THE UNIVERSITY *of* EDINBURGH

The Role of 5α-Reductase Type 1 in Modifying Anxiety, Appetite and the HPA Axis

Emma M Di Rollo

Doctor of Philosophy (Ph.D.)

The University of Edinburgh

2013

Declaration

I declare that this thesis was written by me and that the data presented within it is a result of my own work performed at The University of Edinburgh, with the exception of the procedures listed below:

- Adrenalectomy and surgery for mini-pump implantation was performed by Dr Dawn Livingstone of the Centre for Cardiovascular Science, Queen's Medical Research Institute, The University of Edinburgh/BHF Centre for Cardiovascular Science.

- LC-MS/MS and data analysis were performed by staff of the Wellcome Trust Mass Spectroscopy Core, The University of Edinburgh/BHF Centre for Cardiovascular Science.

- Quantitative PCR for the expression of hypothalamic neuropeptides involved in feeding regulation in WT and 5αR1-KO mice (females only) was performed by senior laboratory technician Karen French.

- Some investigations (where specified) were carried out using archived tissues obtained from previous *in vivo* investigations performed by Dr Dawn Livingstone.

I also declare that this work has not been previously submitted for any other degree or qualification.

Emma Di Rollo

Abstract

Glucocorticoid excess is associated with adverse effects on a number of physiological parameters, leading to obesity, dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis and behavioural changes such as anxiety and impaired learning and memory. Circulating and local tissue glucocorticoid levels are tightly controlled by the HPA axis but an additional level of control exists in tissues such as brain, liver and adipose tissue. In these structures, enzymes including 5α-reductase 1 (5αR1), catalyse the conversion of corticosterone to A-ring reduced metabolites, which have a different spectrum of activities. This thesis investigates the role of 5αR1 in regulating central glucocorticoid actions which control HPA axis function and behaviour in a mouse model with genetic disruption of 5αR1 (5αR1-KO). Preliminary data showed 5αR1-KO mice were susceptible to developing insulin resistance and obesity and had reduced HPA axis responses to acute stress. Additionally, male 5αR1-KO mice were more prone to obesity than wild-type (WT) when fed a high-fat diet whilst female 5αR1-KO mice gained more weight than WT even on a normal chow diet. Intriguingly, female 5αR1-KO mice subjected to social isolation stress lost this extra weight and became comparable to WT controls. This study tested the hypothesis that 5αR1-KO mice are less able to inactivate glucocorticoids in the periphery and within tissues, resulting in a predisposition to metabolic disturbances and behavioural alterations. These were hypothesised to include hyperphagia, weight gain, impaired stress responses, anxiety (exacerbated by environmental stress) and cognitive deficits. It was also thought that many of these features would be more pronounced in female vs. male mice. The main aims of this study were to determine if 5αR1-KO induced weight gain and if this was correlated to altered gene expression of key hypothalamic neuropeptides which regulate appetite, to determine the central mechanisms which underpin attenuated HPA axis responses to acute stress and to determine whether behaviours such as anxiety and learning and memory ability are affected by global 5αR1 loss.

It was hypothesised that female 5αR1-KO mice have increased appetite and reduced locomotor activity compared with WT and male 5αR1-KOs. However, male 5αR1-KO mice (on a mixed genetic background, C57Bl/6j/SvEv/129) were hyperphagic on

a normal chow diet but did not gain extra weight, while female 5αR1-KO mice gained more weight vs. WT despite hypophagia. Free ambulatory activity was unaffected by genotype in either sex. Male 5αR1-KO mice appeared less anxious but responses of female 5αR1-KO mice in tests of anxiety did not differ from WT controls. Mice lacking 5αR1 generally had a poorer metabolic profile with impaired glucose tolerance and hyperinsulinaemia; with hepatic steatosis evident in female mice. There was evidence of compensatory changes in hypothalamic orexigenic and anorexigenic peptides. Phenotypes were sexually dimorphic such that male mice had a poorer metabolic profile vs. females, which was particularly marked in male 5αR1-KO animals.

5αR1-KO mice were previously shown to have attenuated HPA axis responses to acute stress and it was hypothesised that disruption of 5αR1 would result in altered expression of genes related HPA axis regulation with a view to increased negative feedback. Here, male and female 5αR1-KO mice demonstrated altered corticosteroid receptor expression within the hippocampus and the pituitary, two key structures in the HPA cascade. *In situ* hybridisation showed reduced mRNA for *MR* in the hippocampus and for *Crh* in the hypothalamus of 5αR1-KO mice. These modifications along with decreased *Crhr-1* mRNA (CRH's main receptor) may be due to a lack of corticosterone metabolism within the brain resulting in enhanced negative feedback and reduced HPA axial drive.

In order to study behaviour in detail and also to test whether potential central glucocorticoid excess may predispose to cognitive decline with ageing, a separate cohort of female 5αR1-KO backcrossed onto a uniform C57Bl/6j background was studied both when young (6 months) and when aged (14-15 months). Additionally, mice were housed in either groups or singly (social isolation) to investigate the potentially additive effects of environmental stress. It was hypothesised that local glucocorticoid increases in the brains of 5αR1-KO mice would be associated with anxiety and cognitive deficiencies and that these phenotypes would be exaggerated by the stress of social isolation as well as ageing. Behavioural differences were not observed at 6 months of age. However aged, 5αR1-KO mice housed singly showed

increased anxiety and had higher plasma corticosterone levels than group-housed mice. Moreover, aged mice lacking 5αR1 performed less well than WT in tests of memory and had a marginally greater cognitive decline when learning ability at 14-15 months old was compared to that of the same animals tested at 6 months old. Overall, mice with global 5αR1 loss appeared susceptible to anxiety as well as some degree of age-associated cognitive impairment, but only when subjected to social isolation stress which is a known chronic stressor.

The final set of experiments aimed to determine the effect of mouse strain on 5αR1-KO phenotypes. It was hypothesised that glucocorticoid clearance would be attenuated to a lesser degree in 5αR1-KO mice bred onto a congenic C57Bl/6j strain compared to those of the mixed strain and that this would manifest as less disruption of metabolism and less suppression of HPA axis stress responses. Although social isolation again induced weight-loss in female mice and more so in 5αR1-KO animals, mice on the C57Bl/6j background strain did not show dampened HPA axis responses to acute stress as seen previously. It was subsequently shown in adrenalectomised mice that animals bred on the C57Bl/6j strain cleared active corticosterone from plasma and liver faster than mixed strain mice. This may have rendered mixed strain 5αR1-KO mice more susceptible to excessive corticosterone levels producing a more exaggerated phenotype in this group.

In conclusion, these data suggest a role for the enzyme 5αR1 in modifying glucocorticoid concentrations in the brain and liver, influencing not only metabolic and peripheral effects such as weight gain and insulin resistance, but also in modifying cognition, appetite stimulation and affective behaviours. It has been highlighted that outside factors such as housing and age can modify these phenotypes and are important considerations for future studies. This study has also highlighted the importance of choosing an appropriate genetic background for genetically modified animals since phenotypes can be enhanced or attenuated depending on strain. Finally, 5αR inhibitors are used to treat disorders such as benign prostatic hyperplasia in men, and it is important to consider that these drugs may have a wide array of associated side effects both systemically and in the central nervous system.

Dedication

I would like to dedicate this thesis to my wonderful husband, Fraser. Your continuous love and support has been my anchor throughout my Ph.D. and for that I am truly thankful.

x

Acknowledgements

Firstly I would like to sincerely thank my primary Ph.D. supervisor Dr Ruth Andrew for guiding me through this undertaking. She has not only broadened my knowledge, directed me through experiments (at times even scrubbing up and mucking in with the mice!) and helped me get back on track when I have gotten lost, but she has also been an amazing emotional support and a friend when required and for these reasons I am extremely grateful, thank you so much. I would also like to thank my second supervisor Dr Dawn Livingstone for her continuous guidance and support through the years; she too has been instrumental in me completing this thesis and I am grateful to her for all she's done for me.

Next I would like to express my gratitude to the numerous people within The QMRI who have helped and advised me at some point during my Ph.D., particularly past and present members of the 'A-team', Kerry McInnes, Mark Nixon, Carolynn Cairns and Karen French, without whom I would have been completely lost and I'd like to take this opportunity to apologise for my never ending questions! Other amazingly kind and knowledgeable people who have helped me include Val Kelly, June Noble, Joyce Yau and Lynne Ramage amongst many others. Importantly, I would like to thank all the staff in the BRF for their help and for often going out of their way to make my job a little easier wherever possible. In particular I'm very grateful to Jon, Duncan and Keith.

I would also like to thank some more of the amazing people I have met at the QMRI during my Ph.D., some of whom I can honestly say are now life-long friends who have been there through the good, the bad (and the ugly!). I can't possibly name everyone but I want to say a special thank you to Ewa Rog-Zielinska whose desk was seriously a health hazard but whom I love anyway, to James Catterson for literally his life-long friendship and for always having a YouTube video or "giff" to perfectly compliment any occasion and also Rachel Verdon, Lizzie Skinner, Chris White, Nicole Sime, Evatong Zou, Kat Wilson, Michal Janiszewski, Kirsten Rose and Ania Stefanska: what can I say, you are all awesome!

Finally, and most importantly I would like to thank my family; Mum and Dad, Nicola, Carla, Domenic, Sean and Jonny but also my in-laws Linda, Joe and Ashley and all my beautiful nieces and nephews for making me smile widely and often. I come from the most amazing, supportive family for whom I am truly thankful, every single day. I couldn't possibly ask for a more loving group of people and for that I am so grateful. I'd especially like to thank my mum for pushing me to study so hard all throughout my life; she played a big part in getting me to where I am today. One of the most important things she has done though, is my washing during the "writing up" phase whilst my dad cooked dinner for me every night which I mostly ate glued to my computer screen! And last but by no means least, my husband Fraser. I love you and thank you so much for helping me to stay focused and positive, for being there for me when it was tough and for making me laugh when I felt like crying. Completing my Ph.D. and writing my thesis has been the most challenging and difficult thing I have ever done and were it not for the love and support of my family I could never have made it this far, so thank you to you all!

Contents

List of Figures

List of Tables

Abbreviations

2,2,4,6,6,17A,21,21-D_8B	2,2,4,6,6,17A,21,21 – $^{8}[H]_2$ corticosterone
3αHSD	3α-hydroxysteroid dehydrogenase
3βHSD	3β-hydroxysteroid dehydrogenase
5αDHB	5α-dihydrocorticosterone
5αDHP	5α-dihydroprogesterone
5αDHT	5α-dihydrotestosterone
5αR1/2/3	5α-reductase types 1, 2 and 3
5αR1-KO	5α-reductase type 1 knock-out
5αR-Is	5α-reductase inhibitors
5αTHB	5α-tetrahydrocorticosterone
5βR	5β-reductase
11βHSD 1/2	11β-hydroxysteroid dehydrogenase types 1 and 2
11βHSD1-KO	11β-hydroxysteroid dehydrogenase type 1 knock-out
ACTB	β-actin
ACTH	Adrenocorticotropic hormone
ADX	Adrenalectomy
AF-1/2	Activation function 1/2
AgRP	Agouti-related peptide
AKR	Aldo-keto reductase
ALLO	Allopregnanolone
α-MSH	α-melanocyte stimulating hormone
ANOVA	Analysis of variance
AVP	Arginine vasopressin
B	Corticosterone
BBB	Blood-brain barrier
bp	Base pair
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
C	Control diet
CA1-4	Cornu ammonis regions 1-4 of the hippocampus

CART1/2	Cocaine- and amphetamine-regulated transcript variants 1 and 2
CBG	Cortisol-binding globulin
CBP	CREB-binding protein
cDNA	Complimentary deoxyribonucleic acid
CNS	Central nervous system
CORT	Corticosterone
CRH	Corticotrophin-releasing hormone
CRHR-1/2	Corticotrophin-releasing hormone receptor types 1 & 2
CVD	Cardiovascular disease
DBD	Deoxyribonucleic acid-binding domain
DEPC	Diethylpyrocarbonate
DG	Dentate gyrus
DHC	Dihydrocorticosterone
DHT	Dihydrotestosterone
DIO	Diet-induced obesity
DNA	Deoxyribonucleic acid
DPX	Dibutyl phthalate xylene
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EPM	Elevated plus maze
FBGRKO	Forebrain-specific GR knock-out
G6P	Glucose-6-phosphatase
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid receptor type A
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
GDX	Gonadectomy
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element

GR^{ov}	Glucocorticoid over-expressor
GTT	Glucose tolerance test
HET	Heterozygous
HF	High-fat diet
HPA axis	Hypothalamic-pituitary-adrenal axis
HPG axis	Hypothalamic-pituitary-gonadal axis
HPLC	High-performance liquid chromatography
Hsp	Heat shock protein
HRP	Horse-radish peroxidase
Hr	Hour
i.c.v	Intracerebroventricular
IL-10	Interleukin-10
i.p.	Intraperitoneal
IR	Insulin receptor
ITI	Inter-trial interval
ITT	Insulin tolerance test
K_d	Dissociation constant
K_m	Michaelis-Menten constant
KO	Knock-out
LBD	Ligand-binding domain
LTP	Long-term potentiation
MC3/4R	Melanocortin receptor types 3 and 4
MCH	Melanin-concentrating hormone
MDD	Major depressive disorder
mGR	Membrane-bound glucocorticoid receptor
Min	Minute
MR	Mineralocorticoid receptor
$MR^{CamKCre}$	Forebrain-specific mineralocorticoid receptor knock-out
MR-Tg	Mineralocorticoid receptor over-expressor
mRNA	Messenger ribonucleic acid
MW	Molecular weight

NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate-reduced
NEFA	Non-esterified fatty acid
nGREs	Negative glucocorticoid response elements
NPY	Neuropeptide Y
Ob	Obese gene
OFT	Open field test
P450scc	P450 cholesterol side-chain cleavage enzyme
PBS	Phosphate buffered saline
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
POMC	Pro-opiomelanocortin
PPARγ	Peroxisome proliferator-activated receptor γ
PPIA	Cyclophilin A
PR	Progesterone receptor
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
PXR	Pregnane-X receptor
qPCR	Quantitative polymerase chain reaction
RIA	Radioimmunoassay
RIN	Ribonucleic acid integrity number
RMT	Room temperature
RN18S	18s ribosomal ribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RSD	Relative standard deviation
RT	Reverse transcription
s.c.	Subcutaneous
SDR	Steroid displacement reagent
Sec	Second
SEM	Standard error of the mean

SNPs	Single nucleotide polymorphisms
SPA	Scintillation proximity assay
SSC	Saline sodium citrate
STAT3	Signal transducer and activator of transcription 3
TAT	Tyrosine aminotransferase
TBE	Tris/borate/ethylene diamine tetra-acetic acid
TE	Tris/ethylene diamine tetra-acetic acid
TBP	TATA box-binding protein
TBS	Tris-buffered saline
TMB	3,3’,5,5’-tetramethylbenzidine
Tris	Trisma base
TF	Transcription factor
tRNA	Transfer ribonucleic acid
Wks	Weeks
WT	Wild-type

Publications

Papers

Livingstone, D.E., **Di Rollo, E.M**., Yang, C., Codrington, L.E., Mathews, J.A., Kara, M., Hughes, K.A., Kenyon, C.J., Walker, B.R., Andrew, R. (2014). "Relative adrenal insufficiency in mice deficient in 5α-reductase 1." *J Endocrinol* 222(2): 257-266.

Livingstone, D. E., Barat, P., **Di Rollo, E.M**., Rees, G., Weldin, B., Rog-Zielinska, E. A., Macfarlane, D., Walker, B. R., Andrew, R. (2014). "5α-Reductase type 1 deficiency or inhibition predisposes to insulin resistance, hepatic steatosis and liver fibrosis in rodents". *Diabetes*. [Epub ahead of print]

Morton, N.M., Nelson, Y.B., Michailidou, Z., **Di Rollo, E.M.,** Ramage, L., *et al*. (2011) A stratified transcriptomics analysis of polygenic fat and lean mouse adipose tissues identifies novel candidate obesity genes. *PLoS ONE,* 6 (9):e23944. Epub 2011 Sep 7.

Abstracts

Di Rollo, E.M., Livingstone, D.E., Walker, B.R., Andrew, R. (2012) Ageing and social isolation increase susceptibility to anxiety in mice lacking 5α-reductase type 1. *Endocr Rev*, 33 (03_MeetingAbstracts): SAT-523.

Di Rollo, E.M., Livingstone, D.E., Walker, B.R., Andrew, R. (2012) Ageing and social isolation increase susceptibility to anxiety in mice lacking 5α-reductase type 1. *Endocrine Abstracts*, 28, P305.

Di Rollo, E.M., Livingstone, D.E., Walker, B.R., Andrew, R. (2010) Altered signaling via corticotrophin releasing factor may underpin attenuated responses to acute stress in mice lacking 5α-reductase1. Proceedings of the Scottish Society for Experimental Medicine, Poster presentation (P19).

Livingstone, D.E., **Di Rollo, E.M**., Kenyon, C.J., Yang, C.A., Kara, M., Codrington, L.E., Matthews, J.A., Yau, J.L., Walker, B.R., Andrew, R. (2010). Targeted disruption of 5α-reductase 1 in mice attenuates hypothalamic-pituitary-adrenal axis response to stress. International Congress on Hormonal Steroids and Hormones and Cancer, Poster presentation (P33).

Livingstone D.E., Yang, C.A., **Di Rollo, E.M**., Mathews, J.M., Codrington, L., Kara, M., Kenyon, C.J., Walker, B.R., Andrew, R. (2010) Transgenic disruption of 5α-reductase 1 in mice results in a blunted stress response. *Endocrine Abstracts*, 21, P240.

Chapter 1: Introduction

Obesity (and associated metabolic disease) is rapidly becoming a global pandemic. In 2013, The World Health Organisation reported that the incidence of obesity worldwide has nearly doubled since 1980 and records from 2008 show over 1.4 billion adults aged 20 and over were overweight; of these approximately 500 million were medically obese, representing 7% of the global population (The World Health Organisation 2013). Amongst other obesity-related diseases, cardiovascular disease (CVD) is one of the most common, killing an estimated 17 million people each year (Mackay *et al.* 2004), making it the single leading cause of death today, in spite of improved cardiological interventions. Mathers and Loncar predict that by 2030, almost 23.3 million people will die from CVD annually, mainly from heart disease and stroke (Mathers *et al.* 2006). Therefore, understanding the mechanisms which underlie the progression of CVD, and associated disorders, is crucial in minimising the incidence of obesity-related diseases. Moreover, investigating the role of modifiable factors such as steroid hormones is essential in any attempt to curb the rapid progression of metabolic and cardiovascular dysfunction. Here we focus on the role of glucocorticoids in both health and disease.

1.1 Glucocorticoids

The term "glucocorticoids" originates from their role in glucose metabolism, their synthesis in the adrenal cortex and their steroidal structure (**GLUCO**se, **CORT**ex and ster**OID**). Glucocorticoids (cortisol in humans and corticosterone in rodents) are lipophilic ketosteroid hormones with a $\Delta^{4,5}$,3-oxo structure (Figure 1.1). They have the capacity to modulate the expression of approximately 10% of our genes and can act in almost every cell type in the body (Buckingham 2006). Glucocorticoids can therefore influence the function of many physiological processes and organ systems. Glucocorticoids act to maintain or restore physiological homeostasis both basally and following an emotional or physical stressor; rendering them "stress-protective". They do this by mobilising fuel via the breakdown of carbohydrate and protein from storage and also exert a spectrum of effects on lipid breakdown and deposition. Whilst glucocorticoids are well known to influence energy metabolism, they also

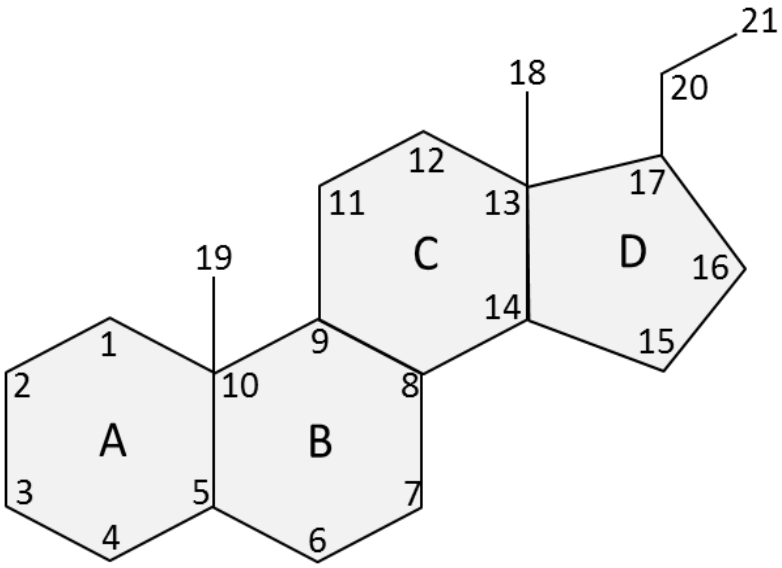

Figure 1.1 The generic keto steroid cyclopentanoperhydrophenanthrene structure
All ketosteroids comprise this basic 3 cyclohexane (A-C), 1 cyclopentane (D) ring structure made up of 21 carbon atoms (numbered 1-21). The specificity of individual steroids is defined by the addition of various side-chains. For example, both corticosterone and cortisol have carboxyl groups at C3 and C20 and hydroxyl groups at C11 and C21 which defines them as glucocorticoids (see Figure 1.6 for illustration).

have highly integrated effects on behaviour and play a role in promoting the resolution of infection and inflammation. This last property has led to their use clinically as anti-inflammatory agents. However, though many glucocorticoid-mediated actions are beneficial and necessary, some can be detrimental, especially when exposure is prolonged or excessive. For this reason, circulating glucocorticoid levels must be maintained within a normal physiological window, achieved mainly through the hypothalamic-pituitary-adrenal axis (discussed later).

1.1.1 Glucocorticoid biosynthesis

Glucocorticoids are synthesised in the zona fasciculata of the adrenal gland from the precursor cholesterol. This synthetic pathway, which also leads to the *de novo* synthesis of other steroids, is catalysed by cytochrome P450 oxidative enzymes in conjunction with other oxidase and reductase, steroidogenic enzymes (Figure 1.2).

1.1.2 Corticosteroid receptors

Glucocorticoids can mediate their effects through different mechanisms of action which can be either rapid (non-genomic in minutes), intermediate (transcriptional effects in hours) or long (nuclear protein-protein interactions which can last days; Keller-Wood *et al.* 1984). The cellular and physiological actions of glucocorticoids are mainly mediated through interactions with two nuclear hormone receptors each with differing affinities, the mineralocorticoid receptor (MR; the type I receptor; *Nr3c2*) and the glucocorticoid receptor (GR; the type II receptor, *Nr3c2*;(Reul *et al.* 1985; Lu *et al.* 2006). GR is widely distributed in the mammalian body and is present in most cell types. Compatible with its ubiquitous expression, GR is involved in many physiological processes from energy mobilisation and stress adaptations to modulation of brain and immune functions (Miller *et al.* 1995). Two GR isoforms (α and β), arise from alternative splicing of the pre-mRNA. GRα is the functional isoform and is expressed in abundance throughout the body. On the other hand, GRβ does not bind glucocorticoids and acts instead as a dominant-negative inhibitor of glucocorticoid action by binding to DNA and interfering with GRα-DNA associations (Hollenberg *et al.* 1985; Oakley *et al.* 1996).

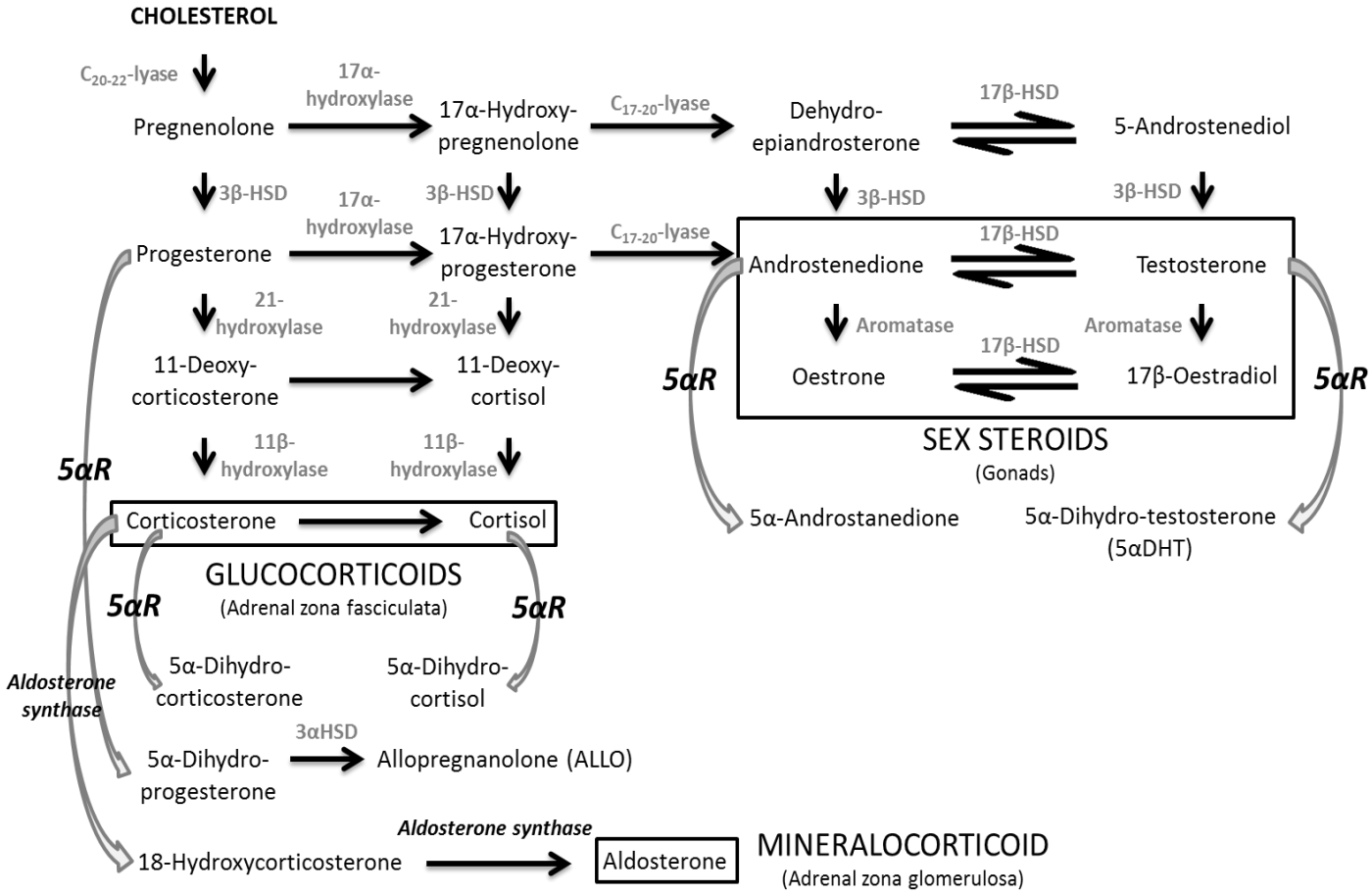

Figure 1.2 The biosynthesis and metabolism of glucocorticoids and related steroids

Pathways involved in production of glucocorticoids in the adrenal cortex and sex steroids in the gonads. 5αR = 5-alpha reductase and HSD = hydroxysteroid dehydrogenase.

1.1.2.1 Tissue distribution and specificity of corticosteroid receptors

Whilst the physiological effects of glucocorticoids are primarily mediated via GR, MR is the higher affinity receptor for endogenous glucocorticoids (approximately 10-fold higher affinity than GR for corticosterone and cortisol; Seckl 1997) and has almost equal affinity for corticosterone and the mineralocorticoid aldosterone (approximate K_d = 0.5 - 2 nM; Reul *et al.* 1985; Arriza *et al.* 1987). *MR* expression is seen mostly in areas involved with Na^+/K^+ balance, such as the distal tubule epithelial cells of the kidney, exocrine glands and the colon (Buckingham 2006). In these tissues where MR activation by mineralocorticoids (as opposed to glucocorticoids) is important for function, the glucocorticoid-metabolising enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) is highly expressed allowing mineralocorticoid-MR activation.

By contrast to MR, GR is widely distributed throughout the body but has a much lower affinity for endogenous glucocorticoids (approximate K_d = 2.5-5 nM for corticosterone; Reul *et al.* 1985), thus receptor activation is dependent on the glucocorticoid load within the tissue. At the circadian nadir the tissue levels of endogenous glucocorticoids are within the affinity range of MR (0.5 - 1 nM) suggesting MR mediates glucocorticoid effects at very low concentrations. By contrast, during a stressful event or at the peak of the circadian rhythm, when free glucocorticoid levels can exceed 300 nM, MR becomes saturated allowing GR occupation and mediation of biological effects (Buckingham 2006).

Both corticosteroid receptor types are expressed in the brain (Reul *et al.* 1985; 1986; de Kloet 1991). *GR* is abundantly expressed throughout the central nervous system (CNS) in most neurons and glia (De Vellis *et al.* 1974; Sousa *et al.* 1989; Ahima *et al.* 1990); indeed it is thought the receptor is present in every cell type in the mammalian CNS (Seckl 1997). However, highest *GR* expression is seen in the paraventricular nucleus (PVN) of the hypothalamus and the hippocampus (Reul *et al.* 1985). MR has a more restricted distribution in the CNS than GR with high expression confined to the hippocampus, lateral septum and some brain stem nuclei (Reul *et al.* 1985; 1986; Arriza *et al.* 1988; Luttge *et al.* 1989; Jacobson *et al.* 1991).

