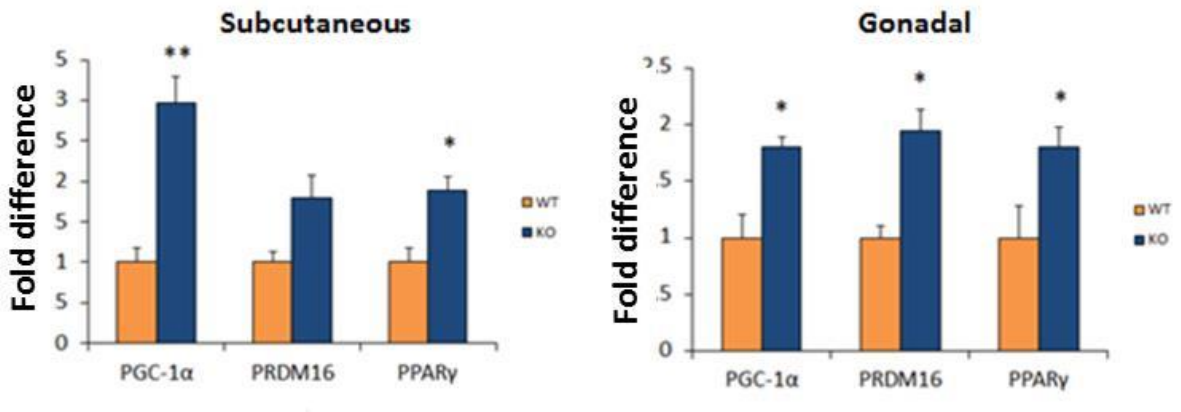

Figure 5-11 Effect of 11β-HSD1 knock-out on the expression of PGC-1α, PRDM16, and PPARγ in primary subcutaneous and gonadal adipose tissue in white adipose tissue
*Knock-out of 11β-HSD1 led to a significant increase in the mRNA expression of PGC-1α and PPARγ expression in subcutaneous primary adipose tissue culture, and a significant increase in PGC-1α, PRDM16, and PPARγ expression in gonadal primary adipose tissue culture. Data was analysed using student T-test. n=3. *p<0.05, **p<0.01. Error bars represent SEM.*

I then proceeded to test the effect of 11β-HSD1 knock-out on the mRNA expression of UCP1, Cox7a1, and Cox8b. I found that there was a significant increase in the mRNA expression of Cox8b ($p<0.01$) and UCP1 ($p<0.01$) in the subcutaneous primary culture with the GKO (figure 5-12). I also found that there was a significant increase in the mRNA expression of UCP1 ($p<0.01$) and Cox8b ($p<0.05$) in the gonadal primary culture, with the GKO (figure 5-12).

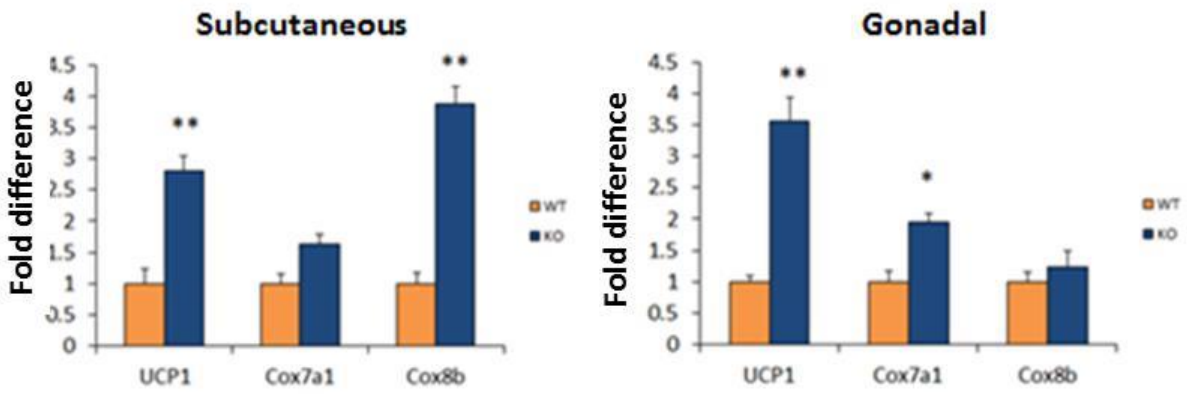

Figure 5-12 Effect of 11β-HSD1 knock-out on the expression of UCP1, Cox7a1 and Cox8b in subcutaneous and gonadal primary culture in white adipose tissue
*Knock-out of 11β-HSD1 led to significant increase in UCP1 and Cox8b expression at in subcutaneous primary adipose tissue culture and a significant increase in UCP1 and Cox7a1 expression at in gonadal primary adipose tissue culture. Data was analysed using student T-test. n=3. *p<0.05, **p<0.01. Error bars represent SEM.*