More modest *MR* expression is also seen in other neuroendocrine brain regions including the PVN where *MR* is expressed at very low levels (Swanson *et al.* 1989; Seckl *et al.* 1993). However, there is evidence to suggest co-localisation of aldosterone and 11βHSD2 in neurons in the CNS, such as those in the nucleus of the solitary tract (Geerling *et al.* 2009), allowing access to the receptor. There remain many questions though, regarding the direct effects of aldosterone-induced MR activation in the brain due to its poor penetration of the blood brain barrier (BBB) and glucocorticoid-dominant occupation of MR (glucocorticoid concentrations in the brain are 1000-fold higher than aldosterone concentrations; Geerling *et al.* 2009).

1.1.3 Glucocorticoid activation of corticosteroid receptors

Intracellular corticosteroid receptors (GR and MR) belong to the nuclear receptor superfamily of ligand-activated transcription factors, which exert their effects by attaching to specific DNA sequences (glucocorticoid response elements; GREs), classically as homodimers (Glass 1994) and altering the transcription of a plethora of target genes. All nuclear receptors have the following structure (see Figure 1.3 for more information): the N-terminal (A/B) domain, the DNA-binding (C) domain (DBD), the hinge region (D-domain) and the ligand-binding (E/F) domain (LBD; Buckingham 2006; Beck *et al.* 2009). The N-terminal domain shows the most variability within the family of nuclear steroid receptors. It contains the transactivation domain, activation function-1 (AF-1), which associates with transcription factors such as CREB-binding protein (CBP) and TATA box-binding protein (TBP) and is crucial for GRs transcriptional activity (Kumar *et al.* 2005). The central DBD targets the receptor towards GREs within the DNA sequence and has two highly conserved zinc finger structures which are unique to nuclear receptors and crucial for dimerisation (Berg 1989; Klug *et al.* 1995). The hinge region facilitates translocation to the nucleus upon ligand binding and receptor dimerisation whilst the C-terminal LBD containing a second transactivation domain (AF-2), comprises of 11 helical structures which form the ligand-binding pocket ensuring ligand recognition and specificity (Bledsoe *et al.* 2002; Buckingham 2006).

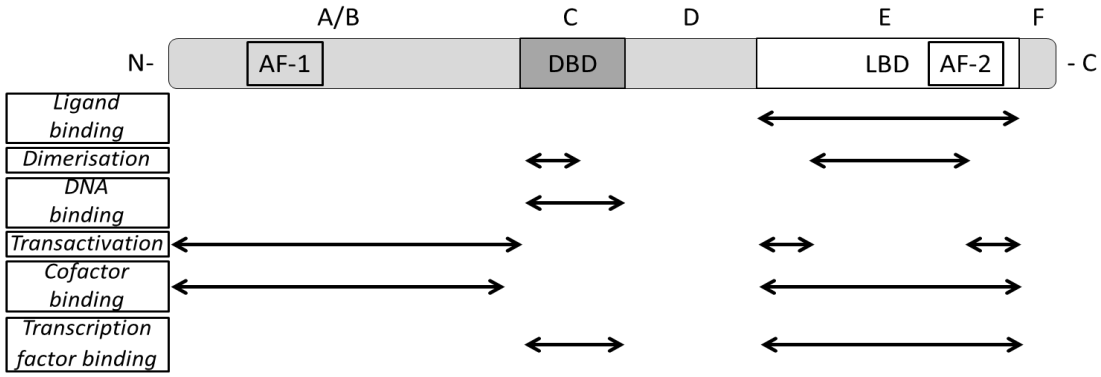

Figure 1.3 Structure of the glucocorticoid receptor

This schematic diagram highlights the N-terminal activation function 1 (AF-1), the DNA-binding domain (DBD) and the ligand-binding domain (LBD). The specific domains necessary for various receptor functions are also shown.

Unliganded, GR is held in the cytoplasm adhered to a protein complex consisting of mainly heat-shock proteins (Hsp) including Hsp70 and Hsp90 as well as other Hsp90-binding proteins which bind to the LBD and work to stabilise the complex inducing a conformation favourable to ligand binding (Pratt *et al.* 2006). Upon steroid binding to the LBD, the Hsp chaperone complex dissociates inducing an allosteric change which shifts the receptor into a transcriptionally active state. Activated corticosteroid receptors then undergo phosphorylation and are generally believed to act as ligand-induced homodimers which translocate from the cytoplasm to the nucleus to bring about transcriptional changes. GR activation results in waves of gene regulation which either positively (transactivation) or negatively (transrepression) affect the transcription of glucocorticoid responsive genes (De Bosscher *et al.* 2003; Newton *et al.* 2007; Datson *et al.* 2008; Beck *et al.* 2009).

1.1.3.1 Genomic effects of glucocorticoids

1.1.3.1.1 Glucocorticoid-induced transactivation

It is accepted that the majority of endocrine and metabolic actions of glucocorticoids are mediated via the direct interactions of corticosteroid receptors with distinct DNA regions which influence transcription (Schäcke *et al.* 2004; Buckingham 2006). Although the DBDs of MR and GR are almost identical, it is noteworthy that there is less than 30% overlap in the genes whose transcription is activated or repressed subsequent to receptor activation (Datson *et al.* 2008). This is somewhat unsurprising since the two receptors show distinct tissue localisation, especially in the brain as highlighted previously (1.1.2.1).

Following ligand binding, dissociation of chaperone proteins and translocation into the nucleus, the activated GR homodimer binds directly to consensus sequences (GREs) in the promoter region of glucocorticoid-sensitive genes to induce transcription (transactivation) of genes such as tyrosine aminotransferase (*Tat*). *Tat* encodes an hepatic enzyme of gluconeogenesis and glucose-6-phosphatase (*G6p)* which catalyses another important step of gluconeogenesis (Jantzen *et al.* 1987; Rigaud *et al.* 1991; De Bosscher *et al.* 2003; Beck *et al.* 2009). By upregulating the expression of genes such as *Tat* and *G6p*, glucocorticoids lead to an increased rate of

gluconeogenesis and therefore induce overall fuel mobilisation (Yoshiuchi *et al.* 1998). GR can also upregulate transcription of target genes in an indirect manner via the tethering of a GR monomer to DNA-bound transcription factors. For example the GR monomer has been shown to bind to and enhance the transcriptional activity of signal transducer and activator of transcription 3 (STAT3), ultimately increasing expression of the gene coding for interleukin-10 (IL-10; Unterberger *et al.* 2008; Beck *et al.* 2009). Moreover, evidence suggests the potential for the formation of GR/MR heterodimers with transcriptional activity at multiple GREs (Trapp *et al.* 1994; Liu *et al.* 1995).

1.1.3.1.2 Glucocorticoid-induced transrepression

As with transactivation, ligand-activated GR can directly or indirectly associate with negative GREs (nGREs) to regulate the transcription of glucocorticoid-responsive genes. In the case of direct transrepression, the binding of GR monomers to the promoter region of specific genes actively inhibits their transcriptional activity as is seen in the repression of the osteocalcin gene in response to glucocorticoids. However, indirect transrepression mechanisms also exist via the tethering of GR monomers to transcription factors recruited to the DNA (Beck *et al.* 2009). Multimers of GR can also form and regulate gene expression. One such example includes pro-opiomelanocortin (POMC; the precursor of adrenocorticotropic hormone; ACTH), the promoter of which is repressed following binding of GR multimers to an nGRE (Drouin *et al.* 1993). This defines one mechanism by which glucocorticoids exert negative feedback upon forward drive of the hypothalamic-adrenal-pituitary (HPA) axis.

1.1.3.1.3 The role of co-regulators

Access of the ligand-receptor complex to GREs is regulated not only by the presence of specific transcription factors but is also influenced by the actions of intracellular co-activator and co-repressor proteins. These co-regulators, which often show cell- or tissue-specific expression patterns, are recruited by the ligand-bound receptor itself and have the ability to influence (both positively and negatively), GR-mediated transcriptional regulation via a process called chromatin remodelling (Fryer *et al.*

1998; Fryer *et al.* 1998; Buckingham 2006). This involves either acetylation or deacetylation of core histone proteins that support the helical structure of the DNA. Histone acetylation, facilitated by co-activators, induces an unwinding and opening of the DNA double helix allowing access of RNA II polymerase and transcriptional complexes thus promoting transcription whilst histone deacetylation induces tightening of the DNA helix around the histone core thus repressing gene transcription (Beato *et al.* 1995; Goulding 2004; Buckingham 2006).

1.1.3.2 Non-genomic effects of glucocorticoids

Although glucocorticoids exert their biological effects mainly through the activation and repression of gene transcription, some of their actions occur too rapidly to be explained by such mechanisms. For example, cortisol hyperpolarises hippocampal neurones within 1-2 mins of ligand binding and dexamethasone infusion can feedback to depress the release of ACTH from the anterior pituitary within a similar time period (Maier *et al.* 2005; Buckingham 2006). The latter has been suggested to occur via the association of membrane-bound GR (mGR) with specific G-proteins in the lipid membrane of cells in the pituitary (Bartholome *et al.* 2004; Maier *et al.* 2005). Moreover, Stahn *et al.* demonstrated that high concentrations of glucocorticoids incorporate into both the lipid and mitochondrial membranes altering ion influx into and out of the cell via interactions with Ca^{2+}, K^{+} and Na^{+} ion channels (Stahn *et al.* 2007). Other research suggests that conventional, intracellular GR is also capable of inducing rapid, non-genomic effects involving stimulation of a complex kinase cascade (Buckingham 2006). Moreover, the activated, intracellular GR complex may rapidly and indirectly affect gene expression by regulating post-transcriptional events such as mRNA stability and translation as well as post-translational processing (Buckingham 2006).

1.2 The hypothalamic-pituitary-adrenal (HPA) axis.

Glucocorticoids exert their effects in the cells of almost every tissue type in the mammalian body. Therefore, maintenance of their levels at normal biological concentrations or return to the physiological set-point post-stress, is vital to sustain or restore homeostasis. This regulation is mainly achieved via the HPA axis which co-

ordinates a complex series of direct influences and feedback interactions between the hypothalamus, the anterior pituitary and the adrenal glands in order to control circulating levels of endogenous glucocorticoids. When perceived negative feedback on the axis is low or during acute stress, corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) are secreted from parvocellular neurons in the PVN of the hypothalamus into the portal blood vessel of the hypophyseal stalk (Plotsky 1987). By these means, they reach corticotrophe cells in the pituitary gland, inducing cleavage of ACTH from its precursor pro-POMC before ACTH is secreted from the anterior pituitary lobe into the systemic circulation (Burns *et al.* 1989; Trainer *et al.* 1995). When ACTH reaches the adrenal glands, it elicits biosynthesis and release of corticosteroids (mainly glucocorticoids and but also mineralocorticoids) from the adrenal cortex into the circulation (Ganguly *et al.* 1984). Corticosterone (rodents) or cortisol (humans) is transported around the body bound to corticosteroid-binding globulin (CBG) and directed to peripheral target tissues, where its bioavailability is dependent upon the activity of tissue-specific metabolic enzymes such as 5α-reductase (5αR) and 11β-hydroxysteroid dehydrogenase (11βHSD); a detailed discussion of which will follow below.

The biological effects of glucocorticoids are also dependent on the extent of their binding (> 90%; Breuner *et al.* 2002) to CBG which is believed to hold steroids in an inactive state in the circulation therefore regulating the amount of hormone which is freely able to enter target tissues (Mendel 1989). For example, although the CBG-knock out (KO) mouse does not exhibit features of enhanced glucocorticoid signalling, HPA axis activity in these mice was increased with an exaggerated response to stress (Petersen *et al.* 2006). This phenotype was apparent even though free corticosterone levels were increased 10-fold suggesting an active role for CBG in bioavailability, transportation and cellular signalling which determine the overall target tissue responsiveness to glucocorticoids. The rapid chain of events from stimulus to adrenal steroid release means that systemic glucocorticoids can rise within 2-5mins (Keller-Wood *et al.* 1984).

1.2.1 HPA axis regulation in health

The HPA axis, like most other physiological body systems, demonstrates circadian rhythmicity (Pincus 1943). Glucocorticoid concentrations rise just prior to wakening (light phase in humans, dark phase in rodents) e.g. the major activity period of the organism, and reach the diurnal nadir before sleep (dark phase in humans, light phase in rodents; Lupien *et al.* 1998; Windle *et al.* 1998). As well as circadian control, corticosteroids are released in hourly pulses (the ultradian rhythm) which essentially maintains the responsiveness of target tissues i.e. to peaks in corticosteroid release following a stressor (Young *et al.* 2004; Lightman *et al.* 2010). In addition to circadian regulation, activity of the HPA axis is also under negative feedback control by glucocorticoids themselves in order to reduce the biosynthesis and release of CRH and ACTH, dampening axial drive (Dallman *et al.* 1992). Within minutes of their release, glucocorticoids act to inhibit further CRH, AVP and ACTH secretory responses in both humans and rodents (Dallman *et al.* 1972; Fehm *et al.* 1979; Keller-Wood *et al.* 1984; Widmaier *et al.* 1984; Dallman 2005; de Kloet *et al.* 2005). This regulation is achieved via conventional actions on GR (and to some extent MR) but also by rapid, non-genomic negative feedback actions (discussed below), mediated by glucocorticoids themselves acting at the levels of the pituitary and the PVN to return circulating levels to a basal set-point post-stress (Widmaier *et al.* 1984; Jacobson *et al.* 1991; Herman *et al.* 1997; Di *et al.* 2003). Glucocorticoids inhibit transcription and cleavage of POMC, therefore preventing ACTH production and release from the pituitary, whilst upstream in the hypothalamus CRH transcription is inhibited (Stewart 2002); the net effect being reduced HPA drive to prevent further glucocorticoid secretion.

Many studies suggest that recovery from and adaptation to stressful stimuli is a coordinated effort involving not only the HPA axis but also parts of the limbic system including the hippocampus, amygdala and prefrontal cortex; projections from which influence functionality of the PVN (Ulrich-Lai *et al.* 2009). The hippocampus, which expresses high levels of both *MR* and *GR*, is particularly sensitive to glucocorticoids and works to reduce hypothalamic stimulation in response to the steroids. For example, following electrical stimulation or lesion of

the hippocampus, systemic glucocorticoid levels are decreased or increased respectively, suggesting an inhibitory role for the hippocampus also (Jacobson *et al.* 1991; Herman *et al.* 1997; de Kloet *et al.* 1998). Moreover, being the memory centre of the brain, the hippocampus facilitates a memory trace of stressful events which can be either facilitated or inhibited by glucocorticoids, depending on concentration.

1.2.1.1 Sex-specific regulation of the HPA axis

There is a sex-related influence over HPA axis function which may act to prevent the deleterious effects of HPA activation on reproductive processes since glucocorticoids are known to inhibit gonadotropin-releasing hormone (GnRH) and thus gonadotropin secretion (Selye 1939; Dubey *et al.* 1985; Ringstrom *et al.* 1985). Basally, female rodents have increased corticosterone secretion compared to males (Critchlow *et al.* 1963; Handa *et al.* 1994).

The magnitude of the HPA axis stress response is also determined by sex. Post-stress ACTH and corticosterone levels are consistently shown to be higher in female preclinical models vs. males (Kitay 1961; Handa *et al.* 1994; Armario *et al.* 1995). This is unsurprising since testosterone has been shown to reduce HPA axis responses to stress (Gaskin *et al.* 1971; Handa *et al.* 1994) whilst oestrogen administration increased both basal and stress-induced activity of the HPA axis in both males and females (Burgess *et al.* 1992; Handa *et al.* 1994).

The effect of sex is not so clear in human subjects since some studies have shown males to have greater increases in cortisol and ACTH following psychological stress compared to female subjects (Kirschbaum *et al.* 1992; Kirschbaum *et al.* 1995; Kirschbaum *et al.* 1995). These studies were done using young human subjects and in an elderly cohort, HPA axis hormonal responses to stress are shown to be higher in females vs. males (Seeman 1995; Seeman *et al.* 2001). There are also disparities within age groups though (Collins *et al.* 1978; Kudielka *et al.* 1998) meaning studies investigating the impact of sex on HPA axis stress responses are largely inconclusive, at least in humans.

1.2.2 The HPA axis and the stress response

Selye (1950) defines “stress” in three stages: an initial alarm reaction, characterised by an immediate sympathoadrenomedullary discharge, a subsequent "stage of resistance" characterised by the activation of the HPA axis (Figure 1.4), and, finally, by exhaustion (Selye 1950). Whilst under normal circumstances the activity of the HPA axis is tightly constrained, during periods of emotional, psychological or physical stress (both chronic and acute) the axis plays a key role in conjunction with a general discharge of the sympathetic nervous system. The hypothalamus is a highly evolved sensing center and is able to receive, integrate and relay information from a number of neural and humoral sources. It is the hypothalamus that initiates the stress response. The stress response is a physiological or emotional consequence of any real or perceived threat or “stressor”, the purpose of which is to maintain or restore homeostasis following any adverse circumstance. Under basal conditions, physiological levels of glucocorticoids are tightly controlled to avoid prolonged periods of glucocorticoid excess which is known to be deleterious to a number of physiological systems.

1.2.2.1 The role of corticosteroid receptors in regulating the HPA axis

MR and GR play distinct roles in regulating HPA axis activity both basally and post-stress. Having high affinity for corticosterone, MR remains activated in between ultradian pulses (hourly bursts of corticosterone release of 20 mins duration) and basally in the absence of stress (Reul *et al.* 1985; Young *et al.* 2004) which allows this receptor to play an active role in the maintenance of homeostasis (Joels *et al.* 2008). In contrast, GR with its much lower affinity only becomes occupied and activated following exposure to stress and at the circadian and ultradian peaks (Kitchener *et al.* 2004; Young *et al.* 2004) when it plays an active role in a) facilitating the stress-response by influencing brain activity and energy mobilisation and distribution and b) recovery from the stress response by mediating negative feedback effect back upon the HPA axis (de Kloet *et al.* 1987; Groeneweg *et al.* 2011).

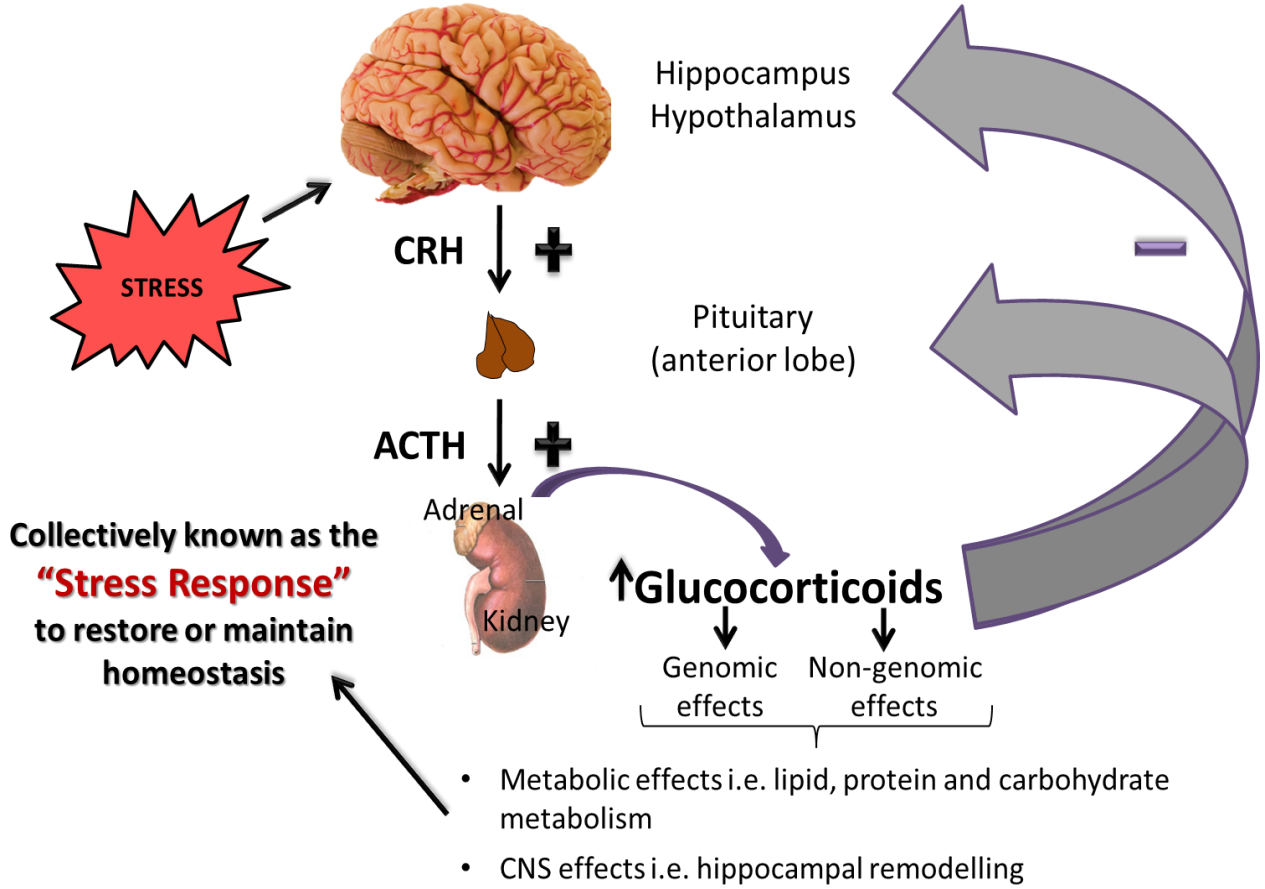

Figure 1.4 Hypothalamic-Pituitary-Adrenal (HPA) axis-regulation of the stress response
Schematic diagram illustrating the roles of hippocampal, hypothalamic, pituitary and adrenal formations involved in the control of the hypothalamic-pituitary-adrenal (HPA) axis. CRH = corticotrophin releasing hormone, ACTH = adrenocorticotrophic hormone, CNS = central nervous system.

Evidence for the crucial role of corticosteroid receptors in HPA axis regulation comes from mutagenic animal studies. Lack of GR activity in a forebrain-specific GR-KO mouse model (GR^{NesCre}) resulted in dysregulation of the HPA axis with raised *Crh* but not *Avp* expression in the PVN, along with increased abundance of transcripts for *Acth* in the pituitary (Tronche *et al.* 1999). Such modifications are consistent with impaired negative feedback regulation of the HPA axis. Moreover, these GR^{NesCre} mice had elevated circulating corticosterone levels at both the circadian peak and nadir but retained similar levels to control post restraint stress (Tronche *et al.* 1999). Conversely, specific GR overexpression in forebrain had no effect on HPA axis activity with morning and evening corticosterone and ACTH levels, and those immediately following a stressor, were comparable to WT controls (Wei *et al.* 2004). There was also no change in the abundance of transcripts for *Crh* in the PVN (Wei *et al.* 2004).

The MR/GR balance hypothesis put forward by de Kloet in 1991 suggests that increased MR relative to GR would be indicative of reduced HPA responsiveness to stressful stimuli (de Kloet 1991). This is suggestive that MR functionality is critical for the sensitivity and feedback of the HPA axis. Indeed, MR activation following corticosterone binding regulates ACTH secretion during both the trough and peak of the circadian rhythm (Dallman *et al.* 1989; Ratka *et al.* 1989; Bradbury *et al.* 1994) and MR antagonism via intracerebroventricular (i.c.v.) administration of RU28318 to rats, resulted in elevated plasma corticosterone basally and prolonged elevations post-stress compared to vehicle (Ratka *et al.* 1989). The same study also showed that specific GR antagonism via i.c.v. administration of RU38486, did not alter basal glucocorticoid levels in the circulation but did prolong stress-related elevations in corticosterone following a novel environment stress (Ratka *et al.* 1989). Taken together these results are suggestive of a largely MR-driven regulation of basal glucocorticoid levels whilst both MR and GR work synergistically to regulate HPA axis activity during and post-stress to maintain and return homeostasis.

1.2.2.2 The role of rapid, non-genomic glucocorticoid effects in HPA axis regulation

Conventionally, regulation of the axis has been thought to occur mainly via negative feedback in a delayed, genomic fashion e.g. corticosterone acting via GR in the PVN (de Kloet *et al.* 1998). However, it is now known that HPA stress responses are regulated, in part, by glucocorticoid mediated feedback which can occur on both fast and slow time-frames (Jones *et al.* 1976; Keller-Wood *et al.* 1984; Dallman 2005). Even as early as 1947, it was shown that "adrenal gland products" inhibited further biosynthesis in the adrenal glands within 30 mins by acting upon extra-adrenal tissues (Sayers *et al.* 1947); consistent with the time-frame of fast, non-genomic effects. Moreover, HPA inhibition has been shown to occur within even shorter time-frames, for example animals exposed to a immobilisation stress followed by another episode 5 mins later, did not elicit a CRH response subsequent to the second stressor (Sakakura *et al.* 1976). In more recent electrophysiological studies rapid negative feedback has been shown to occur via the inhibition of glutamate release onto CRH-containing cells in the PVN and was suggestive of actions at a membrane-bound receptor (Di *et al.* 2003; Malcher-Lopes *et al.* 2006). Further evidence from the same studies revealed this was achieved via endocannabinoid synthesis and retrograde activation of presynaptic cannabinoid receptors (which reduced the release of neurotransmitter from presynaptic terminals), all mediated by mGR.

Rapid, non-genomic actions on HPA function have also been observed at the level of the anterior pituitary where high expression of *GR* and relatively low *MR* has been demonstrated (Reul *et al.* 1990). ACTH release from the pituitary was inhibited following corticosteroid administration; an effect which was not ameliorated by protein synthesis inhibitors suggesting a non-genomic mechanism (Keller-Wood *et al.* 1984). The cellular basis of these rapid effects is somewhat ambiguous and there is opposing evidence for the involvement of GR. Some authors have reported that pretreatment with the GR antagonist RU486 did not prevent rapid, corticosterone inhibitory effects on CRH-induced ACTH release *in vivo* (Hinz *et al.* 2000) indicating that the receptor plays no part in mediating these effects. Conversely, there is evidence suggesting a role for the classical GR in mediating rapid feedback

at the level of the pituitary. For example, annexin-I was required for rapid inhibition of ACTH release which in turn seemed to be dependent upon the actions of GR since this effect was blocked by the administration of the GR antagonist mifepristone (Buckingham *et al.* 2003; Solito *et al.* 2003; Tierney *et al.* 2003).

1.3 Glucocorticoids in health and disease

1.3.1 Metabolic effects of glucocorticoids

In health, glucocorticoids play a role in regulating metabolism and energy homeostasis such that energy is mobilised to be used as fuel. These metabolic effects of glucocorticoid hormones include the upregulation in the expression of enzymes involved in gluconeogenesis (primarily in the liver), resulting in glucose synthesis from non-hexose substrates, such as amino acids and glycerol. One mechanism by which this is achieved is by opposing or blocking the effects of insulin in inducing glucose storage in the liver. Glucocorticoids also stimulate lipolysis which mobilises fatty acids to be used for energy production in muscles, and glycerol as a substrate for gluconeogenesis. This process is beneficial when food is scarce or during periods of acute stress (i.e. the "fight or flight" response) where energy must be mobilised for survival. However, glucocorticoids, when present at concentrations exceeding normal physiological levels (for prolonged periods of time), can be detrimental in a number of aspects (Reaven 1988; Kahn *et al.* 2005).

Glucocorticoid effects mediated through corticosteroid receptors can be described as immunomodulatory, metabolic/endocrine or toxic and although glucocorticoid treatment is of benefit in treating many disorders, side effects are common; the severity of which is dependent upon the dose given and the duration of exposure (Goodwin 1994). Across several decades now, glucocorticoids have been used clinically as anti-inflammatory agents in the treatment of chronic diseases such as asthma, inflammatory bowel disease, rheumatoid arthritis and other autoimmune diseases associated with increased inflammatory gene expression (Barnes 1998). Therapeutic glucocorticoid use can result in metabolic and cardiovascular disturbances associated with glucocorticoid excess including dyslipidaemia, insulin resistance, type II diabetes mellitus, visceral obesity, CVD and hypertension

(Schacke *et al.* 2002; Walker 2007); the aforementioned being symptomatic of Cushing's syndrome. Cushing's syndrome is the collective name used for several rare disorders resulting from chronic glucocorticoid exposure from either endogenous or exogenous sources.

Dysregulation of the HPA axis and its regulatory parameters (e.g. CRH, MR and GR) which results in glucocorticoid excess is also linked to syndromes of metabolic tissue systems including diabetes mellitus types I and II (Ray *et al.* 1993; Tsigos *et al.* 2002; Tsigos *et al.* 2002), obesity (Devenport *et al.* 1989; Hautanen *et al.* 1993; Weaver *et al.* 1993; Pasquali *et al.* 1996; Andrew *et al.* 1998; Rosmond *et al.* 1998) and appetite stimulation (Tempel *et al.* 1994). In turn, the most common effects of long-term glucocorticoid exposure largely affect the normal function of the HPA axis. Obesity, particularly upper body obesity, almost always correlates with HPA axis dysfunction. For example, glucocorticoid secretion is increased in obese patients compared to lean control subjects; however, levels in the peripheral circulation remain unchanged which is suggestive of enhanced glucocorticoid clearance via metabolic enzymes (Boscaro *et al.* 2001; Walker 2007).

Much is now known about the role of 11βHSDs (type I and type II activate and inactivate glucocorticoids respectively) and A-ring reductases such as 5αR1 (reduces glucocorticoid potency) in intracellular glucocorticoid metabolism and their link to metabolic disorders. Enhanced tissue regeneration of glucocorticoids inferred from increased adipose 11βHSD1 expression has been shown in genetic studies of both rodent and human models of obesity (Livingstone *et al.* 2000; Masuzaki *et al.* 2001; Rask *et al.* 2001; Lindsay *et al.* 2003; Wake *et al.* 2003) whilst in transgenic mouse models of 11βHSD1 disruption, glucose tolerance and insulin sensitivity were improved even whilst fed a high-fat diet (Kotelevtsev *et al.* 1997; Morton *et al.* 2001; Morton *et al.* 2004). However, regeneration of active corticosterone by 11βHSD1 in the liver is reduced in obese Zucker rats in comparison to lean controls (Livingstone *et al.* 2000), and this is also seen in comparisons between obese and lean human subjects (Stewart *et al.* 1999). Alterations in glucocorticoid metabolism in a highly tissue-specific manner may influence activation of corticosteroid receptors whilst

also affecting feedback regulation of the HPA axis. Moreover, studies over the last decade have shown both obesity and the metabolic syndrome are associated with chronic inflammation (Hotamisligil 2006). HPA axis activity is stimulated by pro-inflammatory cytokines whilst in turn glucocorticoids downregulate the production of cytokines and other related inflammatory mediators, providing evidence for cross-talk between the HPA axis and the inflammatory cascade (Lee *et al.* 1988; Cronstein *et al.* 1992). This may therefore be the mechanism which underpins the role of HPA axis alterations in the aetiology of obesity.

1.3.1.1 Energy balance and the role of glucocorticoids

As well as the development and advancement of obesity, glucocorticoids are also a key requirement for normal food intake and metabolism. Germano *et al.* observed a significant reduction in food intake following bilateral adrenalectomy (ADX) in rats (Germano *et al.* 2007). Additionally, normal rats infused centrally for 3 days with synthetic glucocorticoids (dexamethasone) demonstrated a sustained increase in food consumption with accompanying body weight gains (Green *et al.* 1992; Zakrzewska *et al.* 1999). In human obesity, especially of visceral distribution, elevated glucocorticoid levels and hyperactivity of the HPA axis are seen (Weaver *et al.* 1993). Furthermore, observational studies have demonstrated the effect of glucocorticoid excess (e.g. in Cushing's syndrome) showing that food intake is also increased in these individuals (Castonguay 1991; de Herder *et al.* 1996). Adrenal glucocorticoids and pancreatic insulin have opposing effects on feeding responses (Strack *et al.* 1995) but insulin secretion is also driven by glucocorticoids. These feeding responses are mediated through modulating the expression of various hypothalamic neuropeptides which are key in the regulation of appetite and energy balance (Solano *et al.* 1999; Savontaus *et al.* 2002; Drazen *et al.* 2003; Coll *et al.* 2005).

1.3.1.1.1 Glucocorticoid control of neuropeptides

Much of appetite regulation (and neuropeptide expression) occurs in structures such as the pituitary and the hypothalamus where 5αR enzymes are also expressed in close proximity to GR (Lakshmi *et al.* 1991; Lephart *et al.* 1991; Gottfried-Blackmore *et*

al. 2008). The hypothalamus is the key structure in regulating appetite, food consumption and satiety. The hypothalamic neuropeptides which mediate these parameters can be described as anorexigenic, causing loss of appetite and reduced food consumption, or orexigenic, an appetite stimulant increasing feeding behaviours and food intake. Examples of orexigenic peptides include cocaine- and amphetamine-regulated transcript (CART), neuropeptide Y (NPY) and agouti-related peptide (AgRP) which is co-expressed with NPY, whilst neuropeptides with anorexigenic properties include melanin-concentrating hormone (MCH) and proopiomelanocortin (POMC); a precursor for the generation of α-melanocyte stimulating hormone (α-MSH) which acts upon melanocortin receptors type 3 and type 4 (MC3R and MC4R) in the hypothalamus and medial amygdala to mediate its anorexigenic effects (see Figure 1.5; Solano *et al.* 1999; Savontaus *et al.* 2002; Drazen *et al.* 2003; Coll *et al.* 2005; Densmore *et al.* 2006; Smart *et al.* 2006). CRH has appetite regulating properties which are less well defined but it is most often described as having anorexigenic properties (Heinrichs *et al.* 1993; Ahima *et al.* 1996; Huang *et al.* 1998).

Glucocorticoids mediate their effects on food intake through cross-talk with feeding regulators including CRH, NPY, leptin and insulin, and can therefore induce either orexigenic or anorexigenic effects depending on which neuropeptide regulatory system is activated (see Figure 1.5 for details). However, expression of *GR* within hypothalamic regions key in appetite regulation (i.e. PVN and the arcuate nucleus), suggest more direct pathways also exist (Morimoto *et al.* 1996). Glucocorticoids can increase appetite and feeding behaviour by increasing NPY secretion and decreasing the release of CRH from the hypothalamus (Zakrzewska *et al.* 1999; Cusin *et al.* 2001), effects which may be counteracted by leptin release from the adipose tissue shortly after a meal (Cavagnini *et al.* 2000). Importantly though, it has been shown that high levels of glucocorticoids induced during periods of psychological stress can lead to hypophagia and weight loss (Marti *et al.* 1994; Valles *et al.* 2000).

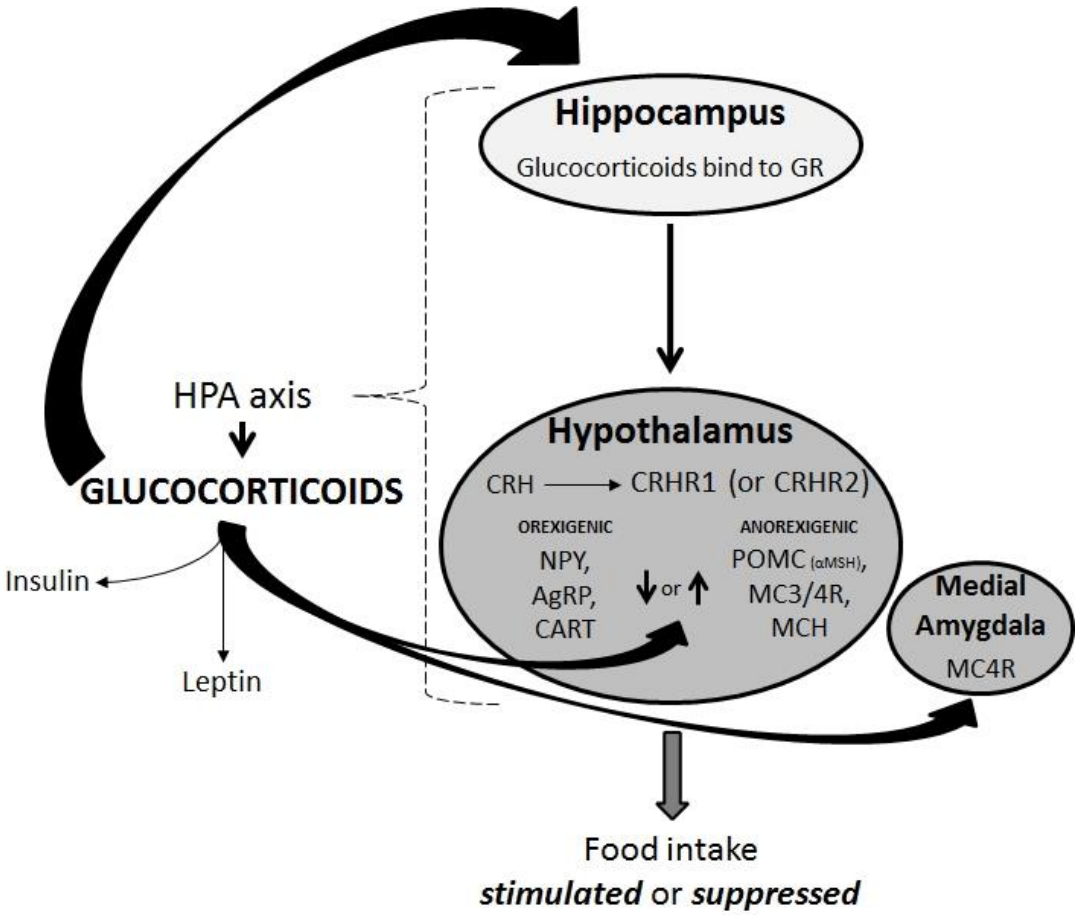

Figure 1.5 Glucocorticoid regulation of appetite and feeding.
Glucocorticoids released from the HPA axis (with or without a stressful stimulus) can directly (through GR) or indirectly (through altering the expression of neuropeptides and hormones) alter food intake. GR stimulation in the hippocampus reduces CRH expression (negative feedback) thus reduces CRHR-1/2 activation in the hypothalamus. Glucocorticoids also alter the expression of insulin and leptin and other neuropeptides (both anorexigenic and orexigenic); the balance between which determines the ultimate effect on food intake. Insulin and leptin mediate their effects on feeding behaviour through hypothalamic appetite regulatory neuropeptides. HPA=hypothalamic-pituitary-adrenal axis, GR=glucocorticoid receptor, CRH=corticotrophin-releasing-hormone, CRHR-1/2=corticotrophin releasing hormone receptor type 1/type 2, NPY=neuropeptide Y, AgRP=agouti-related peptide, CART=cocaine- and amphetamine-regulated transcript, POMC= proopiomelanocortin, α-MSH=α-melanocyte stimulating hormone, MCH=melanin-concentrating hormone, MC3/4R=melanocortin receptor type 3 and type 4. Adapted from (Maniam *et al.* 2012).

1.3.1.1.2 Glucocorticoid control of leptin

Glucocorticoids also induce changes in feeding responses through interactions with the adipose-derived hormone, leptin; a key regulator of appetite and food intake (Cavagnini *et al.* 2000). Leptin is a 166 amino acid protein transcribed by the obese (Ob) gene and is secreted mostly from adipocytes of the white adipose tissue (Masuzaki *et al.* 1995) but also from other organs such as the stomach (Bado *et al.* 1998; Sobhani *et al.* 2000). Leptin is an appetite suppressant and, in health, circulating levels are directly proportional to the total amount of fat in the body. Leptin reduces appetite and food intake through actions such as promoting the synthesis and release of anorexigenic peptides including α-MSH whilst also counteracting the effects of NPY and other orexigenic peptides in the hypothalamus. Glucocorticoids can directly increase leptin levels (Mostyn *et al.* 2001). Furthermore, rats receiving high dose dexamethasone (1 mg/kg/day) for 4 days, had hyperleptinaemia associated with dose-dependent appetite suppression, reduced food intake and resulting weight loss compared to saline controls (Jahng *et al.* 2008). Similar leptin increases have been demonstrated in both lean and obese human subjects (Miell *et al.* 1996; Dagogo-Jack *et al.* 1997). Although leptin is an appetite suppressant, its increased levels in models of obesity points to a role of glucocorticoids in reducing leptin sensitivity (Solano *et al.* 1999).

1.3.1.1.3 Glucocorticoid control of insulin

Increased plasma glucose levels give rise to insulin secretion from β-cells in the pancreas which promotes glucose metabolism and energy storage in tissues such as muscle and fat (Baskin *et al.* 1999). Insulin secretion is in proportion to the degree of adiposity in the body. High levels of ligand activation of the insulin receptor (IR) e.g. in the hypothalamus (Schwartz *et al.* 1992), can desensitise the receptor leading to insulin insensitivity, reduced receptor signalling, insufficient glucose uptake leading to hyperglycaemia and ultimately type II diabetes mellitus, a condition often found concomitantly with obesity

Glucocorticoids and insulin have opposing effects on glucose uptake and storage. For example the main role of insulin is to induce cells to absorb glucose from the

bloodstream in a number of tissues including the liver, skeletal muscle and adipose tissue and promote storage as glycogen in the liver and skeletal muscle or triglycerides in adipocytes (Moloney *et al.* 1955; Wright 1959; Antoniades *et al.* 1960; Slater *et al.* 1961; Dixit *et al.* 1963; Froesch *et al.* 1963; Jefferson *et al.* 1968) . Conventionally, glucocorticoids are known to mediate carbohydrate metabolism (Dallman *et al.* 1993), in the opposite direction to that of insulin. Glucocorticoids have also been shown to have a number of effects on the activity of insulin through the impairment of insulin-dependent glucose uptake in the periphery (e.g. skeletal muscle), potentiating glucose uptake and energy storage in adipose tissues and enhancing gluconeogenesis in the liver (Rizza *et al.* 1982; Rooney *et al.* 1993; Chrousos 2000). Moreover, glucocorticoids also oppose the actions of insulin in terms of food intake where the steroid hormones oppose insulin-induced reductions in central appetite (Chavez *et al.* 1997).

An antagonistic interaction between glucocorticoids and insulin exists in most adipose depots, whereby glucocorticoids promote lipolysis whilst insulin stimulates the synthesis of new fat molecules (Fried *et al.* 1993). During periods of hyperactivation of the HPA axis (e.g. in chronic stress), persistent glucocorticoid excess stimulates gluconeogenesis and inhibits glycolysis resulting in increased insulin secretion from the pancreas and hyperinsulinaemia; a condition which has been associated with an increased incidence of abdominal fat deposition and a poor cardiovascular risk profile (Pasquali *et al.* 2006; Nieuwenhuizen *et al.* 2008). In the presence of insulin, this metabolic consequence of visceral energy storage is thought to inhibit activity of the HPA axis (Dallman *et al.* 2005) e.g. metabolically-induced negative feedback on the axis to reduce glucocorticoids and their antagonistic effect upon insulin-dependent processes. Leptin then attempts to antagonise the effects of high glucocorticoid concentrations by stimulating the release of NPY to increase food intake via CRH inhibition (Cavagnini *et al.* 2000).

Glucocorticoid-stimulated insulin secretion is inversely correlated with CRH mRNA expression in the PVN of the hypothalamus, which contributes to reduced activity of the HPA axis (Warne 2009). Finally, animal models have been used to demonstrate

how glucocorticoids and insulin work synergistically in increasing the intake of palatable food which largely contributes to increases in visceral fat depots (Pecoraro *et al.* 2004; Warne 2009).

1.3.2 CNS disturbances linked to glucocorticoid exposure

1.3.2.1 Glucocorticoid control of behaviour and psychiatric disorders

Glucocorticoids exert an array of biological effects in the brain affecting behaviour as well as neuronal survival, metabolism and neurotransmission. This first became apparent with the discovery of glucocorticoid receptors in the brain many years ago, establishing the CNS as an important target for glucocorticoids (McEwen *et al.* 1968; 1969). Moreover, glucocorticoid levels outside the normal physiological range can be detrimental and indeed treatment with synthetic glucocorticoids is associated with an array of CNS side-effects which are often underestimated or misdiagnosed initially (Sirois 2003; McDonough *et al.* 2008). There is a high incidence (57 - 83%) of psychiatric disturbances amongst individuals with Cushing's syndrome and hypercortisolaemia (Haskett 1985; Hudson *et al.* 1987; Dorn *et al.* 1995; Kelly 1996) with depression being the most common amongst anxiety, mania and psychosis (Dorn *et al.* 1995; Kelly 1996; Kelly *et al.* 1996). The propensity for Cushing's patients to develop depression has stimulated an interest in the role of glucocorticoids (namely in their excess) in the brain and their modulatory effects on behaviour. Glucocorticoid excess can occur from exogenous sources following treatment with synthetic glucocorticoids, from adrenal tumours and from disruption of the HPA axis (e.g. suppressed peak responses or delayed recovery). High glucocorticoid levels are associated with neurological conditions such as anxiety, depression, anorexia nervosa and age-related cognitive dysfunctions (Kaye *et al.* 1987; Rybakowski *et al.* 1992; Andreatini *et al.* 1994; Korte *et al.* 1995; Karanth *et al.* 1997). Paradoxically though there are reports of associations between low salivary glucocorticoids and an increased risk of depression (Strickland *et al.* 2002).

1.3.2.1.1 Glucocorticoids in the aetiology of anxiety

Glucocorticoids have repeatedly been implicated in the development and progression of CNS diseases such as anxiety. One such example includes the 11βHSD1-KO mice which are less anxious in elevated plus maze (EPM) and open field test (OFT; two tests designed to quantify anxiety-like behaviours) possibly due to reduced glucocorticoid amplification centrally (Yau *et al.* 2003). Moreover, mice treated with the synthetic glucocorticoid prednisolone displayed increased anxiety-like behaviours in the above two tests also (Kajiyama *et al.* 2010).

Anxiety is regulated by many neuropeptides including CRH; a 41 amino acid neuropeptide which is widely accepted as a key physiological regulator of stress. CRH is synthesised and expressed in the PVN of the hypothalamus, and amygdala and is an essential component of the stress response mediated via the HPA axis; dysregulation of which is linked to affective disorders. Its neuromodulatory role has been demonstrated in animal studies highlighting that the incidence of anxiety- and depression-like behaviours was increased following administration of either CRH or CRH fragments to rodents, and also in CRH overexpressing transgenic mice (Stenzel-Poore *et al.* 1994).

CRH acts upon its receptors in the pituitary (of which there are two subtypes) to induce ACTH release. CRH predominantly elicits its effects through associations with the CRH receptor type 1 (CRHR-1; a G-protein coupled receptor) which is located on corticotrophes of the anterior pituitary. Encouragingly, several, selective, non-peptide antagonists of this receptor are currently in development to treat anxiety and depression (Holsboer 1999; Kunzel *et al.* 2003; Holsboer *et al.* 2008). However, it is unclear whether the anxiolytic effects of CRHR-1 antagonists are mediated through the HPA axis, extra-pituitary CRHR-1 or both, although some studies have found evidence for the mediating effect of central CRHR-1 (Philbert *et al.* 2012). The specific brain regions involved have yet to be determined.

CRH has modulatory effects on a number of neurotransmitter systems involved in emotion including dopamine, noradrenaline and serotonin (Lavicky *et al.* 1993; Price

et al. 2001; Valentino *et al.* 2005). CRH modulation of these neurotransmitter systems (especially serotonin and noradrenaline) supports the role of neuropeptides in emotional behaviours as these neurotransmitter systems are implicated in affective behavioural responses such as anxiety (both normal and disorderly; Koob 1999; Charney 2004). Therefore, there is strong evidence for the role of CRH in modulating circuits involved in emotion.

Finally, altered expression of *GR* and *MR* has also been linked to elevated glucocorticoids, anxiety and other neurological disorders. Depression is associated with HPA axis malfunction characterised by elevated plasma corticosterone (Carroll *et al.* 1976) and treatment with the antidepressant fluoxetine induced up-regulation of *GR* in hippocampal regions (Yau *et al.* 2004). Forebrain specific GR knock-out mice (FBGRKO) who have increased HPA axis activity due to loss of GR regulation (Boyle *et al.* 2005), also show increased anxiety-related behaviours in the EPM (Wei *et al.* 2004; Boyle *et al.* 2006). Paradoxically, forebrain specific over-expression of *GR* (GR^{ov}) in mice also leads to increased anxiety-like behaviours but in this instance in the absence of changes in circadian or mild stress induced HPA axis activity (Wei *et al.* 2004). Over-expression of *MR* in transgenic mice (MR-Tg) led to reduced anxiety compared to WT controls (Lai *et al.* 2007) even though HPA axis activity was unaffected (Rozeboom *et al.* 2007). On the other hand, mice with MR knock-out in the forebrain specifically ($MR^{CamKCre}$), show normal basal circadian and post-stress HPA axis activity but anxiety levels were no different to control (Berger *et al.* 2006).

1.3.2.1.2 Glucocorticoids, cognition and the caveat of ageing

In neuroscience, long-term potentiation (LTP) is a persisting enhancement in the signal transmission occurring between two neurons which results from their synergistic excitation (Cooke *et al.*). It is this phenomenon that underlies synaptic plasticity, the capacity of chemical synapses to alter their strength. It is widely considered that LTP is one of the major cellular mechanisms underlying learning and memory since memories are thought to be associated with modifications in synaptic strength (Bliss *et al.* 1993). The natural process of ageing brings about various

biological changes including learning and memory deficits in both humans and non-human mammals. Various theories exist which describe the mechanisms which drive the ageing process of the brain; these include the involvement of glucocorticoid hormones and their ability to induce structural and functional changes within the hippocampus when they are present at abnormally high concentrations.

Approximately 30% of human and mammalian populations develop age-associated cognitive impairments, much of which is linked to hippocampal dysfunction. The main established parameter used to study hippocampal function in rodents is spatial memory which can be manipulated using tests such as the Y-maze and Morris water-maze. The Morris water-maze is widely used in behavioural neuroscience to study spatial learning and memory. It was developed by neuroscientist Richard G. Morris in 1981 (Morris 1981) who showed that hippocampal lesions impaired spatial learning in rats (Morris *et al.* 1982). The Y-maze is designed to test rodents in much the same way as the water-maze. However, it is a two-trial recognition memory test and exploits the rodent's natural spontaneous novelty exploration behaviours and their ability to recognise novel areas of the maze using spatial cues. Performance in each of the above mazes has been shown to be modified by glucocorticoids, corticosteroid receptors and ageing (Hellemans *et al.* 2004; Wei *et al.* 2007; Yau *et al.* 2007).

Normal ageing is potentially exacerbated by glucocorticoids since their basal levels are generally higher in aged rodents (Sencar-Cupovic *et al.* 1976; Tang *et al.* 1978; Brett *et al.* 1983; Sapolsky *et al.* 1983; 1983; DeKosky *et al.* 1984; Brett *et al.* 1986; Sapolsky 1992). Indeed, in aged rats basal plasma corticosterone levels are closely correlated with the degree of hippocampal dysfunction and spatial learning impairments (Landfield *et al.* 1978; Landfield *et al.* 1981). Moreover, only rats with elevated plasma corticosterone levels show evidence of spatial memory deficits (Issa *et al.* 1990) and ADX at middle age followed by low level glucocorticoid replacement ameliorates cognitive decline by preventing hippocampal degeneration (Landfield *et al.* 1981). There is also evidence for this in the human population,

however, human data is more ambiguous with cortisol levels being shown to increase, decrease or stay the same during ageing (Lupien *et al.* 1996).

The hippocampus plays an important role in behaviour and is a key locus for neuroendocrine control and cognitive function. There are reports of glucocorticoid-mediated cognitive decline where memory and cognition are negatively influenced at very high glucocorticoid concentrations via direct alterations in hippocampal structure and function (Wolkowitz *et al.* 1997; Yau *et al.* 2007). Hippocampal tissue has been shown to have the highest expression of *GR* and *MR* in the brain (Jacobson *et al.* 1991) and is known to be particularly sensitive to glucocorticoids which can cause a premature aging phenotype in their excess (McEwen 1999; Wei *et al.* 2007; Yau *et al.* 2007); see Hibberd, Yau and Seckl 2000 for review). Indeed, human subjects suffering from Cushing's syndrome have been shown to have cognitive impairments in domains such as auditory attention, working memory and spatial orienting, even after treatment when long-term remission has been achieved (Ragnarsson *et al.* 2012). In rodent models such as the 11βHSD1 knock out mouse (lacking glucocorticoid regeneration), hippocampal LTP and spatial learning was enhanced, even in aged animals (Yau *et al.* 2007). It should be noted that although many of the deleterious (and beneficial) effects of glucocorticoids upon memory are mediated via the hippocampus, other brain structures are also susceptible and studies have shown associations between chronic corticotherapy and cerebral atrophy (Bentson *et al.* 1978; Zanardi *et al.* 2001).

To summarise, the ability of glucocorticoids to cause changes in both central and peripheral phenotypes demonstrates close inter-relationships between glucocorticoid secretion, behaviour, feeding, metabolism and energy storage. Conversely, it is important to mention that glucocorticoid insufficiency (e.g. in the autoimmune disorder Addison's disease) can be also be detrimental in terms of vulnerability to stress, hypotension, weight loss and mood disturbances.

1.4 Steroid metabolising enzymes

With reference to the importance of maintaining physiological glucocorticoid concentrations, an additional level of control of intracellular concentrations involves the metabolism of glucocorticoids by metabolic enzymes. Disruption of these enzymes, either by genetic mutation or pharmacologically, can critically influence GR signalling within tissues (Kotelevtsev *et al.* 1997; Rask *et al.* 2001; Wake *et al.* 2003), thus these pathways have a role to play in modulating the same physiological features and disease states mentioned above.

In addition to the control of the HPA axis via negative feedback loops, glucocorticoid levels in the body and their biological activity are tightly regulated via the action of metabolising enzymes. Glucocorticoid inactivation and clearance is largely a hepatic process and mainly involves the action of non-P450, A-ring reducing enzymes such as 5α-reductase (5αR), 5β-reductase (5βR) and 3α-hydroxysteroid dehydrogenase (3αHSD); referred to as A-ring reductases (Walker *et al.* 2006). However, there is one cytochrome P450 enzyme involved in glucocorticoid metabolism. The cytochrome P450 3A (CYP3A) enzyme family is abundant in the human liver and accounts for roughly 30% of the total P450 content (Watkins 1994). The type 4 enzyme (CYP3A4) shows the highest expression in human liver (Aoyama *et al.* 1989), is transcriptionally activated by glucocorticoids (Ogg *et al.* 1999) and has been shown to contribute towards hepatic metabolism of synthetic glucocorticoids (Moore *et al.* 2012). In addition to 5αR, 5βR and 3αHSD, other non-P450 enzymes include the 11β-hydroxysteroid dehydrogenases (11βHSDs; Figure 1.6) which provide an additional major route of glucocorticoid metabolism, where the two isozymes 11βHSD1 and 11βHSD2 can activate or inactivate local glucocorticoids, respectively (Walker *et al.* 2006). The P450 and non-P450 enzymes involved in steroid metabolism are highlighted in Table 1.1 below.

Non-P450 enzymes	*Cytochrome P450 enzymes*	
	Microsomal	*Mitochondrial*
5α-Reductase	P450c17	P450scc
5β-Reductase	P450c21	P45011b
3α-hydroxysteroid dehydrogenase	P450aro	P450c11AS
3β- hydroxysteroid dehydrogenase	P4507a-Hydroxylase	
11β- hydroxysteroid dehydrogenase	20a-Hydroxylase	
17β- hydroxysteroid dehydrogenase		

Table 1.1 Summary of the enzymes involved in metabolising keto steroids
This table highlights the two main groups of enzymes involved in steroidogenesis; the non-P450 enzymes which are A-ring reductases, and the cytochrome P450 group which comprises of haem-thiolate proteins. Non-P450 enzymes such as 5α-reductase use NADPH as a co-factor to facilitate electron transfer (H+) onto the substrate, resulting in a reduced form of the steroid substrate e.g. 5α-dihydro metabolites, plus NADP+. Microsomal P450 enzymes catalyse steroidogenesis via cytochrome P450 reductase and electron transfer from NAPDH. Mitochondrial systems employ adrenodoxin reductase and adrenodoxin as co-factors for electron transfer from NADPH. HSD = hydroxysteroid dehydrogenase, NADP+ = nicotinamide adenine dinucleotide phosphate, NAPDH = reduced form of nicotinamide adenine dinucleotide phosphate.

1.4.1 5β-Reductase (5βR)

5βR belongs to the aldo-keto reductase (AKR) superfamily and catalyses the reduction of the $\Delta^{4,5}$ double bond within the A-ring of steroids in the same way as 5αRs but the resulting metabolite in this case is *cis* 5β-dihydro steroids (Berseus *et al.* 1965). It is believed that the actions of 5βR are mainly catabolic, as it is thought that 5β reduced products (which are further metabolised by 3αHSD (Usui *et al.* 1986)) are biologically inactive at classical steroid receptors (McInnes *et al.* 2004). The enzyme plays a role in the inactivation of glucocorticoids (corticosterone and cortisol) as well as androgens (androstenedione and testosterone) and progesterone thus regulating their ability to activate their target nuclear receptors (Berséus 1967; Wilson 1987; McInnes *et al.* 2004; Penning *et al.* 2007). Evidence for 5βR expression in the brain is lacking and although small amounts are found in the kidneys, expression is restricted to the liver where its activity is dependent upon nicotinamide adenine dinucleotide phosphate-reduced (NADPH) as an electron donor (Okuda *et al.* 1984; Furuebisu *et al.* 1987; Onishi *et al.* 1991; Kondo *et al.* 1994). The predominant function of 5βR involves the processing of bile acids as a result of cholesterol metabolism within the liver, as demonstrated by congenital defects in this metabolism pathway resulting from mutations in 5βR (Palermo et al. 2008). 5βR also generates a range of steroids with the 5β-pregnane configuration, which are natural ligands for the pregnane-X receptor (PXR; Bertilsson *et al.* 1998; Moore *et al.* 2000). PXR indirectly facilitates the metabolism of a large proportion of drugs in the liver by enabling the induction of CYP3A4 (Shimada *et al.* 1996; Huang *et al.* 2006). However, what is most important for this thesis is that 5βR (in parallel with 5αR1 and 3αHSD) plays a role in the metabolism and excretion of glucocorticoids in the liver (Andrew *et al.* 2002).