5.3 Discussion

Age has a detrimental effect on WAT function due to substantial changes in fat tissue metabolic function. These include declines in insulin, lipolytic, and fatty acid responsiveness (Das et al., 2004). Ageing has previously been shown to lead to a redistribution of fat between different fat depots, especially during middle age where fat redistributes from subcutaneous to visceral adipose depots (Meunier et al 1971). Visceral adipose accumulation has been shown to increase the risk of cardiometabolic diseases, therefore prevention of this redistribution of adipose tissue with age is desirable (Wajchenberg et al 2000).

I found that there was no change in the mRNA expression of 11β-HSD1 within gonadal WAT with age. Previous studies in pre and post-menopausal women have shown that there is an increase in 11β-HSD1 expression within WAT following the menopause (Andersson et al 2009), however it can not be said if these changes were menopause or ageing related. Due to there being no significant change in the mRNA expression of 11β-HSD1 with age it can be inferred that this would mean that there would be no alteration in the quantity of reactivated GC availability at the tissue level, as 11β-HSD1 is responsible for the tissue specific regeneration of active GC (Seckl et al 2001). This inference however is limited as often there is not a clear correlation between mRNA and protein expression and thus activity (Kendrick et al 2014). This is due to post-translational regulation, including co-factor binding, and phosphorylation. Despite this in other results where protein levels and 11β-HSD1 activity are measured alongside mRNA expression, there appears to be correlation.

I proceeded to see if there were changes in transcriptional factors and mitochondrial component expression. I found that there was a significant increase in the mRNA expression of UCP1 with age; however I found no significant changes in the expression of the other genes screened, however I did see a trend towards their increase. This increase was

unexpected given results from previous studies as increases in the expression of these genes is related to improvements in function, which is contradictory to studies showing decreased function with age.

UCP1 is the main protein involved in non-shivering thermogenesis, where it uncouples the electron transport chain from the production of ATP to the production of heat (Houstek et al 1978). Non-shivering thermogenesis is triggered by exposure to cold temperatures; this therefore implies that the increased production of UCP1 with age has been triggered by chronic cold exposure (Himms-Hagen et al 1984). The mice were kept in standard animal house conditions (22°C) which is below the standard thermoneutral temperatures for mice (37°C). I therefore hypothesise that the increase in expression in UCP1 is cold driven. This increase in thermogenic genes following mice being housed in animal house conditions has been shown by Young et al, who also showed an increase in brite cells when mice were housed in standard animal conditions. This increase in non-shivering thermogenesis seen with age may be of benefit to the mouse due to following cold exposure there is mobilisation of fatty acids from WAT which may lead to weight loss and a healthier phenotype (Deiulis et al 2010). Previous studies by Nedergaard and Cannon et al 2013 have showed an induction of 100 fold increase in the expression of UCP1. This is far higher than the induction I observed however my increase still reached significance. Due to the induction of UCP1 expression still being <20% of those seen in classical BAT depots it has been questioned whether there is enough UCP1 present to mediate significant thermogenesis (Walden et al 2012; Nedergaard and Cannon et al 2013; Shabalina et al 2010). However Nedergaard et al have shown that the UCP1 within WAT is thermogenically functional however BAT would still predominate in thermogenesis. It can therefore be concluded that with a chronic lifetime exposure to below thermoneutral temperatures there is an increase in

themo-regulated gene expression, demonstrated here by the increase in mRNA expression of UCP1.

To test my hypothesis that it is temperature which has caused the alteration in gene expression with age I generated primary cultures of WAT from both subcutaneous and gonadal adipose depots. I found no change in the 11β-HSD1 mRNA expression or 11β-HSD1 oxo-reductase activity with changing temperatures. Therefore there would be no change in the intercellular regeneration of GC. I can therefore say that any changes seen in gene expression will be as a consequence of the temperature change.

I investigated the effect of cold on the expression of brite markers within the WAT primary cultures. I found a significant increase in the mRNA expression of PGC-1α, PRDM16, PPARγ, UCP1, Cox7a1 and Cox8b with colder temperatures in the subcutaneous primary adipose line and a significant increase in the mRNA expression of PGC-1α, PRDM16, UCP1, Cox7a1 and Cox8b with colder temperatures in the gonadal primary adipose line, therefore showing a more brown like phenotype following cold exposure. This is in keeping with previous studies which have shown an increase in the expression of thermogenic genes, mitochondrial genes, and transcriptional regulators following cold exposure (Wu et al 2012; Barbatelli et al 2010; Walden et al 2012). In addition this change has been shown to be more striking within subcutaneous as opposed to visceral adipose which is what I showed (Barbatelli et al 2010; Lee et al 2012). This increase in thermogenic and mitochondrial genes is linked with an increase in the incidence of brite adipocytes within the WAT (Barbatelli et al 2010). A process which is termed browning. These increases in mRNA expression following cold exposure confirm my hypothesis that it was the chronic cold exposure which triggered the increase in UCP1 in the aged mice.

Certain genes are influential in the development of brite adipocytes. Firstly PRDM16 has been shown to be key in the differentiation of most brite genes including UCP1, Cox8b, and PPARγ (Seale et al 2014). I have demonstrated an increase in expression of PRDM16 and also its previously demonstrated target genes. In addition PPARγ has been shown to allow preadipocyte cultures to acquire brown–like characteristics (Petrovic et al 2010; Seale et al 2007), this activation is achieved along with PGC-1α which coactivates the PPARγ2/RXRa heterodimer to stimulate brown adipocyte markers including UCP1 (Tiraby et al 2003). In addition PPARγ has been shown to induce the expression of UCP1 and also stimulate mitochondrial biogenesis and uncoupled cellular respiration (Seale et al 2007).

Therefore this potential increase in the expression of brite cells seen following cold exposure may have considerable medical implications as the brown-like phenotype demonstrated in my studies correlates with a reduced propensity for the development of obesity and diabetes in mice (Murano et al 2009; Bachman et al 2002). Further Tsukiyama-Kohora et al 2001 have shown that when there is an increase in brite cells within WAT there is resistance to diet induced obesity and improved glucose metabolism. These results following cold stimulation within primary cultures is in agreement with my hypothesis that the effect I saw with ageing on the expression of UCP1 and other genes is due to a chronic lifetime exposure to cold temperatures within an animal house setting. The increases in mRNA I observed from the primary culture studies are far more striking than those I found with my ageing study. This may be due to the cold exposure that was experienced by the primary cultures was more intense than that experienced by the mice, and also that the mice within the animal house are often group housed with bedding which can allow them access to additional heat other than the ambient temperature of the animal house. Further this study exposes WAT directly to cold stimulus, however when this study is converted to look at

potential in vivo effects it may be argued that the effects of cold would not be as great, as the WAT within an in vivo model would not be directly exposed and therefore may not be exposed to the same degree of cold, and as a consequence the dramatic effects seen within this study may not be fully converted. However a study by Huttunen et al demonstrated an increase in BAT in outdoor workers which was hypothesised to be an effect of cold exposure.

Further to ageing and temperature another factor that effects WAT is GCs. GCs can be reactivated at the pre-receptor level by 11β-HSD1 (Seckl et al 2001), leading to an increase in the local availability of active GC. GCs have also been shown to suppress thermogenesis (Walker et al 1992). Studies have shown that an increase in 11β-HSD1 activity WAT leads to the development of MS with visceral obesity, diabetes, dyslipidemia and hypertension (Masuzaki et al 2001).I tested the effects of exogenous GC administration on primary cultures of WAT and also the effect of GKO of 11β-HSD1 on WAT primary culture and WAT.

Following exogenous GC administration I found no change in the expression of 11β-HSD1 and also no change in 11β-HSD1 activity in subcutaneous adipose tissue. This is contradictory to previous studies which have shown that GC increases the expression of 11β-HSD1 (Low et al 1994), however there was a significant increase in the mRNA expression of 11β-HSD1 in gonadal adipose tissue following GC treatment. If the GC had been left on the primary culture for a longer period of time the change may have reached significance in subcutaneous adipose.

I went on to test the effect of GC administration on genetic markers. I found a significant decrease in the mRNA expression of PGC-1α, PPARγ, PRDM16, UCP1, Cox7a1 and Cox8b within the subcutaneous primary culture. In addition I found a significant decrease in the mRNA expression of PRDM16, PPARγ, UCP1, Cox7a1 and Cox8b within the gonadal primary culture. These results clearly demonstrate the negative effect GCs have on WAT and

how they decrease the amount of browning within the WAT and lead to a stronger WAT phenotype. This has the potential to be detrimental to the mice as it leads to a decrease in the degree of browning, and therefore changes the WAT from an energy expending to an energy storing depot.