1.4.2 3α-Hydroxysteroid dehydrogenases (3αHSDs)

5α- and 5β- dihydro steroids formed via the action of 5α- and 5β-reductases can be further reduced into their 5α- or 5β-tetrahydro forms by 3αHSDs. Moreover, *in vivo,* 3αHSDs have the capacity to function in both directions allowing them to also catalyse the reverse oxidative reaction. One dominant soluble 3αHSD in the rodent which has been identified and cloned (*Akr1c9*; Lin *et al.* 1999) and has been shown

to have 69% sequence identity with each of the human types at the amino acid level. In humans, at least four isozymes exist which share ~84% amino acid homology with each other (Khanna *et al.* 1995; Khanna *et al.* 1995; Penning 1997; Penning *et al.* 2000), but are each transcribed from a different gene: 3αHSD type 1 (*Akr1c4*), 3α(17β)HSD type 2 (*Akr1c3*), 3αHSD type 3 (*Akr1c2*) and 20α(3α)-HSD (*Akr1c*; Penning 1999). Distinct tissue distribution of these enzymes is apparent; the rodent 3αHSD is highly expressed in the brain (Griffin *et al.* 1999; Compagnone *et al.* 2000; Dong *et al.* 2001) whilst in humans both brain and liver express all four isozymes, lung tissue expresses all except 3αHSD type 1 and in the prostate 3αHSD type 3 and 3α(17β) HSD type 2 are predominantly expressed (Penning 1999). For the purpose of this study, in the brain 3αHSD is important in the *de novo* synthesis of neurosteroids such as allopregnanolone (ALLO; Karavolas *et al.* 1991).

1.4.3 11β-Hydroxysteroid dehydrogenases (11βHSDs)

11βHSD type 1 and 2 catalyse the interconversion between active and inactive glucocorticoids. They are microsomal membrane-bound proteins, expressed in a tissue-specific manner allowing them to influence local glucocorticoid exposure within tissues. The type 1 isozyme (11βHSD1) functions mainly as a reductase, converting less active 11-keto glucocorticoids (cortisone in humans, 11-dehydrocorticosterone in rodents) into their biologically active forms (cortisol in humans, corticosterone in rodents). 11βHSD1 is highly expressed in various tissues including liver, adipose, the CNS, lung and ovary (Monder *et al.* 1993; Stewart *et al.* 1999; Napolitano *et al.* 1998). It was more recently established that 11βHSD1 provides an important intracellular amplification of glucocorticoids, as well as contributing to circulating levels of the hormone (Walker 2007). Highlighting the importance role of metabolising enzymes, 11βHSD-KO mice have adrenocortical hypertrophy and the adrenal is hyper-responsive to ACTH stimulation (Kotelevtsev *et al.* 1997). This study also highlighted the resistance of 11βHSD1-KO mice to obesity- and stress-induced hyperglycaemia (Kotelevtsev *et al.* 1997).

Conversely, the type 2 isozyme, 11βHSD2, is known to reduce the biological activity of glucocorticoids by catalysing the conversion of corticosterone/cortisol into their

inert 11-keto derivatives (Stewart *et al.* 1988). This is especially important in pregnancy, when high expression of 11βHSD2 in the placenta and foetus itself (Burton *et al.* 1968) protects the embryo from exposure to high glucocorticoid levels. Excessive foetal glucocorticoid exposure and loss of this metabolic barrier during the early stages of development can result in low birth weight offspring (Holmes *et al.* 2006) with a predisposition towards diseases such as hypertension and cardiovascular disease in adulthood; an effect known as pre-natal programming or "The Barker Hypothesis" (Barker *et al.* 1986; Barker *et al.* 1990; Barker *et al.* 1993).

Whilst 11βHSD1 is highly expressed in an array of different tissue types, including importantly the liver, 11βHSD2 does not show hepatic expression and is localised to mineralocorticoid (aldosterone) responsive tissues such as the kidney, colon and sweat glands (where glucocorticoid metabolism by the enzyme allows access of mineralocorticoids to MR) as well as in the vasculature and placenta (Rundle *et al.* 1989; Cole 1995; Brown *et al.* 1996; Seckl *et al.* 2001). In the absence of 11βHSD2 activity, glucocorticoids are able to illicitly activate MR in the distal nephron of the kidney leading to sodium retention and hypertension which are symptomatic of apparent mineralocorticoid excess syndrome (Ulick *et al.* 1979). Therefore, in addition their role in hepatic steroid clearance, the expression of metabolic enzymes in a wide number of other organ systems provides an additional level of endocrine control specifically within tissues. In this way, active glucocorticoid levels can be manipulated in a tissue-specific manner, independently of the concentration in the systemic circulation. The expression of 11βHSD isozymes in the brain (e.g. in the hippocampus) means that these enzymes, their activity and their expression ratios to each other can influence glucocorticoid load in the CNS allowing them influence over certain behaviours such as memory, which will be discussed later in detail.

The evidence above highlights the important role of metabolic enzymes in many physiological processes and demonstrates the profound impact disruption of their function can have. This thesis focuses on the importance of 5αR type 1 specifically and demonstrates the peripheral and central effects of genetic disruption of this enzyme *in vivo.*

1.4.4 5α-Reductases (5αRs)

There are three isozymes of 5αR, type 1 (5αR1), type 2 (5αR2) and type 3 (5αR3), encoded by three separate genes (in the mouse, *Srd5a1*, *Srd5a2* and *Srd5a3* respectively), each with individual biochemical characteristics and tissue-specific distribution patterns (Andersson *et al.* 1990; Andersson *et al.* 1991; Uemura *et al.* 2008) which has important physiological implications. 5αR type 1 and 2 (first characterised in the 1950s in rat liver sections; Dorfman *et al.* 1956) catalyse the reduction of $\Delta^{4,5}$ double bonds in almost all keto steroids. 5αRs are hydrophobic due to their particular amino acid content and for this reason are membrane-bound and deeply embedded into the lipid bi-layer. Using NADPH as a cofactor and preferentially inserting the 4*S*-hydrogen of the nicotinamide ring into the 5α configuration of the steroid structure (Wilson 1975), these reductases produce 5α-dihydro metabolites from a number of different steroid substrates. Genetic (Imperato-McGinley *et al.* 1974) and enzymological (Fisher *et al.* 1978) studies have given evidence for the capacity of these enzymes to convert a large number of ketosteroids (virtually all steroids with a $\Delta^{4,5}$,3-oxo structure, see Figure 1.1). These include glucocorticoids, progestagens, androgens and mineralocorticoids which 5αRs convert into their A-ring reduced metabolites (Martini *et al.* 1993; Martini *et al.* 1996) which retain varying degrees of activity of the parent steroid (McInnes *et al.* 2004). For most metabolites of steroid hormones, reduction of the $\Delta^{4,5}$ bond, and reduction of their 3-oxo groups, is thought to largely inactivate transcriptional signalling rendering them more susceptible to reduction by 3α- and 3βHSDs. Additionally, following 5α/5β reduction, metabolites are subject to sulphation and glucuronylation which reduces their affinity for binding proteins and renders them hydrophilic in order to facilitate excretion. The 5αR enzymes were first thought to catalyse complete inactivation of the active steroid e.g. corticosterone into 5α-dihydro-corticosterone. However, contrary to what was previously thought, the subsequent metabolite, 5α-tetrahydro-corticosterone has more recently been shown to retain some activity at GR (McInnes *et al.* 2004; Yang *et al.* 2011).

1.4.4.1.1 5αR substrates

The K_m values for the various substrates of rat and human 5αR1 have been summarised in Table 1.2 (Andersson *et al.* 1990; Normington *et al.* 1992).

Glucocorticoids:

5αR1 clears glucocorticoids from peripheral tissues such as liver and adipose, but also in the CNS (Figure 1.6; Lephart *et al.* 1991; Normington *et al.* 1992; Gottfried-Blackmore *et al.* 2008). Glucocorticoids are not the only important substrates of 5αR1 however and this enzyme is also involved in *de novo* steroidogenesis of progestagens.

Progestagens:

It has been shown in humans as well as in rodents that 5αR1 (and 5αR2) in the brain can convert progesterone into 5α-dihydroprogesterone (5αDHP); a major hormone in the circulation of both normal hormonal cycling women and pregnant women (Milewich *et al.* 1975; Milewich *et al.* 1977). In the brains of both humans and rodents, 5αDHP can be further metabolised by 3αHSD to form 3α-hydroxy-5α-pregnan-20-one (ALLO; Hara *et al.* 1988; Pawlowski *et al.* 1991; Hoog *et al.* 1994; Penning *et al.* 2003; Penning *et al.* 2004). ALLO is a neurosteroid which has potent positive allosteric modulatory effects of γ-aminobutyric acid (GABA) binding to the $GABA_A$ receptor (Mellon *et al.* 2002) resulting in regulatory effects upon various psychophysiological phenomena. Additionally, neurosteroids such as ALLO affect HPA axis function: ALLO treatment of ADX and gonadectomised (GDX) rats resulted in reduced ACTH and corticosterone in response to acute stress (Patchev *et al.* 1996) whilst central injection with an ALLO anti-serum prevented the negative effects of ALLO upon the HPA axis resulting in an exaggerated stress-induced corticosterone flux (Guo *et al.* 1995).

Substrate	K_m Value for Rat 5αR1 (μM)
Corticosterone	16.6
Cortisol	18.9
Progesterone	0.5
Testosterone	2.5
Androstenedione	2.8

Table 1.2 A comparison of the K_m values for rat 5αR1

K_m values for the type 1 5α reductase isozyme were obtained from publications by Andersson *et al.* and Normington *et al.* (Andersson *et al.* 1990; Normington *et al.* 1992).

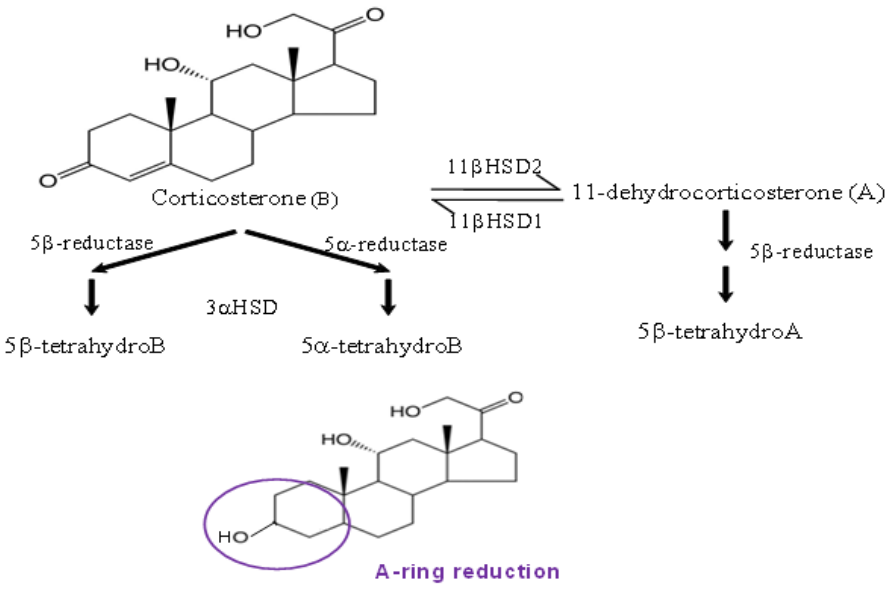

Figure 1.6 Enzymatic glucocorticoid metabolism

Metabolic pathways by which A-ring reductases (5αR and 5βR) and 11βHSD convert active steroid (corticosterone) into its less active metabolites. 5αR = 5α reductase, 5βR = 5 β reductase, 11βHSD = 11β hydroxysteroid dehydrogenase, 3αHSD = 3α hydroxysteroid dehydrogenase.

Androgens:

5αRs are crucial regulators of androgen action. 5αR2 plays the more important role, due to its high expression in the male reproductive organs (Andersson *et al.* 1991), but both the type 1 and type 2 isozymes are capable of metabolising testosterone into the 50 times more potent 5α-dihydrotestosterone (5αDHT) thus 5αR1 also contributes to plasma 5αDHT levels (Gisleskog *et al.* 1998; Amory *et al.* 2007).

Androgens are generally thought to exert negative effects on the HPA axis. Following GDX in male rats, CRH levels in the hypothalamus were significantly increased but this was preventable by androgen replacement therapy (5αDHT) given at the time of castration (Bingaman *et al.* 1994). Similarly, plasma ACTH and corticosterone were higher in GDX vs., intact rats following shock and novel environment stress, and treatment with either testosterone or 5αDHT reduced ACTH and corticosterone back to levels seen in intact controls (Handa *et al.* 1994).

The behavioural effects of androgens and how these may be modifiable by 5αR1 is less well documented. However, Frye *et al.* have reported that the aggression-enhancing effects of testosterone treatment in GDX mice were abolished in 5αR1-KO mice (Frye *et al.* 2002) suggesting that 5αDHT may be the main androgen involved in mediating male aggressive behaviours.

Studies have shown that 5αR1 is the dominant isozyme in the rodent brain (Lephart 1993) where the enzyme is essential in neuroprotective, catabolic processes (Poletti *et al.* 1998). 5αR1 is largely capable of metabolising all ketosteroids, however, the main focus of this thesis is 5αR1 and its capacity to metabolise glucocorticoids, most interestingly in the CNS. It is proposed that 5αR1 may regulate the exposure of not only peripheral tissues but also the brain to glucocorticoid actions and that the level of its activity may determine disease progression or predisposition. Characterisation has largely been carried out using rat tissue and more work is required in the mouse which is the focus of this thesis.

1.4.4.1.2 5α-reductase type 1 (5αR1)

The type 1 isozyme with an alkaline pH optima (6-8.5; Andersson *et al.* 1991; Normington *et al.* 1992) is abundant in liver in both humans (Thigpen *et al.* 1993) and rodents (Normington *et al.* 1992; Mahendroo *et al.* 1999) and brain (Lephart *et al.* 1991) including the nuclei of the hypothalamus (Campbell *et al.* 1989; Pelletier *et al.* 1994), pituitary (Massa *et al.* 1972; Yokoi *et al.* 1996) cerebral cortex and sub-cortical white matter (Poletti *et al.* 1990). It is also expressed at lower levels in adipose tissue (Barat *et al.* 2007; Wake *et al.* 2007). 5αR1 expression shows sexual dimorphism whereby activity and mRNA abundance was 10-20 times higher in females compared to males (Yates *et al.* 1958; Farkash *et al.* 1988; Andersson *et al.* 1989; Normington *et al.* 1992). 5αR1 is found in the adrenal glands (Fraser 1990) where its activity is modifiable by sex steroids. For example, GDX in both male and female rats induces increased adrenal 5αR1 activity and mRNA content (Ando *et al.* 1989; Lephart *et al.* 1991). Tissue distribution also differs between species. For example, 5αR1 and 5αR2 are detected in the rat ventral prostate whilst in human and mouse prostate, 5αR2 shows dominant if not exclusive reductase activity (Russell *et al.* 1994). Moreover, in the mouse liver 5αR1 is exclusively expressed (Mahendroo *et al.* 1996; Mahendroo *et al.* 1997) whilst both 5αR1 and 5αR2 show hepatic expression in humans (Evans *et al.* 2003).

1.4.4.1.3 5α-reductase type 2 (5αR2)

The second 5αR isozyme is the product of a separate gene and differs in its pH optima (a narrow acidic pH centered around 5; Andersson *et al.* 1991; Normington *et al.* 1992) and tissue distribution pattern. In reproductive tissue (i.e. androgen target organs) such as the testes and the ventral prostate, type 2 predominates (Andersson *et al.* 1991) but 5αR2 is also expressed in the human but not rodent liver. 5αR2 is also essential for the conversion of testosterone into the 50-fold more potent 5αDHT (the principle androgenic stimulant within the prostate gland; Kovacs *et al.* 1983; McConnell 1995; Bartsch *et al.* 2002; Carson *et al.* 2003) which determines male phenotypic sexual differentiation (e.g. development of the male external organs and growth of the prostate) but can also induces hyperplasia of the prostate. It was the latter that lead to the use of 5αR inhibitors (5αRIs; both dual [dutasteride] and type 2

specific [finasteride]) in a clinical setting to treat disorders such as benign prostatic hyperplasia (BPH) where the underlying etiology requires the action of 5αDHT.

1.4.4.1.4 5α-reductase type 3 (5αR3)

5αR3 has been less well studied as its discovery is fairly novel (Uemura *et al.* 2008). There remains ambiguity over whether this third isozyme is involved in steroid hormone formation. Some have reported 5αR3 does not play a role in steroid hormone formation and sexual development since homozygous *Srd5a3* gene mutations found via genome-wide linkage analysis and DNA sequencing, did not result in abnormalities usually associated with impaired steroid metabolism (Cantagrel *et al.* 2010). Instead, the same authors suggested a role for 5αR3 in the N-linked glycosylation of proteins since the phenotypes related to *Srd5a3* mutations were strikingly similar to those arising from congenital disorders of glycosylation (Cantagrel *et al.* 2010). However, there is evidence that 5αR3 does have the ability to reduce steroids including testosterone, androstenedione and progesterone but there is no suggestion of a role for the type 3 isozyme in glucocorticoid metabolism (Azzouni *et al.* 2012).

1.5 5α-Reductase type 1 and glucocorticoid action

Many authors investigating 5αRs have focussed on their role in regulating androgen action and the enzymes role in glucocorticoid action is less well studied. Authors investigating human (Andrew *et al.* 1998; Fraser *et al.* 1999) and rodent (Livingstone *et al.* 2000) obesity report increased excretion of cortisol/corticosterone as 5α-reduced metabolites compared to lean controls. In the publication by Livingstone *et al*. rats were also hypercorticosteroneaemic suggesting the activity of 5αR1 was enhanced to facilitate increased glucocorticoid clearance. Therefore, 5αR1 plays a key role in the aetiology of obesity and other metabolic diseases (Tomlinson *et al.* 2008; Baudrand *et al.* 2011). It was hypothesised that global loss of 5αR1 would release the brake on glucocorticoid action leading to metabolic and CNS disturbances due to reduced glucocorticoid clearance both peripherally and centrally.

1.5.1 Generation of the 5αR1-KO mouse model and an outline of the preliminary data leading to this study

In 1996, Mahendroo *et al.* generated a mouse model with global disruption (null allele) of the *Srd5a1* gene, denoted the 5αR1-KO mouse (Mahendroo *et al.* 1996). Since then, this model has been used primarily to study the effect of reduced androgen and progesterone metabolism *in vivo.* Although male 5αR1-KO mice are reproductively sound, females had a serious parturition defect whereby 70% of pregnancies failed to end at term due to impaired cervical ripening resulting in foetal death *in utero* (Mahendroo *et al.* 1996; Mahendroo *et al.* 1999; Mahendroo *et al.* 1999). However, our group is mostly interested in the resulting phenotype of reduced glucocorticoid clearance by 5αR1 which has been much less well studied.

Using the above model, Livingstone *et al.* established a colony of mice heterozygous for the *Srd5a1* allele, allowing generation of a 5αR1-knock-out mouse model at the University of Edinburgh. It is proposed that global loss of this glucocorticoid metabolising enzyme in these animals may lead to the accumulation of active glucocorticoids, most notably within the liver and brain, and indeed phenotypic changes consistent with increased corticosteroid levels were observed.

1.5.2 Metabolic and peripheral phenotypes

Preliminary data has shown that the absence of 5αR1 renders 5αR1-KO mice more susceptible to features of the Metabolic Syndrome. On a high fat (HF) diet, male 5αR1-KO mice demonstrated greater weight gain (Figure 1.7), possibly in part due to increased cumulative food intake compared to wild-type (WT) controls (Livingstone *et al.* 2014a). Moreover, male 5αR1-KO mice were hyperinsulinaemic (insulin measured during a glucose tolerance test) compared to WT mice and deposited more fat in their livers following the HF diet, leading to hepatic steatosis (Livingstone *et al.* 2014a).

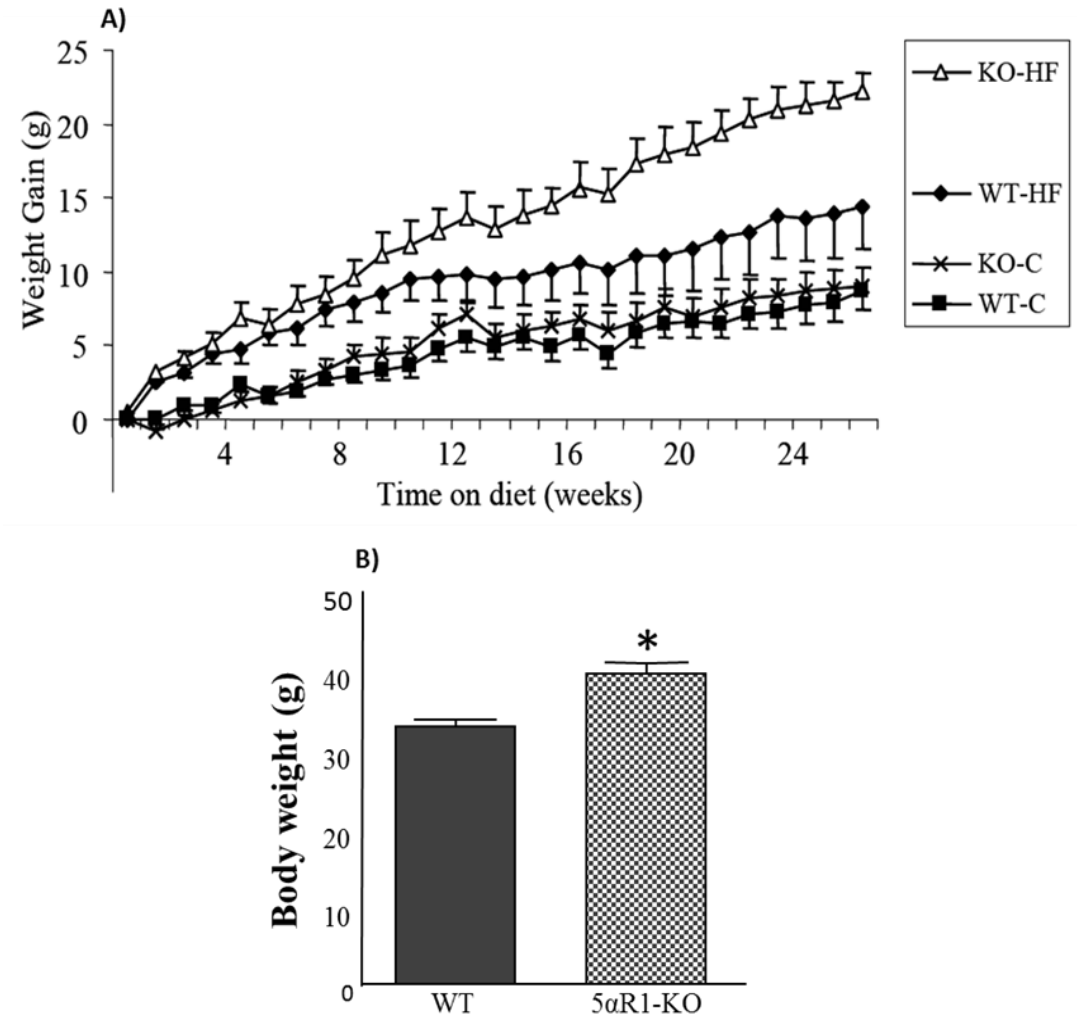

Figure 1.7 Cumulative weight gain in male 5αR1-KO mice

This preliminary data (A) showed that male 5αR1-KO mice were comparable in weight to wild-type (WT) controls when fed a low fat diet (crossed symbols and square symbols respectively). The phenotype changed when the animals were fed a high fat diet where 5αR1-KO mice (triangles) gained significantly more weight than WT controls fed the same food (diamonds). Panel B shows the final weight at cull where 5αR1-KO animals (checkered) weighed more vs. WT (solid) at 12 months of age. KO-HF = 5αR1 knock-out animals fed high fat diet, WT-HF = wild-type animals fed high fat diet, KO-C = 5αR1 knock-out animals fed control low fat diet, WT-C = wild-type animals fed control low fat diet (Livingstone *et al.* 2014a).

Increased weight gain may be of peripheral origin or explained solely by hyperphagia, however the molecular mechanisms underlying increased food intake on a HF diet had not yet been fully studied at the undertaking of this Ph.D. Furthermore, this adverse metabolic phenotype was particularly marked in female 5αR1-KO mice who gained more weight even when fed a normal chow diet (Livingstone *et al.* unpublished). At the outset of this thesis, the reason for this sexual dimorphism in susceptibly remained to be elucidated.

1.5.3 Behavioural and central phenotypes

It appears that the obesity phenotype in female 5αR1-KO mice is modulated by factors influencing behaviour. Intriguingly, this cohort only developed obesity and hepatic steatosis when group-housed. When these females were subjected to social isolation (housed in single cages as opposed to in groups) they lost all of the extra weight and became comparable to WT littermate controls; a phenomenon which was reversible within 4 weeks of reverting to a group-housing scenario (Livingstone *et al.* unpublished).

Although baseline corticosterone levels are maintained in both male and female 5R1-KO mice, plasma corticosterone concentrations were lower in 5αR1-KO mice compared to WT following both minor stress (cage disturbance) and acute restraint stress (Livingstone *et al.* 2014b for male mice and Livingstone *et al.* unpublished for female mice).

1.5.4 Data supporting development of hypotheses

1.5.4.1 Metabolic disturbances

In terms of the metabolic phenotype, loss of the braking effects of 5αR1 upon glucocorticoid action within tissues led to weight gain and the development of symptoms consistent with the metabolic syndrome (Livingstone *et al.* 2014a). This adverse metabolic phenotype is suggestive of a role for this enzyme in regulating tissue glucocorticoid levels and this study aims to determine the mechanisms which underlie the poor metabolic profile of 5αR1-KO mice. In this study, it is proposed that at least some of the phenotypes may be due to altered *GR* and *MR* expression in

tissues. Downregulation of corticosteroid receptor expression may be expected in the presence of excessively high glucocorticoid levels, suggested to form the basis in linking HPA axis function with the aetiology of metabolic diseases and also with psychiatric disorders. Moreover, since changes in both CRH and CRHR-1 are known to influence behaviour as well as both basal and peak corticosterone levels reached post-stress (Muglia *et al.* 1995; Smith *et al.* 1998; Preil *et al.* 2001; Bale *et al.* 2002; Bale *et al.* 2002), these parameters may also be modified in 5αR1-KO mice.

1.5.4.2 The effect of glucocorticoids and 5αR1 in the brain

Female 5αR1-KO mice have been shown to be predisposed to weight gain when housed in groups but weight loss when housed under social isolation stress. An important consideration in terms of this observation is that, in rodents, chow intake is usually decreased under chronic stress (Dallman *et al.* 2001) and this can be modulated by glucocorticoids (Pralong *et al.* 1993; Alfarez *et al.* 2008). Certainly, a large number of studies have used social isolation of rodents as a model of chronic stress which induces detrimental changes in central processes such as locomotor activity, thermoregulation, learning and memory, anxiety and weight gain (Juraska *et al.* 1984; Bates *et al.* 1985; Jones *et al.* 1991; Hellemans *et al.* 2004; Võikar *et al.* 2005). In this model, it is proposed that environmental stress (social isolation) in the milieu of local increases in glucocorticoids within the brain may lead to anxiety, manifesting as weight loss in the 5αR1-KO female mice, which was the starting point for this study.

1.5.4.3 Neuroendocrine control of food intake in 5αR1-KO mice

There is evidence for disruption of glucocorticoid clearance in peripheral tissue as described in the preliminary data section above (1.5.2). There is also some evidence of CNS disturbances such as a sensitivity to stress (single housing). It is therefore possible that the intricate balance in levels of anorexigenic and orexigenic neuropeptides in the brain may be altered in 5αR1 KO mice with glucocorticoid excess, promoting increased food consumption (hypothalamic regions) and thus driving hyperphagia. For example, orexigenic peptides may be increased whilst

anorexigenic peptides including POMC would be down-regulated. However, if global loss of 5αR1 in these mice leads to an anxious phenotype and thus chronic stress during periods of social isolation, this could be a key mechanism behind weight loss in this particular cohort of mice.

1.5.4.4 5αR1, the hippocampus and cognition

Much research has been published under the umbrella of glucocorticoid-mediated learning and memory modifications. For example, 11βHSD1 is upregulated in the aged mouse hippocampus resulting in memory impairments (Holmes *et al.* 2010) whilst mice 11βHSD1-KO mice have reduced intrahippocampal corticosterone levels but normal circulating concentrations and are protected from age-related cognitive impairments (Yau *et al.* 2001). Although 5αR1 has not previously been studied with direct relevance to its role in cognition, these data, taken together with the evidence outlined in section 1.3.2.1.2 suggests that our model of impaired glucocorticoid metabolism and resulting excess may be expected to cause adverse effects upon the hippocampus and learning and memory abilities.

To conclude, since 5αR1 is highly expressed within numerous brain structures including subcortical white matter, the cerebral cortex and the hippocampus (Celotti *et al.* 1987; Melcangi *et al.* 1987; Melcangi *et al.* 1993) it is therefore likely to influence behaviour, as has been shown previously (Agis-Balboa *et al.* 2007). There is supporting evidence from preliminary studies by Livingstone *et al.* for the role of 5αR1 upon CNS function through altered stress responses (reduced HPA axis activation post-stress; Livingstone *et al.* 2014b) and feeding behaviours (hyperphagia and subsequent weight gain; Livingstone *et al.* 2014a). The aim of this thesis is to assess the effects of impaired glucocorticoid inactivation (through manipulating 5αR1) on HPA axis responses and behaviours including anxiety and cognitive ability determined by brain areas such as the hypothalamus and the hippocampus.

1.6 Hypothesis

Female 5αR1-KO mice are more prone to obesity than male mice due to behavioural differences affecting energy balance, including:

- A predisposition to hyperphagia through changes in neuroendocrine signalling.
- Reduced locomotor activity.

Furthermore this predisposition to obesity is modified by the environmental influence of housing, due to 5αR1-KO mice being more susceptible to anxiety-induced weight loss. Reduced ability to clear glucocorticoids predisposes 5αR1 mice to anxiety and features of cognitive decline, becoming more marked with age.