To try to counteract the negative effects of GC on WAT I have tested the effect of GKO of 11β-HSD1 in both subcutaneous and gonadal adipose tissue. I found a significant increase in the mRNA expression of Cox8b within the young cohort and a significant increase in the expression of PRDM16, PPARγ, Cox7a1 and Cox8b within the aged cohort. The increase in significance seen within the aged cohort compared to the young cohort may be due to the increased amount of time that these mice were exposed to a decrease in local GC regeneration and therefore the GKO of 11β-HSD1 had a longer time to exert significant effects on the WAT.

I increased my study into the effects of GKO of 11β-HSD1 by generating primary WAT cultures of subcutaneous and gonadal adipose tissue of both WT and GKO genotype. Within the subcutaneous primary adipose tissue culture I found a significant increase in the mRNA expression of PGC-1α PPARγ, UCP1 and Cox8b within the GKOs. Within the gonadal primary adipose tissue culture I found a significant increase in the mRNA expression of PGC-1α, PRDM16, PPARγ, UCP1 and Cox7a1 within the GKOs. This increase in gene expression with GKO suggests an increase in browning.

Previous studies have shown the benefit of 11β-HSD1 GKO on WAT where they have demonstrated resistance to diet induced visceral obesity and diabetes through improved liver and adipose function. In addition over expression of 11β-HSD1 within adipose tissue has been shown to lead to obesity, dyslipidemia and insulin resistance (Masuzaki et al 2001). In addition, the brown like phenotype I have demonstrated with GKO of 11β-HSD1 has been

shown to correlate with reduced propensity for developing obesity and diabetes in mice (Murano et al 2009) (Bachman et al 2002). These results demonstrate an increased degree of browning seen with GKO of 11β-HSD1 and that manipulation of 11β-HSD1 is a potential therapeutic target for a myriad of diseases.

Overall I have demonstrated that cold exposure leads to an increase of browning of WAT which could confer a beneficial phenotype. In addition to this I have investigated the effect of GKO of 11β-HSD1 and the consequential decrease in local regeneration of active GC. I have demonstrated that following GKO there is an improved genetic profile which implies a more brown like phenotype. The potential increase in brite seen following GKO and cold exposure has potential in combating a myriad of diseases. Therefore 11β-HSD1 represents a promising therapeutic target.

6. Chapter 6 - Conclusions and further studies

6.1 Adipose specific knock-out of 11β-hydroxysteroid dehydrogenase type 1

When in excess GCs have been shown to lead to the development of Cushing's syndrome, metabolic syndrome and type 2 diabetes (Fardet. L. 2007). In addition GCs can also be regenerated at the tissue specific level by 11β-HSD1 (Koteleytsev et al 1997). Animal models incorporating a GKO of 11β-HSD1 have shown protection from glucose intolerance, hyperinsulinemia, hepatic steotosis, adiposity, hypertension, myopathy, and Cushing's syndrome (Morton. N. M 2001). In contrast to this over expression of 11β-HSD1 has been shown to lead to weight gain (predominantly visceral), dyslipidemia, hyperglycaemia, hypertension and insulin resistance (Masuzaki. H. 2003). Further increased 11β-HSD1 activity selectively in adipose tissue results in the development of full metabolic syndrome with visceral obesity, diabetes adyslipidemia and hypertension (Masuzaki. H. 2001). Together these data suggest that adipose 11β-HSD1 plays a key role in the development of the adverse metabolic profile of GC excess, leading to the potential for adipose 11β-HSD1 inhibition as a potential treatment for Cushing's syndrome and metabolic complications within humans.

I showed that GKO of 11β-HSD1 conferred a protective phenotype following GC excess and I wished to attempt to elucidate the tissue responsible for this effect. I generated a novel mouse model which contained an adipose-specific deletion of 11β-HSD1 (FKO) utilising cre flox technology. The KO was confirmed using DNA, RNA and 11β-HSD1 oxo-reductase activity assays.

I wished to test my model to see if it had protective effects against exposure to excess GC. This is due to GC being widely prescribed in medical practice, and this leads to many adverse effects and long term GC therapy can lead to suppression of the HPA axis (Krasner et al 1999), insulin resistance (Lansang et al 2011) and diabetes (Lansang et al 2011) within

humans. This effect of GCs is amplified by 11β-HSD1 which regulates intracellular GC metabolism at the pre-receptor level (Rabbitt et al 2002), via its oxo-reductase activity increasing intracellular GC levels by converting circulating inert GC to active GC (Tomlinson et al 2001; Napolitano et al 1998). Further recent data in our lab has shown that global KO of 11β-HSD1 leads to improved glucose tolerance, insulin sensitivity, and improved systolic blood pressure.

With my adipose specific knock-out of 11β-HSD1 I showed that these mice were not protected from adiposity, decreased lean body mass, glucose intolerance, hyperimsulinemia or reduced grip strength following CORT treatment. This therefore highlights the role played by 11β-HSD1 within other tissues. The tissues where 11β-HSD1 is most highly abundant are adipose, liver, kidney and lung. These tissues are therefore the primary suspects in contributing to the effects of GC excess still shown in my FKO model which are protected from in the GKO model. I hypothesise that the 11β-HSD1 present in these tissues reactivates GC to levels significant enough to ameliorate the proactive effect of 11β-HSD1 KO in adipose. Further the reactivated GC may be able to travel to other tissues apart from those where it is reactivated and therefore exert its effects more widely. This may consequently mean that tissue specific KO of 11β-HSD1 may not be sufficient to ameliorate all of the side effects of GC excess and it may prove necessary to KO 11β-HSD1 globally in order to fully achieve protection.

However these mice were protected from hepatic TAG accumulation, increased serum free fatty acids, hepatic steotosis and increased adipose expression of hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL), liver X receptor a (LXRa), and liver X receptor b (LXRb). These effects were not seen in the LKO mice which suggests that increased

intracellular GC availability within adipose tissue as opposed to the liver is central to the beneficial effects seen in the GKO.

This demonstrates the protective role adipose specific KO of 11β-HSD1 has on the liver by protecting from hepatic TAG accumulation and hepatic steotosis. Further it implies that the KO of 11β-HSD1 specifically within adipose tissue has a central role in the regulation of energy stores, as HSL and ATGL are involved in the breakdown of triglycerides (Kershaw et al 2006; Zimmermann et al 2004).In addition adipose specific KO of 11β-HSD1 confers protection by preventing an increase in expression of LXRa or LXRb which breakdown stored triglycerides, and as a consequence increase free fatty acid levels. However adipose specific knock-out of 11β-HSD1 does not recapitulate the full beneficial profile demonstrated following global KO of 11β-HSD1 and therefore this highlights the role of other tissue in this process.

This study highlights the contributory effect of excess GC on the development of MS and its related disorders, and offers a potential therapy in the form of 11β-HSD1 inhibition. There are currently a wide range of 11β-HSD1 inhibitors including BVT.2733 which lead to a decreased the level of cortisol generated in the liver and adipose tissue, and decrease tissue-specific gluconeogenesis and fatty acid metabolism (Harno et al 2010; Liu et al 2013).

There is however a need for selectivity of these inhibitors in order to remove the negative effects linked with non-selective binding to 11β-HSD2, leading to over activation of MRs, thus causing hypokalemia and hypernatremia, which in turn would lead to hypertension (Lepsin et al 2011).

The further development of specific 11β-HSD1 inhibitors offers a novel therapeutic for the treatment of GC excess within man and a potential counteractive to the negative effects on health of prescribed GCs.

6.1.1 Further studies

In order to elucidate the tissues responsible for the full list of beneficial effects seen with global KO of β-HSD1, further studies could include the generation of other tissue specific KOs of 11β-HSD1 and a repeat of the tests conducted here to produce a full document of individual tissue contributions to the beneficial phenotype conferred with global KO of 11β-HSD1. Further, additional gene expression studies could be conducted to within the adipose tissue to clarify consistency between the models.

6.1.2 Limitations of the study

I showed that there was protection in both the global and adipose-tissue specific knock-out for liver fat accumulation, steatosis, and serum NEFA level. I hypothesise that the protection from increased lipolytic gene expression in adipose tissue explains some of the protective effects observed for the adipose tissue specific knock-out, however I did not conduct similar gene expression studies within the adipose tissue (or liver for CD36) of the global knock-out which would serve to clarify if this is genuine consistency between models. In order to add further weight to my conclusions this study would prove beneficial.

6.2 Effect of 11β-hydroxysteroid dehydrogenase type 1 activity on brown adipose tissue

BAT is the main site for adaptive thermogenesis and responds to fluctuations in environmental factors, for example cold and diet, by increasing energy expenditure through thermogenesis. Following exposure to cold BAT uses lipids and carbohydrates to generate heat by using UCP1 to uncouple electron transport from oxidative phosphorylation, thus playing an active role in energy expenditure and fatty acid oxidation (Cannon. B. 2008).