1.7 Aims

In this Ph.D. thesis, the following research aims will be addressed:

Aim 1: To determine if loss of 5αR1 modifies the expression of genes coding for hypothalamic neuropeptides which may increase food intake, ultimately inducing weight gain in female 5αR1-KO mice.

Aim 2: To investigate the central mechanisms which underpin attenuated HPA axial responses to acute restraint stress in 5αR1-KO mice.

Aim 3: To establish whether 5αR1-KO mice are predisposed to anxiety under the chronic stress of social isolation. Moreover, to investigate if 5αR1-KO mice are susceptible to cognitive impairment and whether this is exacerbated by social isolation and/or ageing.

Aim 4: To determine if genetic background has any influence on the metabolic and HPA axis phenotypes of 5αR1-KO mice and whether these effects are sexually dimorphic.

Chapter 2: Materials and methods

2.1 Materials

2.1.1 Chemicals

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich, Poole, UK. Storage was at room temperature (RMT; 18-22^{o}C) unless specified otherwise, and away from direct sunlight. Solvents for mass spectrometry assays were glass distilled HPLC grade and were obtained from Fisher Scientific UK Ltd, Loughborough, UK.

2.1.2 Radioactivity

All radioactive isotopically labelled chemicals were purchased from GE Healthcare, Buckinghamshire, UK.

2.2 Buffers and solutions

2.2.1 Borate buffer

130 mM Boric acid, 68 mM sodium hydroxide, 3.5 mL concentrated hydrochloric acid (33% v/v) and 5 g bovine serum albumin (BSA; 0.5% w/v); Cohn fraction V) diluted to 1 L with distilled water to intrinsically give pH 7.4. The solution was stored at -20°C and thawed at RMT immediately before use.

2.2.2 Box buffer

20% v/v 20xSaline sodium citrate buffer and 50% v/v deionised formamide were diluted in DEPC-water (see below).

2.2.3 Cresol red

Cresol red (4 mg/mL) in 40% w/v sucrose.

2.2.4 DEPC-treated water

Diethylpyrocarbonate (DEPC) was prepared in double distilled water (300 μL/100 mL), shaken and allowed to stand at RMT for ~16 hrs prior to autoclaving.

2.2.5 Deionised formamide (for box buffer only/non-sterile)

150 mL Formamide was mixed (1 hr) with 15 g Amberlite ion exchange resin (MB-6113; BDH, Lutterworth, UK), filtered twice through no.1 Whatman filter paper to remove Amberlite and stored at RMT in a brown bottle in the dark.

2.2.6 1 M dithiothreitol (DTT)

0.15 mg/mL DTT in DEPC water. The solution was sterilised by filtration and prepared as aliquots, stored at -20°C.

2.2.7 250 mM ethylene diamine tetra-acetic acid (EDTA; pH 8.0)

100 mL DEPC water was added to 46.5 g EDTA. The volume was made up to 500 mL with DEPC-water and the pH adjusted to 8.0 with 5 M sodium hydroxide (NaOH).

2.2.8 Ethanol in ammonium acetate

300 mM Ammonium acetate was prepared in either 50, 70 or 90% v/v ethanol (EtOH) as specified diluted to 1 L in DEPC water.

2.2.9 Ethanolic potassium hydroxide

Two parts ethanol (EtOH):1 part 30% w/v potassium hydroxide (KOH) prepared in distilled water.

2.2.10 2x hybridisation buffer

1.2 M Sodium chloride (NaCl), 20mM Tris-hydrochloride (Tris-HCl), 400 µl 50xDenhardts, 2 mM EDTA, 0.2 mg/mL salmon sperm DNA, 0.2 mg/mL yeast tRNA and 0.4 mM dextran sulphate in DEPC-treated water (final volume 10 mL). 1 mL aliquots were prepared and stored at -20°C.

2.2.11 5 M sodium chloride (NaCl)

5 M Sodium chloride (NaCl) in DEPC water (500 mL). The solution was sterilised by autoclaving.

2.2.12 4% w/v Paraformaldehyde in 0.1M sodium phosphate buffer

20 mM Sodium dihydrogen phosphate (NaH_2PO_4) and 80 mM disodium hydrogen phosphate (Na_2HPO_4) were prepared in 1 L DEPC water and heated to 80°C prior to addition of 1.3 M paraformaldehyde. The solution was stirred (1 hr) to dissolve, allowed to cool and then stored at 4°C for up to 1 wk.

2.2.13 1x phosphate buffered saline (PBS)

140 mM Sodium chloride (NaCl), 20 mM hydrated disodium hydrogen phosphate ($Na_2HPO_4.H_2O$), 1.5 mM potassium dihydrogen phosphate (KH_2PO_4; both VWR International, Leicestershire, UK) and 2.7 mM potassium chloride (KCl) in DEPC water (final volume 1 L). The resulting solution achieved pH 8.0 and was autoclaved before use.

2.2.14 10x PBS

1.4 M Sodium chloride (NaCl), 0.2 M hydrated disodium phosphate ($Na_2HPO_4.H_2O$), 15 mM monopotassium phosphate (KH_2PO_4) and 27 mM potassium chloride (KCl) were dissolved in 1 L DEPC-water and sterilised in an autoclave before use. The pH was intrinsically 8.0.

2.2.15 2x pre-hybridisation buffer

1.2 M Sodium chloride (NaCl), 20 mM Tris-hydrochloride (Tris-HCl), 400 µL 50xDenhardt's, 2 mM EDTA, 0.2 mg/mL salmon sperm DNA, 0.2 mg/mL yeast tRNA in DEPC-water (final volume 10 mL), prepared as aliquots (1 mL) and stored at -20°C until use.

2.2.16 RNase Buffer

0.5 M sodium chloride (NaCl), 10 mM Tris-hydrochloride (Tris-HCl) and 1mM EDTA were dissolved in 10 mL DEPC-water.

2.2.17 10x TBE (Tris/borate/EDTA) buffer

0.9 M TRIZMA base ($NH_2C(CH_2OH)_3$), 0.9 M boric acid (H_3BO_3) and 20 mM EDTA were dissolved in 1 L distilled water. The resulting solution was pH 8.0.

2.2.18 0.5x TBE buffer

10x TBE (50 mL) was diluted in distilled water (950 mL).

2.2.19 Tris-EDTA (TE) buffer

10 mM Tris-hydrochloride (Tris-HCl) and 1 mM EDTA were dissolved in 1 L DEPC-water. This yielded a solution of pH 8.0, which was sterilised in an autoclave before use.

2.2.20 0.1 M Triethanolamine

1.3% v/v Triethanolamine ($C_6H_{15}NO_3$) was dissolved in 1 L DEPC water. The solution was adjusted to pH 8.0 with 10 M hydrochloric acid (HCl). Sterile glassware was used to avoid autoclaving as this solution cannot be autoclaved.

2.2.21 1 M Tris

1 M Tris-hydrochloride (Tris-HCl) in DEPC water (final volume 550 mL). The pH was adjusted to 7.5 with 10 M hydrochloric acid (HCl) and the solution was autoclaved prior to use.

2.2.22 Wash Buffer One (2x saline sodium citrate [SSC] buffer)

60 mL 20x SSC (Invitrogen, Paisley, UK) was diluted to 600 mL with DEPC water.

2.2.23 Wash Buffer Two (0.1x SSC buffer)

3 mL 20x SSC was diluted to 600 mL with DEPC water.

2.2.24 Yeast tRNA

50 mg/mL Yeast tRNA in DEPC water prepared as aliquots for storage at -20^{o}C.

2.3 Molecular Biology

Total RNA was extracted using Qiagen RNeasy kits and DNA digested using Qiagen RNase free DNase sets, all from Qiagen, West Sussex, UK. The following procedures were all performed at room temperature (RMT; 21^{o}C) unless otherwise stated.

2.3.1 RNA extraction and quality control

The principle of the RNeasy extraction method is as follows. First biological samples are lysed and homogenised in a highly denaturing guanidine-isothiocyanate-containing buffer (RLT) which immediately inactivates RNases ensuring RNA in the sample remains intact during purification. The added ethanol provides conditions which favour RNA binding in high salt conditions to the silica-based membrane whilst contaminants including genomic DNA are washed through it and away. Subsequent wash steps remove the ethanol and salt breaking the attraction between the matrix and the RNA and finally purified RNA is eluted into water.

2.3.1.1 RNA extraction from liver and prostate

RNA was extracted from liver using the Qiagen RNeasy mini kit sourced as above. A small piece of tissue (~30 mg) was excised from the sample on dry ice and homogenised in the RLT buffer provided in the kit (600 μL) using a rotor-stator homogeniser (Pro 200; PRO Scientific Inc., Monroe, CT, USA) to create a uniform, homogenous solution. All samples were then subjected to centrifugation (16,000xg, 3 mins, RMT). The supernatant was then removed by pipetting, transferred to a new microcentrifuge tube containing an equal volume of ethanol (50% for liver, 70% for prostate) and mixed well. The entire volume was transferred into an RNeasy mini

spin column placed in a 2 mL collection tube and subjected to centrifugation (10,000xg, 15 secs, RMT). The eluate was discarded and the column washed with RW1 buffer (700 μL) before centrifugation (10,000xg, 15 secs, RMT). If on-column DNAse digestion was required (2.3.1.5), it was carried out at this point. A further 350 μL RW1 buffer was added into the column and subject to centrifugation (9300xg, 15 secs, RMT), the flow through was discarded. The RNA extraction process was then continued as follows. RPE buffer (500 μL) was added to the columns and the centrifugation process repeated. Further RPE buffer (500 μL) was applied to the columns before centrifugation (9300xg, 2 min, RMT) to wash the spin column membrane. The RNeasy spin columns were then placed into new 2 mL collection tubes and subject to centrifugation (16000xg, 1 min, RMT) to eliminate any RPE buffer carryover. Using a new 1.5 mL Eppendorf, RNase-free water (30 μL) was added directly to the spin column membrane and the eluate was collected by centrifugation (9300xg, 1 min, RMT), added back to the column and the process repeated to maximise the concentration of the eluted RNA. Eluted RNA was stored at -80^{o}C.

2.3.1.2 RNA extraction from adipose tissue

Approximately 60 mg of tissue was excised from frozen samples on dry ice and homogenised in Qiazol Lysis Reagent (800 μL, Qiagen) to create a uniform, homogenous solution. Further Qiazol Lysis Reagent was added (200 μL) before incubation (5 min, RMT). Chloroform (200 μL) was added and then all tubes shaken vigorously (15 sec) before centrifugation (12,000xg, 15 mins, 4^{o}C). The aqueous phase was removed by pipette, transferred to a new microcentrifuge tube containing an equal volume of 70% v/v ethanol and mixed well. The entire volume (in two stages) was transferred into an RNeasy mini spin column placed in a 2 mL collection tube. The remainder of the extraction procedure then continued as outlined above for liver and prostate (section 2.3.1.1).

2.3.1.3 RNA extraction from brain

RNA was extracted from brain as described for adipose tissue above (2.3.1.2) with the addition of on-column DNAse digestion (2.3.1.5). Half a brain (cut sagittally) was used for each sample.

2.3.1.4 RNA extraction from isolated hypothalamus, pituitary and optic chiasm

As the hypothalamus, pituitary and optic chiasm are so small, the entire amount of tissue (<20 mg) was used for each sample. The tissue was disrupted in RLT buffer (350 µL) by pipetting the material up and down vigorously. The lysate was added directly into a QIAshredder spin column and subject to centrifugation (16000xg, 2 mins, RMT). The eluate was then added back into the column and the centrifugation process repeated. RNA was extracted from the homogenate as described above (2.3.1.1) with the inclusion of the on-column DNAse digestion step (2.3.1.5).

2.3.1.5 On-column DNAse digestion for brain tissue

Generally speaking, DNase digestion is not required when purifying RNA by these methods as the silica-gel-membrane and spin column technology usually removes most genomic DNA. However, more complete DNA removal is required for certain RNA applications which are sensitive to very small amount of DNA (i.e. qPCR) and also in situations when the weight of the starting sample was very small (i.e. pituitary or hypothalamus). The potential for genomic DNA carry-over was precluded by DNase treatment of samples as follows. The DNase 1 stock solution was prepared in RNase-free water according to the manufacturer's instructions and divided into single-use aliquots (stored at -20^{o}C). RW1 buffer (350 µL) was added to the spin columns before centrifugation (9300xg, 15 secs, RMT) and the eluate discarded. A master mix of DNase 1 and RDD buffer was prepared and gently inverted allowing for 10 µL DNase stock solution and 70 µL RDD per column. DNase 1 incubation mix was then transferred directly onto the RNeasy spin column membrane (80 µL/column) and incubated (15 min, RMT). 350 µL RW1 buffer was added by pipette into the column and subjected to centrifugation (9300xg, 15 secs, RMT). The

flow through was discarded and the RNA extraction process was then continued as described for liver in section 2.3.1.1 from the first RPE buffer wash step.

2.3.1.6 RNA integrity

RNA integrity was evaluated by electrophoresis (100 V, 1 hr, RMT) in agarose (Lonza, Berkshire, UK) gels (1.2% w/v in 0.5x TBE) containing 0.01% v/v SyBrSafe. Loading dye (Promega, WI, USA; 1 in 5 diluted in distilled water; 10 μL) was added to extracted RNA samples (3 μL) before being loaded into the gel. Visualisation of RNA was carried out at 254nm using an UVIPro platform and software, v12.4 (UVItec, Cambridge, UK) to ensure only two bands (18s and 28s ribosomal (rRNA) bands) were present and to demonstrate exclusion of genomic DNA. RNA integrity was satisfactory when each 28s and 18s rRNA band appeared sharp and clear without smearing, and when the ratio of the 28s:18s band intensity was ~2:1 indicating the extracted RNA was intact. The integrity of the extracted RNA was further determined using an Agilent 2100 Bioanalyzer. An RNA integrity number (RIN) between 8 and 10 was taken to reflect RNA of acceptable quality.

2.3.1.7 RNA concentration and quantification

Finally, the concentration of RNA was quantified using a Nanodrop spectrophotometer (ND-1000, Thermo Fisher Scientific, West Sussex, UK). The absorbance at 260 nm (A260; the maximum absorbance of the nucleic acid) determined the concentration of RNA in the sample. RNA purity was assessed by the ratio of the absorbance at 260nm to the absorbance at 280nm (absorbance of any protein contamination; A260:A280). Purity was deemed acceptable if this ratio was ~1.8-2.0.

2.3.2 Reverse transcription (RT) to synthesise cDNA

Total RNA (200 ng/12 μL solution) was reverse transcribed in a 20 μL (final) reaction using a Quantitect reverse transcription kit (Qiagen, West Sussex, UK) and a G-Storm GS1 thermal cycler (GRI, Essex, UK). Firstly, DNA digestion was carried out: gDNA wipeout buffer (2 μL) was added to the 12 μL RNA samples (total volume 14 μL) before incubation (2 min, 42^{o}C) in the thermal cycler and then placed

on wet ice. The following was then added to each sample: 5x quantiscript RT buffer (4 µL/tube), RT primer mix (1 µL/tube), quantiscript reverse transcriptase (with RNase inhibitor; 1 µL/tube) and the RNA mix containing RNA, water and DNA wipeout (14 µL/tube). Negative controls (one lacking reverse transcriptase enzyme and one lacking template to confirm the absence of contamination by genomic DNA or RNA, respectively) were also prepared and volumes were corrected to 20 µl using RNase-free water. Samples were then incubated; 42^{o}C (15 mins; addition of cDNA nucleotides by reverse transcriptase enzyme), 95^{o}C (3 mins; denaturation of the enzyme) and 4^{o}C (1 hr; storage). All samples were prepared concomitantly to compensate for variability in efficiency of reverse transcription, and all negative controls were run simultaneously. RT reaction products were stored at -20^{o}C until use.

2.3.3 Polymerase Chain Reaction (PCR)

Primers were designed to detect the presence of transcripts of interest (Table 2.1). Primer stocks (100µM) were prepared in TE buffer and stored at -20^{o}C. 10x Primer mix (2µM each primer) was prepared in ultrapure water. Using the Multiplex PCR Kit (Qiagen, West Sussex, UK) a master mix was prepared allowing the following per tube: master mix x2 solution (10 µL), Solution Q (2 µL), 10x primer mix (2 µL) and Qiagen water (5 µL). 19 µL of this master mix was added per tube followed by sample cDNA (1 µL). A further negative control was prepared with Qiagen water added in place of cDNA to confirm absence of contamination. The samples were then placed into the PCR block (G-Storm GS1 thermal cycler, GRI, Essex, UK) and processed under the following programme unless otherwise stated:

Heated lid at 105^{o}C
Denaturation: 94^{o}C (15 mins)
Annealing: 30 cycles X (94^{o}C (30 secs, denaturation), 60^{o}C (1.5 min, primer annealing), 72^{o}C (1 min, elongation))
Elongation: 72^{o}C (10 mins, this step ensures elongation of products to full length).
4^{o}C hold.

Gene Accession No.	Primer Sequence		Product (bp)
Srd5a1 (5αR1) *NM_175283*	**F**	5’-ctacaggagctcccttcaat-3’	122
	R	5’-ctttgcacgtagtggatcag-3’	
Srd5a2 (5αR2) *NM_053188*	**F**	5’-aacacagcgagagtgtgtcg-3’	160
	R	5’-gagaagagacccagcagcac-3’	
Hsd11b1 (11β Hydroxysteroid dehydrogenase 1) NM_008288.2	**F**	5'-aaagtgattgtcacwggggccagcaaa-3'	467
	R	5'-atccaragcaaacttgcttgc-3'	

Table 2.1 Details of primers for conventional PCR amplification
(F = forward; R = reverse; bp = base pair). Conventional primer sequences for murine *Srd5a1* and *Srd5a2* were previously published (Yang *et al.* 2011). The *Hsd11b1* primer was designed by Dr Dawn Livingstone.

2.3.3.1 Assessment of PCR products by electrophoresis

Cresol red loading dye (5 µL) was added to each sample and an aliquot (15 µL) was electrophoresed (100 V, 1 hr, RMT) on a 1.5% w/v agarose (Lonza, Berkshire, UK) 0.5x TBE gel using GelRed fluorescent nucleic acid dye (8 µL/100 µL gel; Cambridge BioScience Ltd, UK). The gel was visualised and photographed using the UVIPro platform and software as before. The presence of one strong band of the appropriate size (as compared to a 1Kb ladder; Promega, Southampton, UK) was determined as a positive result. The presence or absence of a band in the positive and negative control wells respectively, was confirmed by eye.

2.3.3.2 Purification of PCR products

A GENECLEAN *Turbo* Kit (ABgene, Surrey, UK) was used for the purification of cDNA PCR products in preparation for sequencing. Purification was achieved through DNA binding to the silica membrane of the column in a high salt concentration. When the salt concentration is lowered during washing, rehydration of the silica matrix breaks the attraction between the matrix and the DNA, allowing only the purified DNA to be eluted in either water or a low salt buffer. For purification from PCR products, the whole solution was placed in a 1.5 mL microcentrifuge tube before the GENECLEAN *Turbo* salt solution (5 volumes) was added and gently mixed. This mixture (≤600 µL) was transferred to a GENECLEAN *Turbo* cartridge placed inside a cap-less catch tube and subject to centrifugation (14,000xg, 5 secs). The catch tube was emptied. The wash buffer was added to the cartridge (500 µL) before further centrifugation (14,000xg, 5 secs). The catch tube was emptied and the cartridges subject to centrifugation once again (14,000xg, 4 mins) to drive the last of the wash buffer through the membrane and into the catch tube. cDNA was eluted into a clean catch tube via the addition of the kit's elution solution (30 µL) directly onto the membrane before incubation (5 mins). Samples were subjected to centrifugation (14,000xg, 1 min) to elute the purified cDNA which was then stored at -20^{o}C until use.

2.3.3.3 Sequencing

Purified DNA samples were quantified using a Nanodrop spectrophotometer (ND-1000). The absorbance at 260 nm (A260) determined the concentration of DNA in the sample and the purity was assessed by the ratio of absorbance at 260 nm to the absorbance at 280 nm (A260:A280); acceptable if ~1.8. Each cDNA sample was diluted to provide 1.3 ng DNA/5 µL sample and added to 3.2pmol of each primer (in separate tubes) used in the initial PCR reaction in 200 µL volume strip-tubes. Samples were sequenced by Sanger Sequencing at The Gene Pool, King's Buildings, The University of Edinburgh using a BigDye Terminator Cycle Sequencing ready Reaction Kit which determines the presence of each deoxynucleotide base in turn by its unique, identifying fluorescent colour.

2.3.4 Quantitative real-time polymerase chain reaction (qPCR)

The abundances of selected transcripts were quantified by qPCR using gene specific primers and probes (Table 2.2) and the Roche LightCycler® 480 master mix and detection system (Roche Diagnostics, Burges Hill, UK). To prepare the standard curve, equal volumes of all cDNA samples to be compared were pooled and serially diluted (1/8 through to 1/512) with RNase-free water. cDNA samples were also diluted (1/20) with RNase-free water. Where possible, custom primers (Table 2.2) were designed in order to use the Universal Probe Library provided by Roche Diagnostics, Burgess Hill, UK. Custom primers were synthesised by Life Technologies, Paisley. When using these primers the reaction mix consisted of: LightCycler® probemaster mix (5 µL), Roche qPCR grade water (2.7 µL), forward primer (0.1 µL), reverse primer (0.1 µL) and the appropriate probe (0.1 µL). Master reaction mix (8 µL) for each gene was added to the 384-well plate, followed by diluted cDNA samples (2 µL), standard, or negative control, all in triplicate. Excellent qPCR assay efficiency would demonstrate a change of roughly 3.3 cycles between 10 fold dilutions of template.

Gene Accession No.		Sequence	UPL Probe No.
Agrp (Agouti-related protein) NM_007427.2	F	5’-caggctctgttcccagagtt-3’	84
	R	5’-tctagcacctccgccaaa-3’	
Akr1c6 (3α hydroxysteroid dehydrogenase) NM_030611	F	5’-tggggtgtccaactttaacc-3’	78
	R	5’-tccctgattgagataaggatcac-3’	
Akr1d1 (5β Reductase) NM_145364.1	F	5’-gaaaagatagcagaagggaaggt-3’	103
	R	5’-gggacatgctctgtattccataa-3’	
Avp *(*Arginine vasopressin) NM_009732	F	5’- gctgccaggaggagaactac-3’	84
	R	5’- aaaaccgtcgtggcactc-3’	
Cartpt1/2 (Cocaine and amphetamine-regulated prepropeptide transcript variants 1 and 2) NM_013732 (1) NM_001081493.1 (2)	F	5’-cgagaagaagtacggccaag-3’	108
	R	5’-ctggccccttcctcact-3’	
Crh (Corticotropin releasing hormone) NM_205769	F	5’-gaggcatcctgagagaagtcc-3’	34
	R	5’-tgttaggggcgctctc-3’	
Gapdh (glyceraldehyde-3-phosphate dehydrogenase) NM_008084	F	5’-gggttcctataaatacgcactgc-3’	52
	R	5’-ccattttgtctacgggacga-3’	
Hsd11b1 (11β Hydroxysteroid dehydrogenase 1) NM_008288.2	F	5’-tctacaaatgaagagttcagaccag-3’	1
	R	5’-gccccagtgacaatcacttt-3’	
Mc3r (Melanocortin 3 Receptor) NM_008561	F	5’-aaagccctcaccttgatcg-3’	84
	R	5’-gaacatcacgccgcagat-3’	
Mc4r (Melanocortin 4	F	5’-tctgagcagtgtacttcccaac-3’	34

Receptor) NM_016977	R	5’-gatattttcaaccaaagcattacaca-3’	
Npy (Neuropeptide Y) NM_023456	F	5’-ccgctctgcgacactacat-3’	9
	R	5’-tgtctcagggctggatctct-3’	
Nr3c1 (Glucocorticoid Receptor) NM_008173	F	5’-tgacgtgtggaagctgtaaagt-3’	56
	R	5’-catttcttccagcacaaaggt-3’	
Nr3c2 (Mineralocorticoid Receptor) NM_001083906	F	5’-caaaagagccgtggaagg-3’	11
	R	5’-tttctccgaatcttatcaataatgc-3’	
Srd5a1 (5α Reductase 1) NM_175283	F	5’-gggaaactggatacaaaataccc-3’	86
	R	5’-aaacgagctccccaaaata-3’	
Actb (β-actin) *NM_007393*		Taqman ABI (Mm00607939_S1)	
Crhr1 (Corticotropin releasing hormone receptor 1) NM_007762		Taqman ABI (Mm00432670_m1)	
Ppia Cyclophilin A *NM_008907*		Taqman ABI (Mm02342429_G1)	
Rn18s (18s ribosomal RNA) NR_003278.1		Taqman ABI (HS99999901_S1)	
Tbp (TATA box-binding protein) *NM_013684.3*		Taqman ABI (Mm00446973_m1)	

Table 2.2 Custom primers and probes and ABI primer/probe assays used for qPCR
Custom primers were designed using the Universal Probe Library (Roche Diagnostics, Burgess Hill, UK) and manufactured by Life Technologies, Paisley, UK.

Whenever it was not possible to custom design primer sets, ready-made Taqman assays were purchased from Applied Biosystems, Warrington, UK. When using Taqman® assays the reaction mix was prepared as follows: LightCycler® probemaster mix (5 μL), Taqman® combined primer and probe mix (0.5 μL) and Roche qPCR grade water (2.5 μL per well).

Plates were sealed and subject to centrifugation (2,000xg, 2 mins, RMT) to ensure all solutions were mixed thoroughly in the wells and to remove any air bubbles. Reaction conditions were as follows: pre-incubation/ enzyme activation (5 mins, 95^{o}C), amplification (50 cycles x (10 secs at 95^{o}C, 30 secs at 60^{o}C and 1 sec at 72^{o}C)), followed by cooling (30 secs, 40^{o}C). The LightCycler® software provided absolute quantification relative to the standard curve using Cp (crossing point) values. The Cp refers to the point at which the fluorescence in the reaction reaches the maximum of the second derivative of the amplification plot. This corresponds to the point where the fluorescent signal has reached its maximum acceleration which should always be in the middle of the log-linear portion of the amplification curve. Cp is also defined as the point at which the fluorescence in the sample exceeds background levels. Triplicates were averaged and deemed acceptable when the standard deviation of the Cp values was < 0.5 cycles. A standard curve (y axis crossing point, x axis log concentration) was generated for each transcript, fitted with a straight line and deemed acceptable if reaction efficiency was 1.7-2.1. The abundances of five reference genes, Cyclophilin A (*Ppia*), 18s ribosomal RNA *(Rn18s*), TATA box-binding protein (*Tbp*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and β-actin (*Actb*) were quantified. The abundance of transcripts of interest were normalised against the abundance of the reference gene (or combinations of reference genes) which showed the least variability between experimental groups with no statistically significant differences.

2.3.5 Quantification of transcripts via *in situ* hybridisation

2.3.5.1 Sectioning of frozen tissue

Brains were sectioned using a Leica cryostat (10 μm thick slices). Regions of interest were identified by periodic staining of representative sections using Pyronin

Y before being viewed under the microscope. Sections were mounted onto electrostatic slides and stored in pre-frozen plastic slide boxes at -80^{o}C until use. *In situ* hybridisation was performed using radiolabelled probes to hybridise to transcripts of interest in various brain regions.

2.3.5.2 Labelling of radioactive probes

Prepared cDNA templates were provided by Senior Technician Karen French. To synthesise radiolabelled probes the following reagents (Promega, Southampton, UK) were added in the order specified to an RNase-free Eppendorf: 2 μL 5x Transcription buffer, 1 μL of a master mix of 10 mM ATP, 10 mM CTP and 10 mM GTP, 0.5 μL of 200 mM DTT, 0.4 μL RNase inhibitor, 1 μL template (antisense or sense), 1 μL RNA polymerase (T7, T3 or SP6) and 4 μL ^{35}S–UTP. Antisense probes were designed to be complimentary to the mRNA of interest allowing binding during the hybridisation phase. Sense probes have an identical sequence to the mRNA of interest and therefore will not hybridise and are used as a negative control on a representative selection of slides.

The above prepared mixture was gently stirred and incubated (60-90 mins, 37^{o}C [T7 and T3], or 40^{o}C [SP6]). After incubation, 1 μL RNase-free DNase 1 (Promega) was added before further incubation (15 mins, 37^{o}C). Both the sense and anti-sense probes were purified through Nick size exclusion columns (GE Healthcare) using 400 μL TE buffer) to elute the probe. The eluted probe (1 μL) was added to 1 mL scintillation fluid (Meridian, Epsom, Surrey, UK) and the β emissions counted in a liquid scintillation counter (145 MicrobetaPLUS, Walla, PerkinElmer, UK). A minimum of 10 million counts/mL was required to be used per slide for hybridisation. The remainder was stored at -20^{o}C until use.

2.3.5.3 Tissue fixation

Using sterilised metal racks and glass troughs (baked in an oven to approximately 170-200^{o}C), slides were initially incubated in 4% w/v paraformaldehyde in 0.1M sodium phosphate (10 mins, 4^{o}C). They were then washed twice in 1x PBS (2 x 5 mins) and incubated in 0.1 M triethanolamine containing 0.25% v/v acetic anhydride

(10 mins), agitating slightly throughout. Slides were washed in 1x PBS (5 mins) before sequential dehydration in 70, 80 and 95% v/v ethanol (2 mins each).