With its role in energy expenditure BAT has a central role in the regulation body fat stores and may have potential in opposing obesity and diabetes within humans due to its vast metabolic capacity (Enerback. S. 2010), further evidence to support this is that mice deficient

in BAT develop obesity with insulin resistance and other metabolic abnormalities typical of obesity (Lowell. B. B. 1993; Hamann. A. 1995). However many factors have a negative impact on BAT, two main players are GCs and ageing, where increased GC exposure and increased ageing lead to a decrease in BAT function. Age is strongly negatively correlated with BAT mass, and the metabolic effect of BAT, by comparing its relationship to BMI, decreases with age (Tam et al 2012). Ageing also affects BAT tissue where there is a decrease in BAT over time and this may contribute to thermal dysregulation and energy imbalance (Heaton et al, 1972; Cypress et al 2009). The effect of GC is further increased via the 11β-HSD1 enzyme, which converts inactive cortisone to active cortisol. Increasing age has been shown to have negative effects on BAT and may contribute to thermal dysregulation and energy imbalance. Increasing age shows a strongly negative correlation with BAT mass and BAT's metabolic effect, further there is a higher incidence in the increase of BAT following cold exposure in a young rather than an aged cohort (Pfannenberg et al; Saito et al 2009).

I initially tested the effect of age on BAT and showed that with ageing there is as significant increase in BAT weight/body weigh ratio, mRNA expression of 11β-HSD1, PRDM16, PPARγ, UCP1, Cox7a1, Cox8b, NRF1 and TFAM, the protein expression of UCP1, mitochondrial copy number and the protein expression of mitochondrial electron transport chain complex 4. These increases in markers of BAT function are contradictory to previous studies that have shown ageing to have a negative effect on BAT function (Pfannenberg et al 2010). However I conclude that these increases are a consequence of animal conditions (22°C) which are lower than thermoneutral temperatures for the mouse (37°C). This has previously been highlighted by Golozoubova et al 2004. I therefore conclude that the constant thermal stress incurred from animal house conditions severely effects the

animals BAT development and function and leads to an increased capacity for non-shivering thermogenesis.

In addition I investigated the role of GC on BAT I did this by exposing BAT primary culture to GC and also by testing the effect of GKO of 11β-HSD1 on BAT genetic markers. GCs have previously been shown to play a crucial role in adipocyte biology, where they are central to the differentiation of pre-adipocytes to mature adipocytes (Hauner. H. 1987). In addition previous studies have shown that GCs suppress BAT thermogenesis, and that UCP1 expression negatively correlates with GC concentration (Soumano. K. 2000). Further to this when 11β-HSD1 is over expressed within adipose tissue there is development of full metabolic syndrome with visceral obesity, diabetes, dyslipidemia and hypertension (Masuzaki. H. 2001). Conversely, mice that are deficient in 11β-HSD1 are insulin sensitised and resist the adverse effects of a high-fat diet, showing a cardio-protective phenotype. I therefore hypothesise that a GKO of 11β-HSD1 will be beneficial to BAT function. To test this I measured the effects of KO of 11β-HSD1 on various markers of BAT function.

I found that with 11β-HSD1 GKO there is a significant increase in the mRNA expression of PGC-1α, PRDM16, PPARγ, UCP1, Cox7a1, Cox8b, CoxIV, and NRF1 a significant increase in the protein expression of UCP1, a significant increase in mitochondrial copy number and a significant increase in the protein expression of mitochondrial electron transport chain complexes 2 and 4 with GKO in aged mice. I also found a significant increase in the mRNA expression of PGC-1α, PRDM16, PPARγ, UCP1, Cox7a1, Cox8b, and NRF1 in KO with BAT primary culture following GKO. Therefore overall I found that with GKO of 11β-HSD1 there is an increase in markers of overall BAT function. This is in keeping with previous studies that have shown that GCs are detrimental to BAT function and also that GKO of 11β-HSD1 leads to beneficial effects. I hypothesise that the increase in markers of

BAT function in the GKO mice is due to the consequential decrease in local GC reactivation by the 11β-HSD1 enzyme. This therefore points to a possible therapeutic role for the targeted disruption of the activity of 11β-HSD1 in ameliorating obesity and its metabolic complications.

Further to this I analysed the effect of exogenous GC administration on BAT. This is of primary importance for the conversion of the study into humans as it attempts to mimic the effect of GC treatment within humans. I found that there was a significant increase in 11β-HSD1 expression following GC administration which would lead to an increased capacity for reactivation of GC at the tissue specific level. I further found that there was a significant decrease in mRNA expression of UCP1 in the BAT tissue of mice treated with exogenous GC. This was mirrored in the primary BAT culture experiments which showed a significant decrease in the mRNA expression of UCP1 following treatment with exogenous GC. In addition to this there was a significant decrease in the mRNA expression of Cox8b, NRF1 and TFAM in primary BAT culture following treatment with exogenous GC. Overall utilising in vivo and in vitro experiments I showed that following exposure to GC, there is a negative effect on BAT function which is further exacerbated by the significant increase in the expression of 11β-HSD1, which would lead to the potential for further increases in active GC regeneration and further to this a decrease in the expression of BAT genes. This can be inferred to show that with exposure to exogenous GCs there is a decrease in BAT function and therefore highlights GCs as a negative effector on BAT.

These studies clearly demonstrate the negative effect of GC on BAT and that a GKO of 11β-HSD1 has a positive effect on BAT. Due to BAT being linked to a decrease in obesity it would be beneficial to have increased concentrations of BAT within the body. Therefore a potential 11β-HSD1 targeted inhibitor (discussed above) which would lead to a decrease in

the local reactivation of GC could offer a therapeutic. Having a potential target for the prevention of obesity is of huge current importance due to the global rise in obesity and its related complications.

6.2.1 Further studies

To further investigate this area I could perform tests on mitochondrial function. This could be performed using Seahorse technology where the oxygen consumption rate is measured. This would be beneficial as I have currently demonstrated that there is an increase in mitochondrial copy number and mRNA expression of gene, however have not tested how well these mitochondria are functioning. By performing mitochondrial function tests it would allow us to say whether the interventions are truly of benefit to the adipose tissue function. In addition to this I could perform lipid quantification studies on the adipose tissue tested to see if the structure/content of the tissue has been improved. In addition to this I could expand my cold stress studies on the cell culture models by performing experiments at an array of differing temperatures and for differing durations in order to ascertain the optimum cold exposure for BAT function. In addition to this I could perform histological studies to see if the structure of the BAT is altered by the changing temperature. This could allow us to see if there is an increase in mitochondrial number and mitochondrial size. Further I could investigate if long term exposure to cold prevent the age associated negative effects on BAT structure.

6.2.2 Limitations of the study

The mice used within this study were all housed within standard animal house conditions which are below thermoneutral conditions. This places a background cold stress on the mice which may impact on the results. In order to combat these experiments could be repeated at thermoneutral temperatures. In addition to this a large chunk of the data is centred on mRNA

measurements, which do not necessary mirror protein expression or activity. Further studies could be conducted to address this as discussed earlier with Seahorse technology.

6.3 Effect of 11β-hydroxysteroid dehydrogenase type 1 activity on white adipose tissue

11β-HSD1 is responsible for the intracellular regeneration of active GC, and up regulation of this activity within adipose appears to be a key contributor to central obesity and metabolic dysfunction in both men and women (Morton. N. M. 2008; Paulmyer-Lacroix. O. 2002; Rask. E. 2002). This is further emphasised by studies on mice which are deficient in 11β-HSD1 where these mice are insulin sensitised and resist the adverse metabolic effects of a high-fat diet, showing a cardio-protective phenotype (Wamil. M. 2011; Ataab. C. A. 2010) and diabetes through improved insulin sensitivity (Morton. N. M. 2004).

Studies have demonstrated that white adipocytes contain two types of cells; pure white adipocytes and brite cells. Brite cells have a more brown-like phenotype and have the ability to express UCP1, however otherwise do not posses the full molecular characteristics of brown adipocytes (Petrovic. N. 2009). These brite cells are induced following a given stimulus for example cold exposure, or β-adrenergic receptor agonists (Petrovic. N. 2009).

The white/brown plasticity of adipose tissues might have considerable medical implications, since the brown-like phenotype seems to correlate with a reduced propensity for developing obesity and diabetes in mice (Bachman. E. S. 2002). Further an increase in expression of PGC-1α within WAT may lead to the induction of the expression of UCP1 and the oxidation of fatty acids, which together result in a reduction in adipose mass (Tiraby. C. 2003). Further Tsukiyama-Kohora et al 2001 have shown that there is an increase in brite cells within WAT in GKO strains that show resistance to diet induced obesity and improved glucose metabolism.

I showed that with age there is a significant increase in the mRNA expression of UCP1, I hypothesised that this increase in UCP1 with age is a consequence of the non-thermoneutral temperatures in which the mice were housed. This would lead to activation of non-shivering thermogenesis (Himms-Hagen. J. 1984) and a consequential increase in UCP1 quantity as previously demonstrated by Young et al. This increase in non-shivering thermogenesis seen with age may be of benefit to the mouse due to the mobilisation of fatty acids from WAT which may lead to weight loss and a healthier phenotype (Deiulis. J. A. 2010). I hypothesise that this increase in UCP1 represents and increase in the quantity of brite cells present.

This increase in the expression of brite cells seen following cold exposure may have considerable medical implications as the brown-like phenotype demonstrated in my studies correlates with a reduced propensity for the development of obesity and diabetes in mice (Murano et al 2009; Bachman et al 2002). Further Tsukiyama-Kohora et al 2001 have shown that with an increase in brite cells within the WAT there is resistance to diet induced obesity and improved glucose metabolism.