2.3.5.4 Prehybridisation

The prehybridisation mixture (200 µL) was added to each slide covering all the sections equally. Slides were then placed in RNase-free, plastic boxes lined with 3M paper and containing box buffer before incubation (3 hrs, 50^{o}C).

2.3.5.5 Hybridisation

The hybridisation mixture comprised of DTT (1M, 10 µL), the calculated volume of radioactive probe to give counts of 10,000,000 cpm/mL on each slide, volumes of 2x hybridisation buffer appropriate to provide enough solution for all slides and deionised formamide (500 µL/mL of final mix; sterile solution from Sigma-Aldrich, Poole, UK). The deionised formamide, 2x hybridisation solution and probe were mixed and incubated (10 mins, 75^{o}C) then cooled on wet ice (1 min) before the DTT (10µL, 1M) was added and mixed well. Slides were removed from the oven and excess pre-hybridisation buffer was drained off. Hybridisation mix (200 µL) containing the labelled probe was added evenly to each slide. Additional slides were used as controls where a mixture containing the sense probe for each transcript was added to separate slides (as described above) to indicate the degree of non-specific binding. All slides were incubated again in the RNase-free boxes (16 hrs, 50^{o}C).

2.3.5.6 RNase treatment and washes

Slides were removed from the oven and excess hybridisation mix was drained off. The slides were washed in 2x SSC (3x 5 mins). RNase enzyme (3 µL/mL, Promega, Southampton, UK) was added to the appropriate volume of RNase buffer required to cover all slides with 200 µl. RNase buffer (50 mL) was poured into plastic incubation boxes lined with 3M paper. The RNase-containing solution (200 µL) was evenly applied onto each of the slides, which were carefully placed into the prepared boxes and incubated (1 hr, 37^{o}C). The slides were removed from the oven and loaded into plastic racks for washing. The first wash was in 2x SSC (60 mins). Slides were then washed in 0.1x SSC (60 mins, 60^{o}C). For the third wash, slides

were placed in 0.1x SSC (60 mins, pre-heated to 60^{o}C and then left to cool to RMT). Finally, to dehydrate the sections, the slides were passed through 50, 70 and 90% v/v ethanol in ammonium acetate (0.3 M; 2 mins each), before being allowed to air dry (16 hrs) in the fume hood and placed against Kodak HyperMax film (GE Healthcare, Buckinghamshire, UK) in a light tight cassette for 1 wk. The films were developed using a Konica SRX-101 developer.

2.3.5.7 Dipping, developing and staining

In the darkroom, NTB2 emulsion (Anachem, Bedfordshire, UK) was placed in a water bath (42^{o}C) to melt to liquid form. The emulsion was then diluted 1:1 with water and allowed to stand (30 mins), allowing any air bubbles to disperse. Each slide was dipped in turn into the emulsion/water solution, placed in a rack and allowed to dry in the darkroom (16 hrs). The following day the slides were packed into light-tight boxes, wrapped in aluminium foil and stored (4 wks, 4^{o}C). The slides were unwrapped in the dark room and allowed to return to RMT. Four troughs were prepared containing Kodak developer diluted 1:1 with tap water, fixative diluted 1:4 with tap water (both H. A. West Medical Ltd, Edinburgh, UK) and two troughs of water only. All liquids were cooled to 15^{o}C. The slides were processed as follows: 4 mins developer, 10 secs tap water, 5 mins fixer, 5 mins tap water. The solutions were changed after processing two racks and the slides were then allowed to dry. Slides were stained (3 mins) in 1% Pyronin Y (1 g / 100 mL) filtered through Whatman paper. The slides were then passed through three troughs of ultrapure water (3x 1 min) and allowed to dry (16 hrs). They were then passed through acetone (2 mins), acetone/xylene, 1:1 (2 mins), xylene (2 mins) and xylene (2 mins) before being mounted in dibutyl phthalate xylene (DPX; VWR Int, Poole, UK). Cover-slips were applied and the slides were stored at RMT until analysis.

2.3.5.8 Quantification of mRNA abundance

Using Zeiss KS300 3.0 computer software, silver grains were quantified in the dentate gyrus (DG), pyramidal cells at CA1, CA2, CA3 and CA4 of the hippocampus, and the PVN of the hypothalamus. The number of silver grains in each region is representative of the abundance of mRNAs of the gene of interest.

Only one side of the brain/section/slide was counted for each animal. The final "count" was the mean from six randomly placed circles (radius = 43μm) minus background count. This was representative of the count/neuron (pyramidal neurons in the hippocampus and parvocellular neurosecretory neurons in the PVN).

2.4 Biochemical assays

All wash steps were performed using an electronic AW1 microplate washer (Rosys Anthos [Biochrom], Cambridge, UK). Incubations requiring agitation were performed using an electronic plate shaker (iEMS incubator/shaker, Thermo Scientific, Northumberland, UK). For all assays, the fitting of a best-fit straight or sigmoidal line and the reproducibility of duplicates were acceptable when r^2 was greater than 0.98 and when the results of duplicates were within 10% of the mean, respectively. Intra-assay relative standard deviations (RSD) were calculated using the equation shown in section 2.7.

2.4.1 Quantification of plasma insulin concentrations by enzyme-linked immunosorbent assay (ELISA)

Plasma insulin concentrations were quantified using an Ultra-Sensitive Mouse Insulin Enzyme-Linked Immunosorbent Assay (ELISA; Crystal Chem Inc., Illinois, US). This is a basic sandwich ELISA where insulin in the sample binds to the surface of the well coated with guinea pig, anti-insulin antibodies. The secondary, horse-radish peroxidase- (HRP) conjugated, anti-insulin antibody binds to the guinea pig anti-insulin antibody/mouse insulin complex immobilised to the microplate well. Addition of 3,3',5,5'-tetramethylbenzidine TMB substrate produces a colourometric reaction.

Using the insulin stock solution and sample diluent supplied, a series of standards were prepared (0.1 – 12.8 ng/mL) according to the manufacturer's instructions. Sample diluent (95 μL) and then standards or sample (5 μL) were added in duplicate to wells pre-coated with anti-insulin primary antibody (guinea pig) and incubated (2 hrs, 4^{o}C). Sample diluent was used for the blank measurement. After incubation the supplied wash buffer was diluted (1 in 20) with distilled water and used to wash

the plate (5 x 300 μL) to remove unbound materials prior to the second incubation. HRP-conjugated, anti-insulin antibody (100 μL) was added to all wells before incubation (30 min, RMT). To remove unbound HRP-conjugate, the plate was then washed for a second time (7 x 300 μL) using the diluted wash buffer as before. 100μl of the TMB-containing enzyme substrate solution (light sensitive) was added to each well and incubated once more (40 min, RMT, in the dark) before the stop solution (0.5 M sulphuric acid, 100 μL) was added to arrest the reaction. The absorbance at 450 nm (L1) and 630 nm (L2; background count resulting from scratches or artefacts on the plate) were recorded using a spectrophotometer (OPTImax tunable microplate reader, Molecular Devices). L2 values were subtracted from L1 values to remove background counts and thus increase the accuracy of the reading. A standard curve (x-axis concentration, y-axis absorbance) was drawn and a best-fit straight line applied from which the concentration of unknowns could be interpolated.

2.4.2 Quantification of plasma glucose concentrations using a hexokinase assay

Glucose levels were quantified from plasma using an Infinity Glucose Hexokinase Liquid Stable Reagent (Thermo Scientific, ABgene, Surrey, UK). The principles of this assay are as follows. The reagent supplied contains hexokinase, which catalyses the phosphorylation of glucose by ATP, producing ADP and glucose-6-phosphate. The latter is then oxidised to 6-phosphogluconate with the associated reduction of NAD+ to NADH by glucose-6-phosphate dehydrogenase. The concentration of NADH formed is proportional to the concentration of glucose in the sample. Using the glucose standard supplied in the kit (200 mg/dL) and distilled water, a series of dilutions (50 – 300 mg/dL) were prepared. Distilled water was also used as a blank. Samples and standards (2 μL, in duplicate) were added to 96-well plates before addition of the hexokinase reagent (supplied). Plates were incubated (10 mins, RMT) and then the absorbance recorded at 340 nm spectophotometrically (OPTImax tunable microplate reader, Molecular Devices). A straight line was fitted to the standard curve where the x-axis = concentration (mg/dL) and the y-axis = absorbance at 340 nm.

2.4.3 Quantification of liver triglycerides

2.4.3.1 Extraction

To create a saponified, neutralised extract of liver, ~100 mg of frozen tissue was cut and weighed and added to ethanolic potassium hydroxide (KOH; 200 µL) before incubation (14 hrs, 55^{o}C). Samples were vortexed until a homogenous solution was made. 50% ethanol was added (400 µL) before samples were subjected to centrifugation (15,000xg, 5 mins, RMT). 200 µL of the supernatant was transferred to a new tube before 1M magnesium chloride ($MgCl_2$) was added (215 µL) and the samples vortexed and incubated (10 mins, on wet ice). After the incubation, samples were again subjected to centrifugation (15,000xg, 5 mins, RMT) and the supernatant transferred to a new tube. An assay of triglyceride content was then performed as follows below.

2.4.3.2 Quantification

Liver triglycerides were quantified using an *Infinity* Triglycerides Liquid Stable Reagent (Thermo Scientific, ABgene, Surrey, UK). The assay is based on the following: lipoprotein lipase hydrolyses triglycerides into glycerol and non-esterified fatty acids (NEFAs). Glycerol is then phosphorylated by ATP with glycerol kinase to produce ADP and glycerol-3-phosphate. Oxidation of the latter by glycerolphosphatase produces hydrogen peroxide which reacts with 4-aminoantipyrine and 3, 5-dichloro-2-hydroxybenzenesulfonate to produce a red colour. The absorbance of this red-coloured dye is proportional to the concentration of triglycerides in the sample.

Serial dilutions of the triglyceride standard provided were prepared (0.565 – 11.3 M) using distilled water. Distilled water was also used as a blank. Samples or standards (duplicate; 2 µL) were added to wells in a 96-well plate, followed by the reagent (200 µL). The plate was incubated (5 min, 37ºC, in the dark) before the difference in absorbance between 600 nm and 500 nm was recorded for each well. A standard curve was drawn (x axis concentration, y axis absorbance) and a straight line fitted.

2.4.4 Quantification of corticosterone in plasma using an enzyme-linked immunosorbent assay (ELISA)

A commercially available corticosterone ELISA kit (Enzo Life Sciences, Exeter, UK) was used to quantify the concentration of corticosterone in plasma. The corticosterone ELISA uses a polyclonal antibody for corticosterone to competitively bind corticosterone in the standard, sample or an alkaline phosphatase molecule which has corticosterone covalently attached to it. After incubation, substrate is added to induce the colourometric reaction.

2.4.4.1 Method development

Samples were thawed from -20^{o}C and subject to one of three preparation methods (A, B or C) to determine the optimal protocol to yield the most reproducible results.

- Method A used equal volumes (10 μL) of plasma and the steroid displacement reagent (SDR; diluted 1/100 with assay buffer) provided in the kit. The mixture was incubated (5 mins, RMT) in sterile glass tubes before the final volume was adjusted to 500 μL with the assay buffer 15 (AB15) provided.
- Method B was identical to method A except after incubation with SDR, samples were subject to extraction as follows. AB15 (280 μL) and ethyl acetate (3 mL) were added to each sample, vortexed vigorously, allowed to stand for 2 mins and then vortexed once more. The uppermost, clear, organic layer was removed using a glass pipette and bulb and transferred to a fresh glass tube before the samples were reduced to dryness under oxygen-free N_2 (60^{o}C). When dry, samples were stored (16 hrs, 20^{o}C). The following day samples were reconstituted into 500 μL AB15 and vortexed vigorously.
- Method C was identical to method B apart from the addition of water (10 μL) instead of SDR in the initial incubation step.

Method B was selected as the most reproducible with the best, most robust set of replicates.

2.4.4.2 Corticosterone ELISA procedure

Using AB15 as the diluent, standard solutions were prepared fresh on the day of the experiment and within 1 hour of use, from the 200,000 pg/mL stock solution provided. Serial halving dilutions were made to achieve a series of concentrations ranging from 20,000 - 10 pg/mL. 100 µL of standards and samples were added to appropriate wells along with the blue conjugate and the yellow antibody solutions (50 µL of each) as per manufacturer's instructions. The plate was incubated on a plate shaker (400 rpm, 2 hrs, RMT) and then washed using the electronic plate washing machine and the wash buffer provided (3 x 300 µL). The pNpp substrate (200 µL) was added to every well and incubated (1 hr, RMT) until the stop solution (50 µL) was added. The absorbance was recorded spectrophotometrically (TECAN, infinite M1000) at 405 nm with correction at 580 nm (background). The absorbance at 580 nm was subtracted from that measured at 405 nm. The absorbance measurements were inserted into the Assay Zap software program from which the standard curve (inverse sigmoidal) could be plotted (semi-log plot: x-axis log [corticosterone], y axis % binding; B/B_o).

2.4.5 Quantification of corticosterone in plasma by radioimmunoassay (RIA)

Plasma corticosterone was quantified using an in-house RIA (Al-Dujaili *et al*. 1981). In this assay, radio-labelled corticosterone ($[^3H]_4$) competes with corticosterone in the sample for antibody sites. Scintillation occurred when scintillation proximity assay (SPA) beads bound to the fraction of primary antibody which was associated with $[^3H]_4$-corticosterone. Due to the competition between binding of unlabelled and labelled corticosterone to the primary antibody, the extent of scintillation decreased as the concentration of unlabelled corticosterone increases.

Standards were prepared from a stock solution of corticosterone (32µM in ethanol) using borate buffer to obtain a diluted series (0.6-320 nM). Plasma was thawed on wet ice, diluted with borate buffer (10% v/v) and incubated (30 mins, 80^oC,) to denature CBG, before being placed immediately onto wet ice. Sample (20 µL), standard or blank (borate buffer) was added in duplicate to appropriate wells.

Volumes of borate buffer/1,2,6,7-[^{3}H]$_4$-corticosterone (0.034 MBq/μL; approx. 6 mL buffer plus 2 μL [^{3}H]$_4$-corticosterone stock) were mixed in appropriate proportions to achieve radioactivity counts of around 8-12000 cpm/50μL. The primary antibody stock (anti-mouse raised in sheep, kindly provided by Dr Chris Kenyon) was stored at 1/100 dilution. This solution (60 μL) was added to buffer/[^{3}H]$_4$ corticosterone mixture (6 mL) to give a final primary antibody dilution of 1/10,000. An aliquot (50 μL) was added into each well, followed by SPA beads (50 μL; coated with anti-sheep secondary antibody; GE Healthcare Life Sciences, Buckinghamshire, UK and prepared in borate buffer). The plate was sealed, inverted to mix and allowed to incubate (16-20 hrs, RMT) prior to scintillation counting in a β-counter (2 min cycle/well; 145 MicrobetaPLUS, Walla, PerkinElmer, UK). A semi-log, inverse sigmoidal standard curve was fitted with GraphPad Prism software using B/B_o (bound/bound at zero; y axis) vs. log [corticosterone, M] (x axis).

2.4.6 Quantification of plasma leptin levels by ELISA

Plasma leptin concentrations were quantified using a mouse leptin ELISA kit (Crystal Chem Inc., Illinois, US) which is based on the following reactions. Wells are pre-coated with rabbit anti-leptin antibody which first binds endogenous mouse leptin in the sample. In the first reaction, the anti-leptin IgG of the guinea pig anti-serum added to the plate, binds to the mouse leptin immobilised to the base of the microplate well. In the second reaction, HRP-conjugated anti-guinea pig IgG antibody is then bound to the guinea pig anti-leptin IgG of the immobilised "rabbit anti-leptin/mouse leptin/guinea pig anti-leptin IgG" complex. During the enzyme reaction, the bound HRP-conjugated antibody is detected by the addition of the 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution.

From the lyophilised leptin stock solution, a series of standards were prepared (0.1 – 12.8 ng/mL) in the sample diluent supplied and according to the manufacturer's protocol. First, all wells were washed with the diluted wash buffer (2x 300μL). Into each well, sample diluent (45 μL) and guinea pig anti-mouse leptin serum (50 μL) were added. Standards or samples (5 μL) were added in duplicate to wells and incubated (16-20 hrs, 4^{o}C). Sample diluent was used as a blank measurement. After

incubation, the plate was washed (5x 300μL) to remove unbound leptin before the second incubation. HRP–conjugated anti-leptin antibody (100 μL) was added to all wells before incubation (3hrs, 4^{o}C). To remove unbound HRP-conjugate, the plate was then washed for a second time (7x 300μl) using the diluted wash buffer as before. 100 μL of the TMB-containing enzyme substrate solution (light sensitive) was added immediately to all wells and incubated once more (30 mins, RMT, in the dark) until the stop solution (0.5 M sulphuric acid, 100 μL) was added to arrest the reaction. Within 30 mins, the absorbance at 450 nm (L1) and 630 nm (L2; background count) was recorded using a spectrophotometer (OPTImax tunable microplate reader, Molecular Devices). L2 values were subtracted from L1 values to remove background counts and thus increase the accuracy of the reading. A standard curve (x-axis concentration, y-axis absorbance) was drawn and a straight, best-fit line applied from which the concentration of unknowns could be interpolated.

2.5 Determination of tissue steroid levels by liquid chromatography-mass spectrometry (LC-MS/MS)

LC-MS/MS was used to measure corticosterone (B) concentrations in liver and brain.

2.5.1 Steroid extraction from tissues

Steroid extractions were performed using glass tubes to prevent absorption by plastic.

2.5.1.1 Liver

A stock solution of corticosterone (1 mg/mL) and standards ranging from 0.1 ng to 100 ng were prepared in methanol. An internal standard of 2,2,4,6,6,17α,21,21-[^{2}H]$_{8}$B (d8-B; 100 ng; Cambridge Isotopes, MA, USA) was also prepared (1 mg/mL) and added to all standards and samples after homogenisation. ~300 mg of tissue was weighed into 2 mL glass tubes on dry ice and methanol:water (7:2, v/v, 2 mL) was added. The tissue was then homogenised using a rotor-stator homogeniser (Pro 200) and stored on dry ice before the internal standard (100ng) was added. Samples and standards were then processed as follows. Tubes were shaken using an IKA

Vibramax (x1000, 15 min) and then subject to centrifugation (3200xg, 45 min, 4^{o}C). The supernatant was transferred to a fresh tube and reduced to dryness under N_2 (60^{o}C) then reconstituted in methanol (10 mL) and vortexed. Hexane (10 mL) was added and the solution mixed prior to removal of the top organic layer using a glass Pasteur pipette attached to a vacuum. The remaining methanol layer was transferred to a clean tube and reduced to dryness under N_2 (60^{o}C). Once the extracted steroids were dry, they were reconstituted in 30% methanol (v/v, 5 mL). Varian C18 columns (C18; 2 g) were prepared by twice adding 10 mL methanol and allowing it to be drawn through the column over a vacuum tank to clean the columns. This process was repeated with 2 x 10 mL water to prime the columns. The reconstituted steroids and samples (5 mL) were then passed through the columns followed by 2 x 10 mL water and the eluate discarded. Steroids were eluted into glass collection tubes by the addition of methanol (5 mL). Samples were reduced to dryness under N_2 (60^{o}C) and reconstituted in LCMS mobile phase (50 µL; 50:50; v/v; methanol + 0.1% v/v formic acid: water + 0.1% v/v formic acid) before storage at -20^{o}C and analysis.

2.5.1.2 Brain

Stock and standard solutions and the internal standard were prepared as in section 2.5.1.1. Half of each sagittally-sectioned brain (~200 mg) was weighed and placed into a 2 mL glass tube on dry ice before ethyl acetate:ethanol (1 mL; 1:1, v/v) was added. The tissue was then homogenised using a rotor-stator homogeniser (Pro 200) and stored on dry ice before the internal standard (100 ng) was added and mixed. 10 mL of a prepared solution of ethanol: acetic acid: water (95:3:2 v/v) was added to fresh glass tubes and chilled on dry ice before the brain homogenate (or standard) was added drip by drip using a glass Pasteur pipette to form a crystal-like structure). Samples were stored (16 hrs, -20^{o}C).

Samples were brought to 4^{o}C on wet ice before sonication (8 x 15 sec bursts, placed on ice between bursts) in ice-cold water and then subject to centrifugation (3000xg, 30 mins, 4^{o}C). The supernatant was transferred to a fresh tube and reduced to dryness (under N_2, 60^{o}C) before re-suspension in methanol (10 mL) and storage (16

hrs, -20^{o}C). The protocol then continued exactly as described in 2.5.1.1 from the addition of hexane (10 mL) following removal of the supernatant to a clean glass tube.

2.5.2 Analysis

The following was performed by staff of the Wellcome Trust Mass Spectroscopy Core, University of Edinburgh/BHF Centre for Cardiovascular Science. Analysis was performed on a QTrap 5500 Mass Spectrometer (AB Sciex, Warrington, UK), interfaced with an Acquity UPLC (Waters, Manchester, UK) and operated with Analyst software (v1.5.1). Samples were injected onto an X-Select HSS PFP column (2.5 μm, 3 mm, 100 mm, Waters, Riccarton, UK) maintained at 25^{o}C. Initially the mobile phase consisted of water:methanol (each with 0.1% formic acid), 50:50, flowing at 0.3 mL/min and the proportions changed across a linear gradient to 40:60 between 1 and 2.5 mins, and then 35:65 by 5 mins, and maintained until 7 mins before re-equilibrating.

Selected reaction monitoring of mono-protonated species was performed to quantify steroids of interest as follows: corticosterone, m/z 347.1 - 91.1 and d8-corticosterone 355.1-337, using a de-clustering potential (66, 116V), collision energy (69, 19V) and collision cell exit potential (8, 14V) respectively. The source conditions were as follows: curtain gas (N_2) 35psi, the nebuliser current 3μA, temperature 450^{o}C and ion source gas 35 psi.

2.6 *In vivo* experiments

2.6.1 Animal breeding

All mice (WT and 5αR1-KO littermates) were obtained from established in-house breeding colonies. 5αR1-KO mice were originally generated by global disruption of the *Srd5a1* gene on a mixed genetic background (C57BL/6j/SvEv/129; denoted mixed strain; Mahendroo *et al.* 1996). These mice were also bred onto the uniform C57BL/6j strain (mice from Harlan Olac, Bicester, UK; denoted C57Bl/6j strain) by continuous back-crossing for 10 generations. Heterozygous female mice were bred with heterozygous males to generate the wild-type (WT) and 5αR1-KO mice used

experimentally. Both mixed strain and C57Bl/6j mice were used *in vivo* and their tissues *in vitro* where specified. Sex and genotype ratios were all produced at the expected Mendelian ratios and all mice were bred under licensed approval from the U.K. Home Office.

2.6.2 Genotyping for WT and 5αR1-KO mice

2.6.2.1 DNA extraction

DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen; West Sussex, UK). The basic principles of the DNeasy extraction method consist of cell lysis using the enzymatic action of proteinase K. The addition of guanidine hydrochloride and ethanol provide optimal conditions for the binding of DNA to the spin column membrane whilst simple centrifugation forces contaminants such as proteins to pass through. Remaining contaminants and enzyme inhibitors are removed in the two wash steps before the low salt buffer is used to elute the pure extracted DNA from the membrane.

Samples obtained from ear marking (<25 mg tissue) were digested overnight (16 hrs, 55^{o}C, under rotation) in 180 µL supplied buffer ATL plus 20 µL of the supplied proteinase K (>600 mAU/ml solution). The subsequent steps were all performed at RMT. Following digestion, samples were vortexed (15 secs) before buffer AL was added (200 µL) and vortexed again thoroughly. 100% ethanol was then pipetted into the samples (200 µL) and mixed thoroughly. The mixture was pipetted into DNeasy mini spin columns placed in 2 mL collection tubes and subject to centrifugation (6000xg, 1 min). Spin columns were placed in new 2 mL collection tubes before buffer AW1 was added (500 µL) and subjected to centrifugation (6000xg, 1 min). Again placing the columns into new 2 mL collection tubes, buffer AW2 was added (500 µL) before centrifugation (20,000xg, 3 mins). The columns were placed into a 1.5 mL Eppendorf and the DNA eluted using AE buffer (200 µL) by centrifugation (6000xg, 1 min) after a brief incubation (1 min, RMT).

2.6.2.1.1 PCR

PCR was performed as described in section 2.3.3 using the following primers: 5αR1-KO primer (5'-GATTGGGAAGACAATAGCAGGCATGC-3'), common primer (5'-CCAGACACGAACTTCCACGCTTCTG-3') and the WT primer (5'-ATGGAGTTGGATGAGTTGTGC-3'). This primer combination results in the amplification of a 400bp band in the presence of the WT allele and a 360bp band in the presence of the disrupted allele. Bands were visualised as in section 2.3.3.1. WT mice were identified when one band was seen at 400bp, 5αR1-KO mice when a band was seen at 360bp and heterozygous (HET) mice when two bands were seen, one at both 360 and one at 400bp (both the WT and the 5αR1-KO allele present; see Figure 2.1 for clarification).

2.6.3 Animal husbandry

Animals were studied in accordance with the guidelines of the UK Home Office (Project Licence P60/3962 Holmes, Personal Licence P60/12166 Di Rollo). WT and 5αR1-KO mice on each background were bred from in-house colonies (generated as described above). After weaning, mice were housed in groups of 4-6 animals according to their genotype, unless specified otherwise. They were given food (chow, RM1 801002; Special Diet Services, Witham, UK) and water *ad libitum,* room temperature was maintained at 22-23^{o}C and lights in the facility were on between 0700 and 1900 hours. All animals were weighed weekly.

2.6.4 Body weight and food intake

Body weight was measured once weekly throughout the experiment and again just prior to cull using a set of CS 200 scales (OHAUS, UK) which weigh accurately to 2 decimal places. To determine food intake, chow pellets were weighed and placed in the cage food hopper. The weight of the chow pellets remaining in the food hopper the following week was subtracted from the initial food weight to calculate weekly consumption in grams which was then multiplied by the calorific content in 1g of the standard mouse chow to calculate kcal consumed per week, cumulatively. Food was also weighed using CS 200 scales.

Figure 2.1 Genotyping result of experimental animals

Representative image of gel electrophoresis result for the purpose of genotyping. In WT animals one band was seen at 400 bp, 5αR1-KO mice had one band at 360 bp and HET mice had a band at both 400 and 360 bp, representing the presence of both the WT and 5αR1-KO allele. WT = wild-type mouse, HET = heterozygous mouse and KO = 5α-reductase type 1 knock-out mouse, bp = base pair.

2.6.5 Plasma collection

Basal (non-stressed; 0730 – 0830 hrs) blood samples were obtained by tail venesection and collected into EDTA dipotassium salt-coated vials (Microvette CB300, Sarstedt, Numbrecht, Germany). The same process was also performed during any experiment where blood was collected via tail venesection. At cull, trunk blood was collected following decapitation and immediately mixed with EDTA solution (0.5M, 50µl). In both cases, the plasma fraction was then separated by centrifugation (2000xg, 4°C, 15 min), carefully transferred to a fresh Eppendorf and stored at -20°C.

2.6.6 Insulin Tolerance Test

Mice were fasted for 4 hours (1000 hrs – 1400 hrs) before being weighed and immediately prior to injection of the insulin bolus, a basal blood sample was taken (time 0). One drop of blood was placed onto a Freestyle glucose strip and inserted into a Freestyle blood glucose monitoring system which gave an automatic read out of glucose concentrations. Mice were then given an intraperitoneal injection of insulin (0.5 mU/g body weight for males and 0.75 mU/g body weight for females). Subsequent blood samples were taken at 15, 30, 60 and 90 mins post-injection; each time the glucose concentration was measured using the hand-held Freestyle blood glucose monitoring system.

2.6.7 Glucose Tolerance Test

Mice were fasted in individual cages for 6 hrs (0800 hrs – 1400 hrs) before animals were weighed and a basal blood sample (time 0) was taken. Glucose (40% w/v in distilled water) was administered by intraperitoneal injection to give a dose of 2 mg glucose per kg body weight. Blood was collected via tail nick (section 2.6.5) at 15, 30, 60 and 90 mins after the glucose load. Animals were then transferred to clean cages with free access to food and water. Plasma was prepared as described (section 2.6.5) and used in an ELISA to quantify insulin concentrations (section 2.4.1) across the time course. The concentration of glucose both basally and across the time course of the experiment were also quantified from the collected plasma via a glucose hexokinase assay (section 2.4.2).

2.6.8 Behavioural analyses.

2.6.8.1 Dry Mazes

All animals were moved to a designated quiet room for 48 hrs before behavioural tests designed to investigate anxiety and learning and memory were initiated. Tests were carried out during the light period; 0900 to 1300 hrs. On the day of the experiment, animals were brought into the behavioural suite and allowed to acclimatise at the rear of the room in dim light for 30 mins prior to testing. All behavioural tests were recorded using the ANY maze tracking system and computer software (Stoelting, Illinois, US) which enabled full data analysis. The maze was dimly lit with free standing lights and a Lux meter was used to ensure all areas of the maze apparatus were illuminated by equal light intensities (between 10 and 12 lux). The observer was blinded to genotype during all behavioural experiments, which were carried out in the order specified below (from the test most sensitive to stress to the most anxiogenic test). The apparatus was wiped clean with ethanol between testing of different mice.

2.6.8.1.1 Elevated plus maze

In the elevated plus maze (EPM; a test of anxiety), four arms were designated either "closed" or "open", and the "central zone" comprised the area where the four arms met (see Figure 2.2, I for clarification). The mouse was initially placed into the central zone facing one of the open arms and recording began. The time spent in each zone, number of open and closed arm entries, speed and distance were recorded over 5 mins when the animal was allowed to explore freely. Relative movement and time spent in each zone was considered a reflection of the anxiety level of the animal due to the natural preference of rodents for small, dark and enclosed spaces.

2.6.8.1.2 Open field test

To further assess anxiety, exploratory behaviour in the open field test (OFT) was investigated. The animals were placed one at a time into the centre of the open field box, placed on the floor, and allowed to roam freely for 5 mins (Figure 2.2, ii further highlights the OFT protocol used). Parameters measured from observed recordings were ambulation (number of segments crossed fully by all four paws or entries into

each zone), time spent in each zone (inner or outer), speed and distance travelled in each zone (Lai *et al.* 2007). General activity was assessed by total movement, and general movement within the inner zone was considered a reflection on the anxious state of the animal.

2.6.8.1.3 Y maze

Spatial memory-was assessed using the Y-maze apparatus which consisted of three enclosed arms made of black Plexiglas (see Figure 2.2, iii for details) and was elevated onto a round table sitting about 50 cm above the ground. The arms were labelled A, B or C whilst "centre" was the triangular area where are three arms met. Spatial cues (e.g. stuffed toy, Lego model etc.) were set up around the maze and positioned appropriately to allow the animals to visualise them clearly whilst inside the maze. The end of each arm was also labelled with a black and white image. The maze floor was spread with sawdust from each of the animal's cage and mixed in between trials to block olfactory cues. The complete test consists of two trials separated by a time interval (inter-trial interval; ITI) of either 1 min or 2 hrs.

1 min ITI – on the first day the animal was placed at the end of a randomly assigned arm and allowed to roam freely for 5 mins. During this **acquisition trial** the entrance to one of the arms (again randomly assigned) was prevented with a plastic stopping block. After the elapsed time the animal was taken out for 1 min (placed on its cage lid) and then put back in for a further 2 mins, this time allowing the animal to freely explore all three arms of the maze **(retention trial**). The extra-maze spatial cues should aid the animal's ability to recognise the novel arm and due to their naturally inquisitive nature, should spend more time exploring this arm during the retention trial. The animal is expected to freely explore the novel arm more than the other two arms during this time. The purpose of the 1 min ITI test was to ensure the apparatus, testing environment and computer software was all set up appropriately and that all animals were capable of completing the task with a short ITI of only 1 min.

2 hr ITI – 4-5 days after the 1 min ITI trial, animals were re-tested in the maze with an ITI of 2 hrs. The animal was placed into a novel start arm with a novel closed arm and the cues arranged outside the maze were different compared to initial 1 min ITI test. The animal was in the maze for 5 mins, taken out and returned to the home cage for 2 hrs before being put back into the maze for a final 2 mins with all arms open during this final retention trial.

The time spent in the novel arm post-ITI was calculated as a percentage of the total test time (2 mins). Values were compared with a random "chance" level (33%) for time spent exploring each of the three arms (Yau *et al.* 2007).

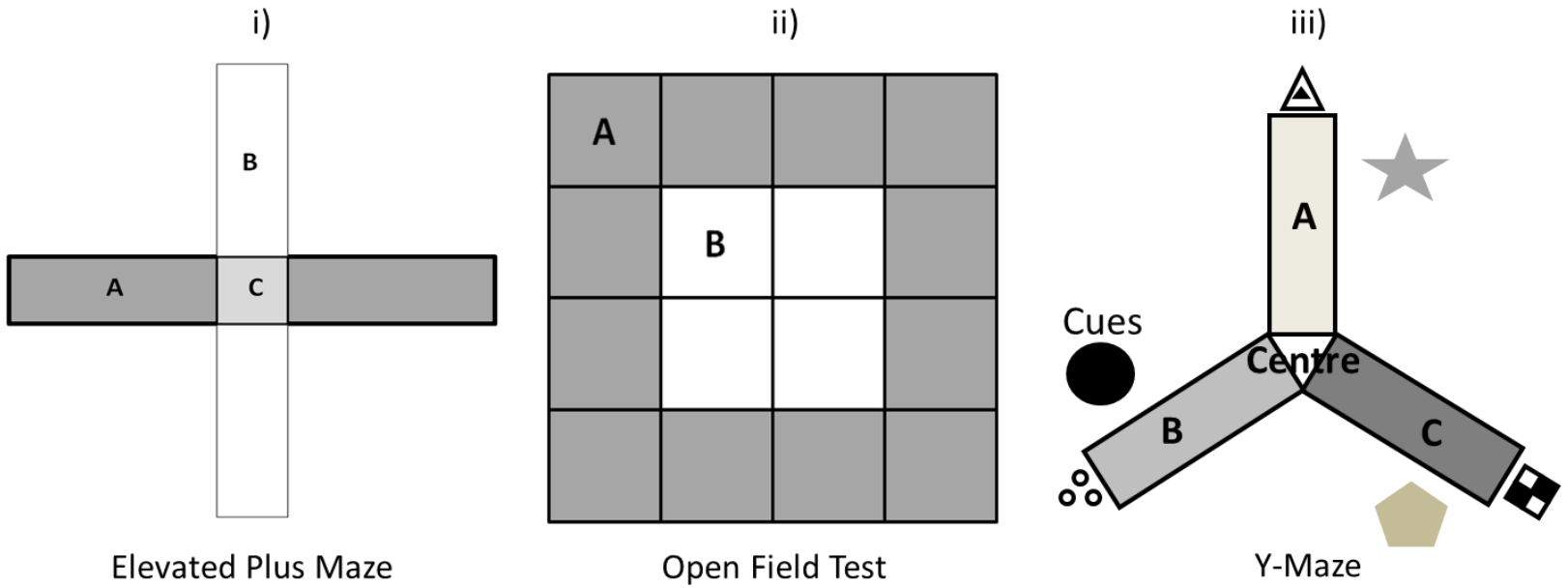

Figure 2.2 Schematic diagram of the mazes used to assess behaviour

i) shows the elevated plus maze (measure of anxiety) where the apparatus, elevated 50 cm from the ground consisted of two opposing open arms (30x5 cm), two opposing closed arms (30x5x15 cm) and a central area of 5 cm^2. Closed arms (A, dark grey, least anxiogenic), were enclosed by opaque Perspex walls 15 cm high. Open arms (B; white) were considered most anxiogenic. The central zone (C; light grey) although neutral is more anxiogenic than A but less than B. ii) shows the open field test (a test of anxiety) where the apparatus (50x50x25 cm) was divided into 16 equal squares. The perimeter of the test field was designated "outer zone" (A, grey, least anxiogenic) and the centre of the test field constituted the "inner zone" (B, white, most anxiogenic). iii) shows the Y-maze apparatus (a test of memory) consisting of 3 arms (50x11x10 cm) A, B or C (progressively darker grey tones) made from black Plexiglas and set at 120^o to each other. Plastic images (triangle, circles or square) were permanently in place at the end of each arm but the cues placed around the maze to aid spatial memory were changeable. A small, black, Perspex block could be slotted between the centre zone and either A, B or C to block off a designated arm.

2.6.8.2 Morris water maze

To further asses learning ability and spatial memory retention, mice were trained in an open-field water maze (120 cm diameter) surrounded by prominent extra-maze visual cues (from which they can build a spatial memory of the area). These cues were placed at various points around the room and maintained in a fixed position throughout testing. The pool was filled with water (26°C) and made opaque using liquid latex (Strathbond Ltd, Ayrshire, UK). The task required the mouse to navigate towards and climb onto a hidden platform (diameter 15 cm) submerged 1-2 cm under water. Like the extra-maze cues, the platform remained in a fixed position throughout testing (in the centre of one of four quadrants in the pool). The animals were given 4 trials/day and released into the pool from a different compass point (North, East, South or West) at the beginning of each trial. When the mouse was placed into the pool facing the wall the trial began and was ended when the platform was located. If however, the animal did not find the platform within the allocated time (120 secs) it was placed there by the experimenter and left for 30 secs. After each trial the animal was briefly dried with a towel and returned to its home cage placed on a heating pad. Each trial was performed on all the animals before the next trial was started. The latency (to the platform) and swim paths of the mice were monitored by a video camera mounted on the ceiling and by a computerised tracking system (Water maze software; Actimetrics Inc., IL, US). Initially the mice had been subjected to non-spatial training (2 days) where curtains were pulled around the perimeter of the pool. On this occasion the location of the platform was made visible by placing a Lego block (8 cm tall) onto the platform. This tested for visual, motivational or motor deficits in all groups of mice and if any animals were unable to locate the platform within 30s by the end of day 2 they were excluded from the spatial memory training.

The animal's spatial learning ability was then tested where the curtains were pulled back to reveal the extra-maze cues located around the room. This time the platform was completely invisible whilst submerged under water. The animals were then tested in the same format as above (for 5 consecutive days), using their spatial memory to locate the platform. At the end of day 5 (1 hr after the last training trial)

and on day 6 (24 hrs after the last training trial), mice were subject to a retention (probe) test where the platform was removed from the pool and the mice allowed to swim freely for 60 secs. The swim path was recorded along with the total time spent in the platform ("training") quadrant and platform crossings during the 60 sec test. Each training day, the average latency (in seconds) of all 4 trials was calculated for each animal. The mean of this average latency was then calculated for each group/day and graphed.

2.6.8.3 Free ambulatory activity

AM524 single layer X, Y, IR Locomotor Activity Monitors and associated Amonlite software were used to measure basal nocturnal activity (Linton Instrumentations, Norfolk, UK). The AM524 uses an array of infrared beams to determine activity and mobility of a subject. The beams are arranged on a single level in an 8 x 16 pitched grid which allows normal X,Y movement to be measured. Pilot studies determined that equal locomotive activity was recorded from the same mice on three consecutive days demonstrating no acclimatisation period was necessary before experimental testing began. On the evening of testing, group-housed mice were placed into single cages before being positioned within the infrared frames. Nocturnal activity was then recorded for 14.5 hrs to encompass the dark phase (1800 hrs – 0830 hrs the following day). When the IR light beams were broken by the animals' movement, total activity counts were recorded by the Amonlite software. Total active time as well as distance covered was also recorded. During testing, animals had free access to chow and water and were left alone in the room for the duration of testing in order to minimise external visual, auditory and olfactory stimuli that might alter their normal activity.

2.6.9 Assessment of HPA axis function and glucocorticoid clearance

2.6.9.1 Acute Restraint Stress

In order to determine differences in HPA axis responses between WT and 5αR1-KO mice on a C57Bl/6j background, animals were subjected to a highly stressful restraining event to elicit a rapid but acute stress response. On the day of the

experiment, a basal (0730 hrs; time 0 mins) blood sample was taken by venesection as described in section 2.6.5 1 hr prior to restraint stress before the animals were allowed to recover quietly in their home cage. At 0830 hrs the animals were placed one by one into a small Perspex tube, small enough that they could not move or turn around, before a stopper was placed into the end to prevent escape. Mice were held in position for 15 mins after which a blood sample was taken prior to recovery in their home cages. Further blood samples were taken throughout the recovery phase at 30, 60 and 90 mins post-stress. Plasma samples were then prepared and stored as described (section 2.6.5) to be used to quantify circulating corticosterone levels by ELISA (see section 2.4.4.2 for details).

2.6.9.2 Adrenalectomy surgery

The following was performed by Dr Dawn Livingstone of the University of Edinburgh/BHF Centre for Cardiovascular Science. Immediately prior to surgery mice were injected subcutaneously with the analgesic buprenorphine (0.05 mg/kg body weight; B. Braun Melsungen AG, Germany) before bilateral adrenalectomy was carried out through dorsal incisions under inhaled isoflurane anaesthesia (2-4%; Merial Animal Health Ltd, Essex, UK). Following surgery, wounds were closed with internal sutures and external staples and mice were placed into a heated compartment. When consciousness was restored, animals were transferred to a clean cage to recover fully for two weeks before experimentation began to allow clearance of all endogenous adrenal steroids. For the remainder of the experiment, adrenalectomised animals were given 0.9% saline in their drinking bottles.

2.6.9.2.1 Mini-pump loading and implantation

The following was performed by Dr Dawn Livingstone of the University of Edinburgh/BHF Centre for Cardiovascular Science. Solutions of vehicle or drug were prepared immediately prior to loading of mini-pumps. Osmotic mini-pumps (Model 2002, Alzet, CA, USA) were prepared as per manufacturer's instructions and loaded to deliver 100 μg of corticosterone per day in 1:1 DMSO:propylene glycol. The pumps were then primed in saline at 37°C for 24 hrs before surgery. These pumps perform due to differences in osmotic pressure between the loading

compartment within the pump and the tissue environment in which the pump is implanted. This causes an influx of water into the area around the loading compartment, displacing the test solution from the pump at a controlled rate (0.5 μL/hr for 14 days). To implant the mini-pumps, animals were injected subcutaneously with the analgesic buprenorphine (0.05 mg/kg body weight; B. Braun Melsungen AG, Germany) and anaesthetised with inhaled isoflurane (2-4%). Loaded osmotic mini-pumps were implanted subcutaneously between the scapulae through dorsal incisions which were closed with staples. Following surgery, animals were allowed to recover on a heat mat before being transferred to individual clean cages.

2.6.10 Terminal procedures and tissue collection

Animals were killed by decapitation in the morning (0800 hrs-1100 hrs) and trunk blood was collected as described above (2.6.5) before tissues were excised, wet-weighed and snap frozen on dry ice within 10-15 mins of the cull: whole brain, liver, prostate, adipose depots (gonadal/epididymal, omental, mesenteric and subcutaneous), kidney and one adrenal. In some experiments, the excised whole brain, the hypothalamus and pituitary were further dissected. Whilst dissecting the hypothalamus from the brain, extra care was taken to ensure the optic chiasm (white neuronal matter which highly expresses the 5αR enzymes) was not collected along with the sample. The optic chiasm (which shows high *Srd5a1* expression) when removed was collected into a separate Eppendorf. All tissues were stored at -80^{o}C.

2.7 Data Analysis

Data are presented as mean values ± standard error of the mean (SEM). All statistical comparisons were conducted using Graph Pad Prism or Statistica software to perform appropriate statistical tests as detailed in specific experimental chapters. Where significance was found by 2-way ANOVA using Graph Pad Prism, Bonferroni post-hoc tests were used to determine if particular pairs of values were significantly different from each other. However, this test is conservative and often lacks statistical power so occasionally data would instead be analysed by 2-way ANOVA and Fisher's LSD post-hoc tests using the Statistica programme. The level

of significance was taken as $p<0.05$ (*) and trends were identified where the p value was between 0.05 and 0.1. Where samples were run in duplicates or triplicates the RSD was calculated as follows:

(Standard deviation of replicates ÷ mean of the duplicates) × 100

Chapter 3: The role of 5αR1 in regulating metabolism and behaviour

3.1 Introduction

Obesity and other features of the metabolic syndrome may be associated with subtle changes in HPA axis activity in combination with altered glucocorticoid metabolism in extra-adrenal tissues (Pasquali *et al.* 2000; 2000; Pasquali *et al.* 2006). Even in the absence of exogenous stress, HPA activity is stimulated by both feeding and fasting (Dallman *et al.* 1993) showing food intake pathways and HPA axis activity are interlinked. A propensity towards developing the metabolic syndrome was observed in previous investigations in our group when glucocorticoid clearance was impaired by disruption of 5αR1, and notably, the weight gain phenotype of 5αR1-KO mice was particularly marked in females, which develop obesity even when fed a standard chow diet. The same was not true for male mice which only developed obesity compared to WT controls during a period of high-fat feeding. The reason for this sexual dimorphism in susceptibly is not yet understood but given that glucocorticoids are not the only substrates of 5αR1 and sex steroids are also subject to metabolism by the enzyme, sex differences are not altogether surprising.

Corticosterone is not absolutely necessary for the diurnal rhythm of the HPA axis and rhythmic activity of the HPA axis also follows the pattern of food consumption. For example, overnight food deprivation in rats leads to dampened HPA axis responses to acute restraint stress and reduced corticosteroid feedback the following morning (Akana *et al.* 1994; Hanson *et al.* 1994). Furthermore, administration of the selective murine 11βHSD1 inhibitor BVT .2733 reduced food intake and inhibited body weight gain in both lean and diet-induced obesity (DIO) mice (Wang *et al.* 2006). Similarly, the 11βHSD1-KO mouse is also resistant to DIO (Morton *et al.* 2004; Densmore *et al.* 2006) and 11βHSD1 knock-down reduces chow intake (Li *et al.* 2011). In light of this, a lack of glucocorticoid metabolism by 5αR1 may lead to hyperphagia, underpinning weight gain in 5αR1-KO mice.

As early as the 1940s, it was shown that lesions of the hypothalamus resulted in a hyperphagic and obesigenic phenotype (Hetherington *et al.* 1942). In this chapter, 5αR1-KO mice were investigated for altered expression of hypothalamic

neuropeptides which regulate appetite and feeding as a potential driver of hyperphagia and weight gain.

In addition to increased food consumption, altered activity (most likely reduced locomotion) is a potential mechanism for obesity in 5αR1-KO mice. CRH is a key regulator of the HPA axis but also plays a role in regulating locomotor activity and feeding (Sutton *et al.* 1982; Krahn *et al.* 1986). Reduced food intake and increased locomotor activity in an unfamiliar environment have been shown following intracerebroventricular CRH administration (Contarino *et al.* 2000). Additionally, the same authors reported CRH increased activity in WT mice but not CRHR1-KO mice. In the same study, food intake was decreased in both WT and CRHR1-KO mice following CRH treatment, suggesting a role for the CRHR2 receptor in mediating CRH-regulated feeding behaviours. Given that HPA activity (at least in response to stress) is dampened in 5αR1-KO mice where glucocorticoid clearance is impaired (Livingstone *et al.* 2014b), expression of transcripts which positively drive the axis (such as CRH and AVP) may be downregulated in 5αR1-KO mice (explored in further detail in Chapter 4). Potential reductions in CRH signalling would be hypothesised to result in reduced physical activity in conjunction with increased food intake; the net result of which could be significant weight gain in mice lacking 5αR1.

It was noted previously, that female 5αR1-KO mice gained more weight but were susceptible to losing it again during a period of social isolation. The underlying cause is unclear but may relate to increased susceptibility to stress and anxiety; certainly it has been noted that these mice display suppressed stress responses (Livingstone *et al.* unpublished for female mice). Many authors have reported instances of glucocorticoid-induced anxiety, either through increased ligand availability or up-regulation of corticosteroid receptors (Seckl 2004; Kajiyama *et al.* 2010). For example, anxiety was reduced in a forebrain-specific GR-KO mouse model (Boyle *et al.* 2006) whilst prednisolone-treated mice displayed more anxiety-like behaviours in the EPM and OFT, possibly related to hippocampal remodelling and damage (Kajiyama *et al.* 2010). Moreover, repeated administration of corticosterone in rodents induces depressive-like behaviours (Hill *et al.* 2003;

Kalynchuk *et al.* 2004; Gregus *et al.* 2005; Zhao *et al.* 2008) whilst 50% of humans who are diagnosed with Cushing's syndrome (a model of glucocorticoid excess within tissues) show an associated development of major depression (Sonino *et al.* 1998). Therefore, 5αR1-KO mice may be predisposed to developing affective disorders, although other explanations of weight loss on single housing should also be considered e.g. maladaptive thermogenesis.

3.1.1 Hypothesis

Our overall hypothesis was that female 5αR1-KO mice have increased appetite and reduced locomotor activity compared with WT and male 5αR1-KOs, and that female 5αR1-KO mice are more prone to anxiety.

3.1.2 Aims

The main aims of this set of experiments were:

- To investigate whether changes in the balance of hypothalamic anorexigenic and orexigenic peptides, indicative of increased appetite, were associated with weight gain in the 5αR1-KO mice.
- To investigate whether locomotor activity of 5αR1-KO mice was less than WT mice.
- To determine if 5αR1-KO mice display more anxiety-like behaviours than WT mice under basal (group housed) conditions.
- To assess if the parameters above were influenced by sex.

3.2 Methods

3.2.1 Metabolic and behavioural effects of 5αR1-KO

3.2.1.1 Experimental outline

Male and female, WT and 5αR1-KO mice (C57BL/6j/SvEv/129 [mixed] strain) were maintained on chow diet and assessed for body weight and food consumption. At 6 months, mice were assessed for anxiety first, followed by free ambulatory nocturnal activity using infrared cage frames. To confirm that 5αR1-KO mice demonstrated similar insulin resistance and steatosis as previously described, glucose and insulin tolerance tests were performed *in vivo*. Following cull, the expression of hypothalamic neuropeptides which are known to drive feeding behaviours were quantified by qPCR.

3.2.1.2 Animal maintenance

All animals were obtained from an in-house breeding colony (2.6.1), housed in groups of 4 animals/cage and maintained as described in methods section 2.6.3. Four experimental groups were studied:

- Male WT mice (n = 19)
- Male 5αR1-KO mice (n = 18)
- Female WT mice (n = 20)
- Female 5αR1-KO mice (n = 20)

3.2.1.3 Body weight and food intake

Between 5 and 7 months old, animals and their remaining food were weighed once weekly in the afternoon to quantify body weight gain and calorific intake (see section 2.6.4 for details). This time frame encompassed the periods of experimental investigation.

3.2.1.4 Glucose tolerance test (GTT)

A glucose tolerance test (2.6.7) was used to determine how quickly WT and 5αR1-KO mice could clear excess glucose from the circulation therefore indicating their sensitivity to exogenous insulin. Plasma insulin and glucose, basally and at 15, 30,

60 and 90 min intervals, were quantified by ELISA (2.4.1) and hexokinase assay (2.4.2) respectively.

3.2.1.5 Insulin tolerance test (ITT)

The acute effects of insulin injection on blood glucose levels were assessed in WT and 5αR1-KO mice by glucometer over a 90 minute time course (2.6.6). Basal glucose levels were also determined prior to injection of the insulin bolus.

3.2.1.6 Assessing relative anxiety levels in the elevated plus maze (EPM)

To determine if lack of 5αR1 led to an anxious phenotype, WT and 5αR1-KO mice were tested in the EPM (2.6.8.1.1) at 6 months of age.

3.2.1.7 Free ambulatory activity

Nocturnal activity of WT and 5αR1-KO mice was assessed using a non-invasive procedure whereby frames containing infrared beams in a 2D plane (X-Y) were placed around the animal's cage overnight (14 hrs). Further details are highlighted in section 2.6.8.3.

3.2.1.8 Terminal procedures

Animals were culled and tissues collected as described in methods section 2.6.10.

3.2.1.9 Quantification of liver triglyceride content

Triglycerides were extracted from the livers of WT and 5αR1-KO mice and quantified as described (2.4.3).

3.2.1.10 Quantification of plasma leptin levels by ELISA

Trunk blood was collected from WT and 5αR1-KO mice at cull (2.6.10) and used to determine non-fasted plasma leptin levels by ELISA (2.4.6).

3.2.1.11 Quantification of mRNA expression by qPCR

Total RNAs were extracted from snap frozen hypothalami from male WT and 5αR1-KO mice (2.3.1.4) and used to synthesise first strand cDNA by RT (2.3.2). qPCR was performed (2.3.4) to quantify the expression of transcripts for hypothalamic feeding peptides including the orexigenics *Npy* and *Agrp* and the anorexigenics *Cartpt1/2, Mc3r* and *Mc4r*. The abundances of transcripts of interest were expressed as a ratio to the mean of the transcript levels of the reference gene. These combinations of reference genes were selected due to their lack of change in expression between experimental groups (WT and 5αR1-KO; see Table 3.1 for p values). Primer sequences and probe numbers can be found in Table 2.2.

3.2.2 Statistical analyses

All data are presented as mean ± SEM and were compared using Student's unpaired t-test, 2-way ANOVA or 2-way repeated measures ANOVA with Bonferroni post-hoc testing where appropriate. All statistical tests were done using GraphPad Prism software.

Tissue	Reference Gene	p Value	
Hypothalamus	Glyceraldehyde-3-phosphate dehydrogenase *(Gapdh)*	Genotype	
		Male	Female
		0.322	*0.314*

Table 3.1 Reference gene used for hypothalamic qPCR

Gapdh was used as the reference gene for transcripts of interest in the male and female hypothalamus. P value indicates the results of a Student's unpaired t-test, for each sex, showing there were no significant differences in reference gene expression between WT and 5αR1-KO mice.

3.3 Results 1 – Metabolic profiling

Concomitant to behavioural assessment, indices of metabolism were assessed to ensure continuity of the original phenotype as described in the chapter introduction (3.1) i.e. a propensity towards features of the metabolic syndrome such as obesity and insulin resistance.

3.3.1 Body and tissue weights

There was no difference in the body weight of male 5αR1-KO mice vs. WT littermates either expressed as weight over time (Figure 3.1, A) or at the time of cull (Figure 3.1, B). Female 5αR1-KO mice were significantly heavier than WT across the period (Figure 3.1, C, $p<0.01$) and at the time of cull (Figure 3.1, D, $p<0.01$). There were no significant differences in wet tissue weights between male WT and 5αR1-KO mice.

In females, absolute wet weight of the gonadal fat pad was significantly increased in 5αR1-KO mice vs. WT (Table 3.2, $p<0.05$) with a trend towards the same pattern in mesenteric adipose tissue (Table 3.2, $p=0.076$). Total fat mass (i.e. the sum of the gonadal, mesenteric, omental and subcutaneous adipose tissue mass) was increased in female 5αR1-KO mice compared to WT controls reaching significance as absolute total adipose weight (Table 3.2, $p<0.05$) with a trend when total adipose weight was corrected for body weight (Table 3.2, $p=0.072$).

3.3.2 Glucose tolerance

3.3.2.1 Plasma glucose

Fasting plasma glucose was not different between WT and 5αR1-KO mice in either males (Figure 3.2, A) or females (Figure 3.2, B). Plasma glucose levels in 5αR1-KO mice following intraperitoneal (i.p.) injection of an exogenous glucose solution did not differ vs. WT in either male or female mice (Figure 3.2, C). However, there was an overall sex difference whereby plasma glucose levels were higher post-glucose injection but not basally in males vs. females overall (Figure 3.2, C, $p<0.001$).