Another effector on adipose are GCs. GCs can be reactivated at the pre-receptor level by the 11β-HSD1 enzyme, leading to an increase in the local availability of active GC. (Seckl et al 2001) I tested the effects of exogenous GC administration to primary cultures of WAT and also the effect of GKO of 11β-HSD1 on WAT primary culture and WAT.

I found a significant decrease in the mRNA expression of PPARγ, PRDM16, UCP1, Cox7a1 and Cox8b within the subcutaneous and gonadal primary culture. These results clearly demonstrate the negative effect GCs have on WAT and how they decrease the amount of browning within the WAT and lead to a stronger WAT phenotype. This has the potential to

be detrimental to the mice as it leads to a decrease in the degree of browning, and therefore changes the WAT from an energy expending to an energy storing depot.

To try to counteract the negative effects of GC on WAT I have tested the effect of GKO of 11β-HSD1 in both WAT and subcutaneous and gonadal primary adipose tissue culture. Within the WAT I found a significant increase in the expression of PRDM16, PPARγ, Cox7a1 and Cox8b with GKO. In addition I found a significant increase in the mRNA expression of PGC-1α PPARγ, and UCP1 within the KOs in both subcutaneous and gonadal primary cultures. This increase in gene expression with GKO suggests that there is an increase in browning within the GKO cohort. Previous studies have shown the benefit of 11β-HSD1 GKO on WAT where they have resistance to diet induced visceral obesity and diabetes through improved liver and adipose function. In addition over expression of 11β-HSD1 within adipose tissue has been shown to lead to obesity, dyslipidemia and insulin resistance (Masuzaki et al 2001). In addition the brown like phenotype I have demonstrated with GKO of 11β-HSD1 has been shown to correlate with reduced propensity for developing obesity and diabetes in mice (Murano et al 2009; Bachman et al 2002).

Overall I showed that an excess of GC has negative effects on WAT and that GKO of 11β-HSD1 and the consequential decrease in local reactivation of GC protects against these negative effects of excess GC. This therefore points the possible role of 11β-HSD1 in the treatment and prevention of disorders related to GC excess.

These studies mirror those seen previously clearly demonstrating the negative effect of GC and that a GKO of 11β-HSD1 has a positive effect. Therefore a potential 11β-HSD1 targeted inhibitor (discussed above) which would lead to a decrease in the local reactivation of GC could offer a therapeutic. Further to this if the effects seen with the decrease in temperatures could be replicated within humans by potentially stimulating the same pathways

artificially this could be of huge benefit to treat obesity and its associated complications by increasing brite cells and as seen above, BAT cells.

6.3.1 Further studies

I could expand this work by testing mitochondrial function. This could be performed using Seahorse technology where the oxygen consumption rate is measured. This would be beneficial as it would allow us to measure how well the adipose tissue is functioning. By performing mitochondrial function tests it would allow us to say whether the interventions are truly of benefit to the adipose tissue function. In addition to this I could perform lipid quantification studies on the adipose tissue tested to see if the structure/content of the tissue has been improved. In addition to this I could expand my cold stress studies on the cell culture models by performing experiments at an array of differing temperatures and for differing durations in order to ascertain the optimum cold exposure for WAT. In addition to this I could perform histological studies to see if the structure of the WAT is altered by the changing temperature. In addition I could expand my study to differing WAT depots to investigate if there are differing levels of effects in different WAT depots.

Further studies could also include investigating the pathways which are stimulated following the cold exposure and investigate whether these pathways could be stimulated artificially.

6.3.2 Limitations of study

As highlighted within the discussion there is an inference that 11β-HSD1 activity correlates with mRNA expression, and therefore no change in mRNA expression is assumed to reflect no change in 11β-HSD1. This assumption may be incorrect as often there is no true correlation between mRNA expression and activity. To improve this study and add further weight to the results further studies measuring protein concentration and 11β-HSD1 activity

would be beneficial. Further the mice studied were all housed within standard animal house conditions which are below thermoneutral conditions. This induces a cold stress on the mice which may affect the results. To assess the affect of this the experiments could be repeated at thermoneutral temperatures.

A further limitation to this study is that temperature changes in a cell culture model were used to infer the in vivo effects of a decrease in temperature. Within an in vivo model the cold temperature would not have direct access to the adipose tissue and therefore potentially would not be cooled to the extreme as what is seen in the study. This would lead to potentially exaggerated results in our model as to what would be seen in vivo. This limitation leads to potential further studies examining the effects of a decrease in temperature on an in vivo model in an attempt to reproduce the effects demonstrated in this study.

7. Chapter 7 - References

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