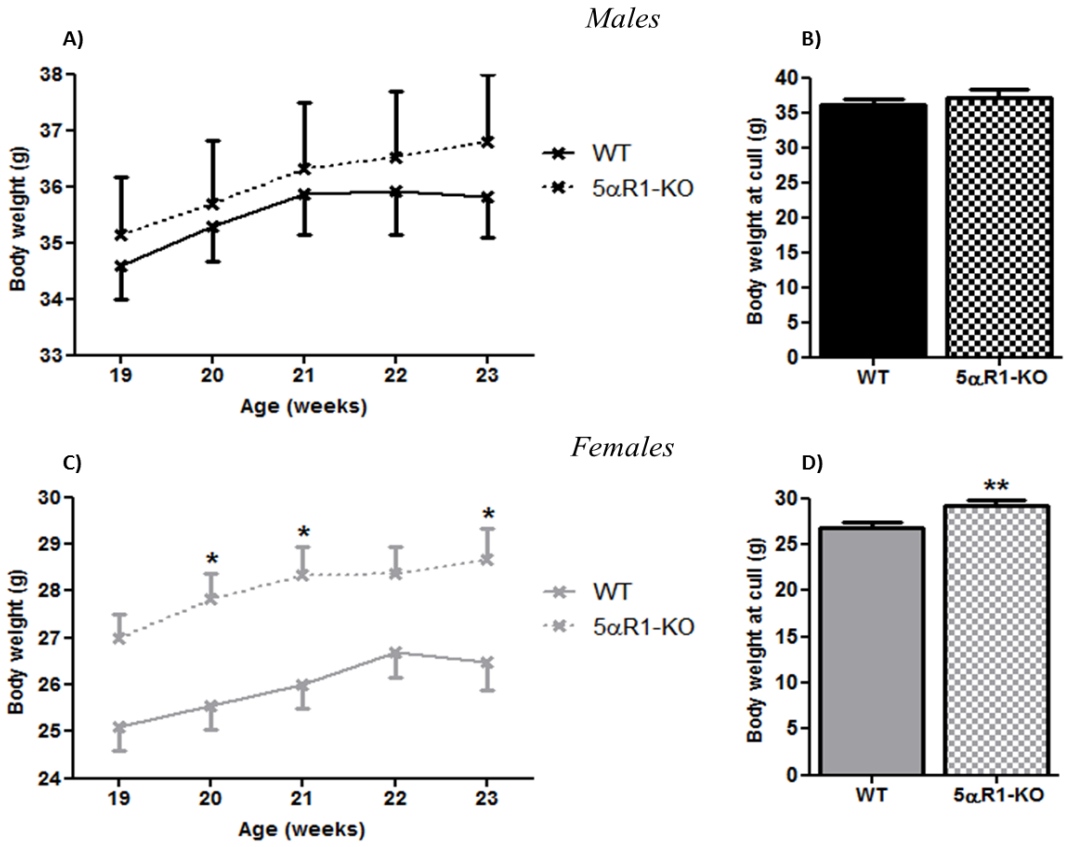

Figure 3.1 Body weight gain and final weight at cull

Male 5αR1-KO mice (dashed black line) did not differ from WT (solid black line) in body weight when measured between 19 and 23 weeks old (A). At cull, there was no effect of genotype on body weight in male mice (B). Female 5αR1-KO mice (grey dashed line) were heavier than WT (sold grey line) throughout the measurement period (C, $p<0.01$). Panel D shows female, 5αR1-KO mice (grey checkered) weighed significantly more than WT controls (sold grey) at the time of cull (D, $p<0.01$). Data are mean ± SEM for n=19-20/group. Comparisons were made by 2-way ANOVA for repeated measures with Bonferroni post-hoc tests where appropriate or Student's unpaired t-test * = $p<0.05$, ** = $p<0.01$. Mice were aged 7 months at cull. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

Body and Tissue Weights							
		Male			Female		
		WT	5αR1-KO	p value	WT	5αR1-KO	p value
Body (g)		36.11±0.78	37.22±1.12	0.419	26.78±0.60	29.25±0.60	**0.006**
Absolute Tissue Weight (mg)							
Liver		1273.83±75.74	1270.47±106.19	0.979	994.96±64.29	1083.29±59.26	0.319
Adipose Depot	Gon	1098.40±97.89	1284.02±107.68	0.209	806.94±65.06	**1016.14±57.05**	**0.021**
	Omen	31.75±2.93	39.11±5.52	0.239	40.78±18.52	29.70±3.31	0.559
	Mes	534.38±26.24	556.71±50.55	0.693	391.14±25.28	448.10±26.06	0.125
	S/C	629.64±72.85	617.59±67.45	0.904	561.19±53.51	**717.95±67.27**	**0.076**
	Total	2294.16±179.33	2497.42±196.01	0.449	1800.05±115.54	**2211.88±119.95**	**0.018**
Tissue Weight/Body Weight (mg/g body weight)							
Liver		35.71±2.25	33.94±2.70	0.616	36.76±2.13	36.96±1.91	0.943
Adipose Depot	Gon	29.78±2.20	34.06±2.50	0.205	30.03±2.30	34.76±1.88	0.119
	Omen	0.89±0.09	1.03±0.13	0.352	1.44±0.61	1.01±0.10	0.487
	Mes	14.72±0.51	14.64±1.04	0.944	14.42±0.79	15.24±0.75	0.453
	S/C	16.96±1.70	16.09±1.37	0.696	20.63±1.80	24.23±1.99	0.188
	Total	62.34±3.73	65.82±3.78	0.517	66.52±3.41	**75.25±3.25**	**0.072**

Table 3.2 Body and tissue weights at cull

In male animals, there were no differences in either body or tissue weights between WT and 5αR1-KO mice but female 5αR1-KO mice weighed significantly more than WT controls at cull ($p<0.01$). Also in females, absolute mass of the gonadal ($p<0.05$), subcutaneous (trend, $p=0.076$) and total of all the fat pads combined ($p<0.05$) was increased in 5αR1-KO mice vs. control. When tissue weights were expressed as a ratio to body weight, the increased total adipose tissue weight in female 5αR1-KO mice vs. WT was a trend only ($p=0.072$) and there were no differences in weight of any other tissues. Data are mean ± SEM for n=19-20 per group. Comparisons between WT and 5αR1-KO were made by Student's unpaired t-test, in each sex. Mice were aged 7 months at cull. Gon = gonadal adipose tissue, omen = omental adipose tissue, mes = mesenteric adipose tissue, S/C = subcutaneous adipose tissue, total = sum of the four adipose depots combined, WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

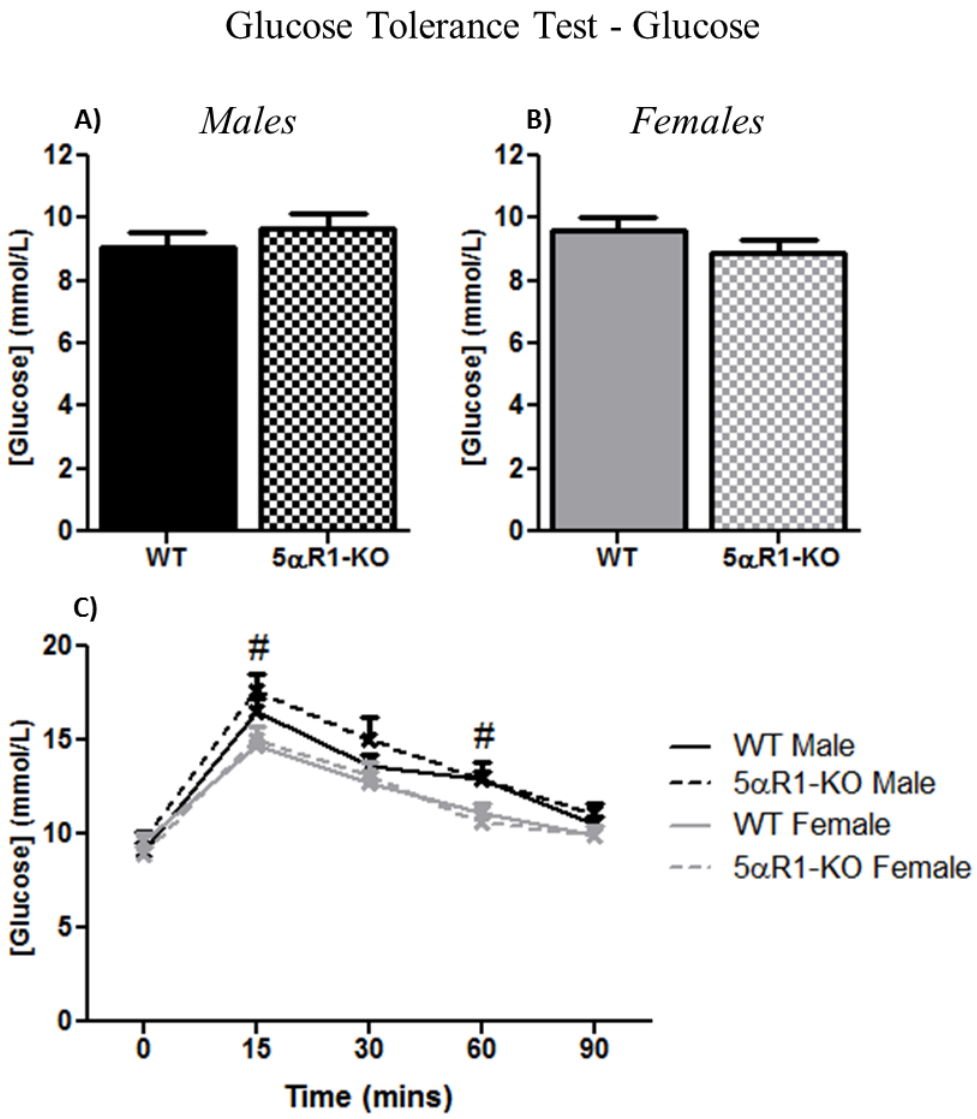

Figure 3.2 Plasma glucose levels during a glucose tolerance test

Basally, fasting plasma glucose concentrations were not different between WT (solid) and 5αR1-KO (chequered) mice in both males (black, A) and females (grey, B). Following glucose injection, there were no genotype differences in plasma glucose concentrations for either of the two sexes (C). However, there was an overall sex effect whereby plasma glucose was higher in males vs. females (C, $p<0.001$). Post-hoc testing showed significantly higher plasma glucose in 5αR1-KO males (black dashed) vs. 5αR1-KO females (grey dashed) at 15 and 60 mins post-glucose injection (C, $p<0.05$). Data are mean ± SEM for n=19-20 per group. Comparisons were made by Student's unpaired t-test or 2-way ANOVA for repeated measures with Bonferroni post-hoc tests where appropriate and # = $p<0.05$ for the effects of sex. Mice were aged 6 months. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

3.3.2.2 Plasma insulin

Male 5αR1-KO mice had significantly higher fasting insulin levels basally compared to control (Figure 3.3, A, $p<0.05$), whilst female 5αR1-KO mice also showed a trend towards higher fasting plasma insulin concentrations vs. WT (Figure 3.3, B, $p = 0.053$). Overall, male mice had higher fasting plasma insulin vs. female mice (Figure 3.3, C, $p<0.0001$). The release of insulin following a single glucose bolus was measured to determine glucose tolerance. Female 5αR1-KO mice produced significantly higher concentrations of insulin compared to WT (Figure 3.3, C, $p<0.05$). Male 5αR1-KO mice also trended towards an exaggerated insulin response vs. WT controls following glucose injection (Figure 3.4, C, $p = 0.069$). Sex differences were apparent such that male mice reached a higher insulin peak vs. females for the WT genotype (Figure 3.3, C, $p<0.05$) and insulin was higher across all time-points in males vs. females for the 5αR1-KO genotype (Figure 3.3, C, $p<0.001$-$p<0.01$).

3.3.3 Insulin tolerance

The clearance of glucose from the circulation following a single insulin bolus was measured to determine insulin sensitivity. Figure 3.4, A and B show fasting glucose levels prior to the insulin injection. Male 5αR1-KO mice had significantly higher fasting glucose concentrations vs. WT (Figure 3.4, A, $p<0.05$) whereas female mice did not (Figure 3.4, B). Following injection with exogenous insulin, there were no differences in glucose clearance from the circulation between WT and 5αR1-KO mice of either sex (Figure 3.4, C).

3.3.4 Liver triglyceride content

Liver triglycerides were not different between male WT and 5αR1-KO mice (Figure 3.5, A). Female, 5αR1-KO mice had significantly higher liver triglyceride content compared to WT littermates (Figure 3.5, B, $p<0.05$).

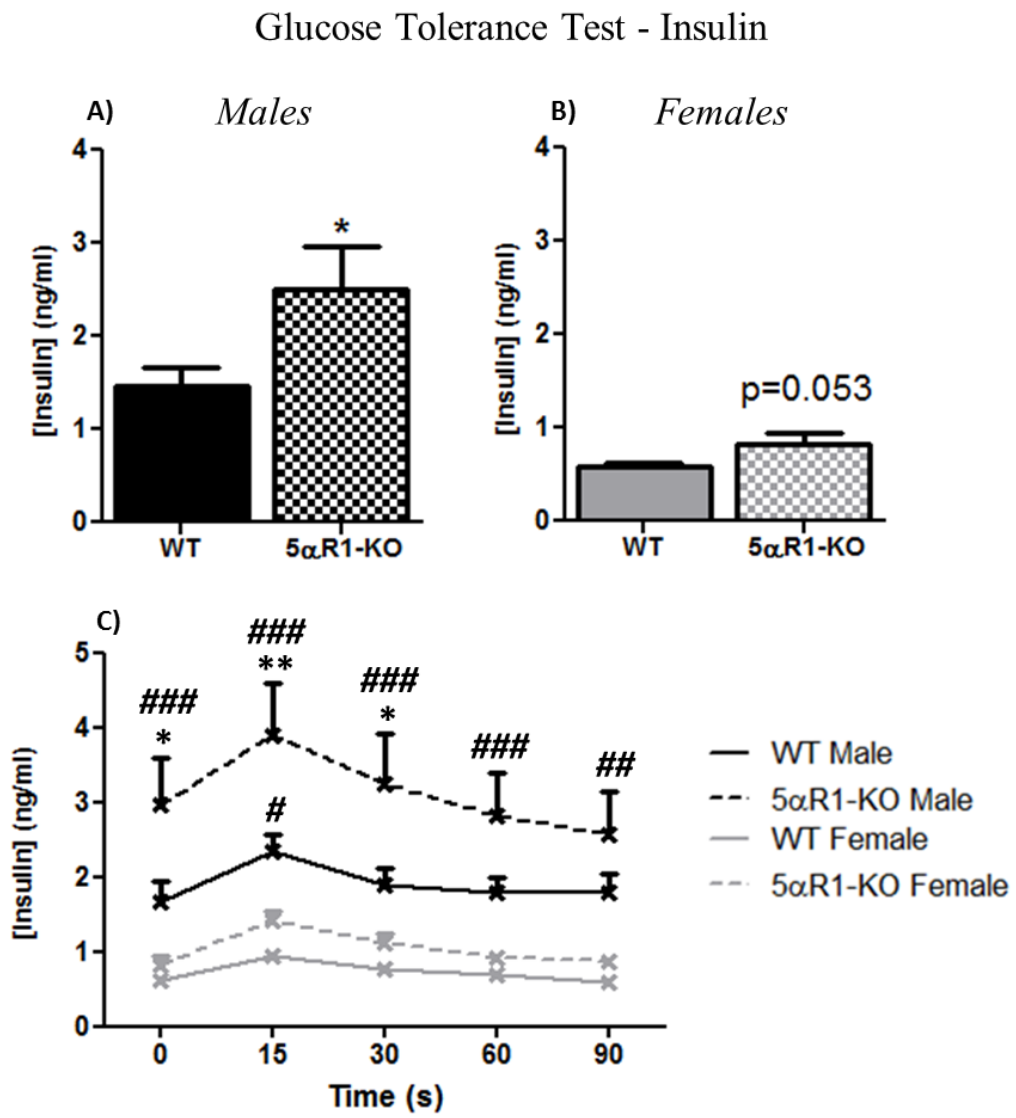

Figure 3.3 Plasma insulin levels during a glucose tolerance test
Fasting insulin levels were increased in male 5αR1-KO mice (chequered black, A, $p<0.05$), whilst there was a trend towards elevated fasting plasma insulin in female 5αR1-KO mice (chequered grey, B, $p=0.053$,) all vs. WT controls (solid fill). Additionally, male mice had higher basal insulin levels in plasma ($p<0.0001$). C shows the insulin release curve in response to a single glucose injection for both male and female mice. Compared to WT littermates (solid lines), 5αR1-KO mice (hatched lines) of both sexes released significantly more insulin during the glucose tolerance test and reached a higher peak at 15 mins, with a slower recovery back to basal levels ($p=0.069$ for males and $p<0.05$ for females). Moreover, there were strong sex differences. Between WT animals, male mice reached a higher insulin peak at 15 mins vs. females (C, $p<0.05$) but between 5αR1-KO mice, the male insulin response to the glucose bolus was greatly exaggerated compared to female genotype equivalents across all time points (C, $p<0.001$–$p<0.01$). Points/bars are mean ± SEM for n=19-20 mice/group. Comparisons were made by 2-way ANOVA for repeated measures with Bonferroni post-hoc tests where appropriate, or Student's unpaired t-test. * = $p<0.05$ and ** = $p<0.01$ and determines genotype differences. # = $p<0.05$, ## = $p<0.001$ and ### = $p<0.001$ for the determination of sex differences with matched genotype. Mice were 6 months old. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

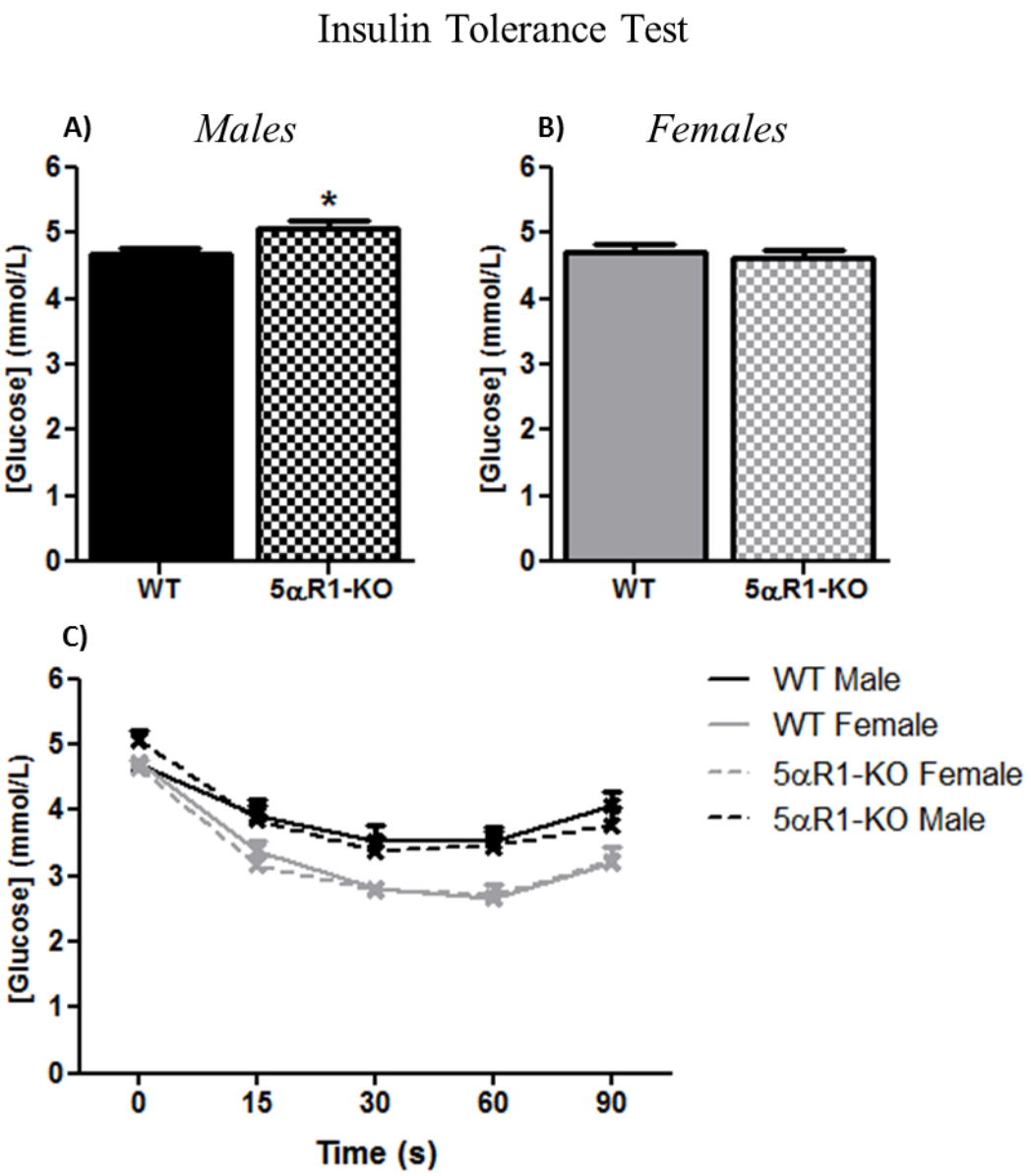

Figure 3.4 Plasma glucose levels during an insulin tolerance test

Male (black), 5αR1-KO mice had significantly elevated fasting plasma glucose levels vs. WT (A, $p<0.05$), but fasting plasma glucose was not different in female (grey), 5αR1-KO mice vs. WT littermates (B). Glucose clearance over time following a single bolus injection of exogenous insulin was not different in 5αR1-KO mice compared to WT littermates (dashed and solid lines respectively; C). This was true for both sexes. Points/bars are mean ± SEM for n=19-20 mice/group. Comparisons were made by 2-way ANOVA for repeated measures, or Student's unpaired t-test where * = $p<0.05$. Mice were aged 6 months. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

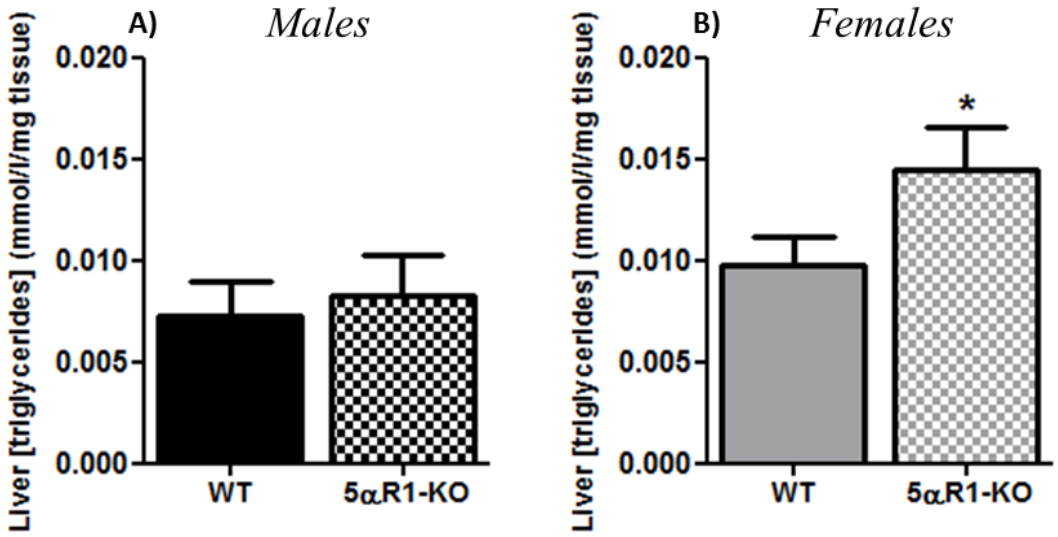

Figure 3.5 Liver triglyceride levels

Panel A shows liver triglyceride content was not different between male WT and 5αR1-KO mice. However, female 5αR1-KO mice had a significantly higher concentration of triglycerides in the liver in comparison to WT littermates (B). Data are mean ± SEM for n=19-20 mice/group. Comparisons were made by Student's unpaired t-test where * = $p<0.05$. Mice were aged 7 months. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

3.3.5 Food intake

Male 5αR1-KO mice consumed significantly more calories cumulatively vs. WT littermates (Figure 3.6, A, $p<0.01$) and also on an average weekly basis (Figure 3.6, B, $p<0.001$). Female WT and 5αR1-KO mice consumed the same number of calories cumulatively (Figure 3.6, C) and when expressed as an average weekly consumption (Figure 3.6, D).

3.3.6 Abundance of transcripts for appetite-regulating genes

There was reduced expression of orexigenic peptides in 5αR1-KO mice. *Npy* mRNA was significantly downregulated in the hypothalamus in 5αR1-KO mice vs. WT (Figure 3.7, A, $p<0.05$). There was also a trend towards lower *Agrp* mRNA expression in 5αR1-KO animals compared to control (Figure 3.7, B, $p = 0.09$). Anorexigenic peptides, *Cartpt1/2* and *Mc3r* were not different between genotypes (Figure 3.7, C-E) but a trend was found in *Mc4r* transcripts being more abundant in the hypothalamus in 5αR1-KO mice vs. WT controls (Figure 3.7, F, $p = 0.067$).

In females, expression levels of all the hypothalamic neuropeptides quantified were not different in 5αR1-KO mice compared to WT (Figure 3.8, A-E).

3.3.7 Plasma leptin levels

There was no effect of genotype on plasma leptin levels (Figure 3.9) however female mice had lower leptin concentrations in comparison to male mice overall (Figure 3.9, $p<0.01$)

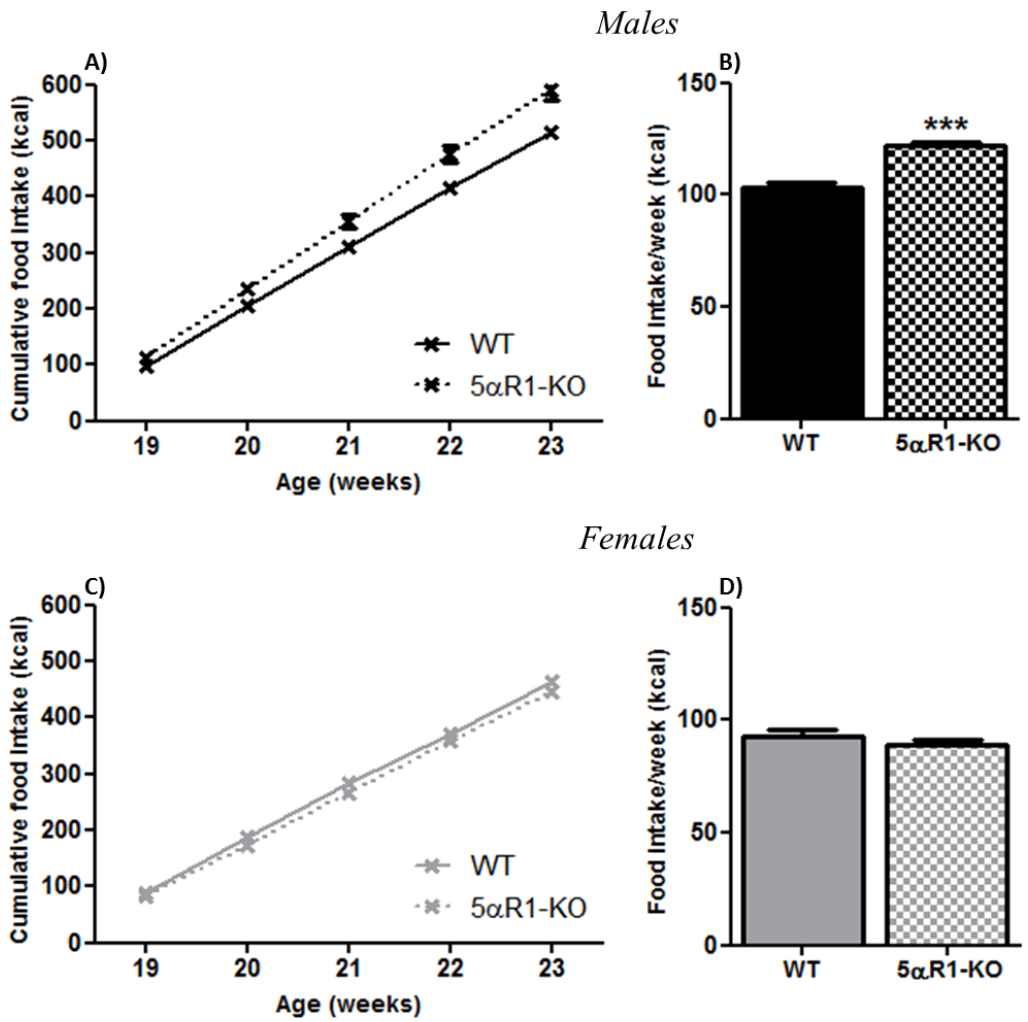

Figure 3.6 Cumulative and weekly calorific consumption

Male 5αR1-KO mice (black dashed) consumed significantly more calories vs. WT (solid black) both cumulatively (A, $p<0.01$) and as a weekly average intake (B, $p<0.001$). However, in females there was no difference in cumulative (C) or weekly (D) calorific consumption between 5αR1-KO (grey dashed) and WT (solid grey) mice. Data are mean ± SEM for n=4-5/group. Comparisons were made by 2-way ANOVA for repeated measures with Bonferroni post-hoc tests or Student's unpaired t-test where appropriate. *** = $p<0.001$. Mice were aged 6 months. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

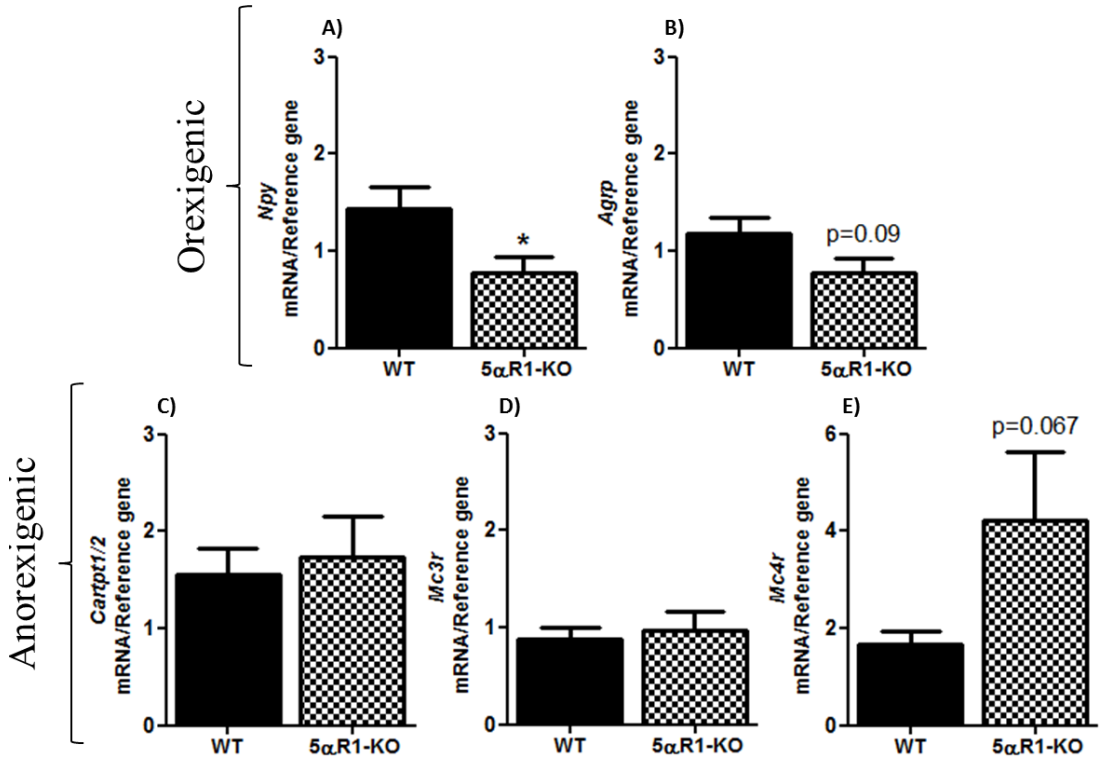

Figure 3.7 Expression of transcripts for hypothalamic neuropeptides involved in appetite regulation (Males)

Npy mRNA abundance was downregulated in 5αR1-KO mice (checkered) vs. WT (solid, A, $p<0.05$). There was a trend towards lower *Agrp* mRNA expression in 5αR1-KO animals compared to control (B, p=0.009). *Cartpt1/2* and *Mc3r* were not different between genotypes (C-E) but trends were found in *Mc4r* transcripts being upregulated in 5αR1-KO mice vs. WT controls (F, p=0.067). Data are mean ± SEM for n=19-20/group and comparisons were made by Student's unpaired t-test where * = $p<0.05$. Mice were aged 7 months. WT = wild-type, 5αR1-KO = 5α-reductase type 1 knock-out, *Npy* = neuropeptide-Y, *Agrp* = agouti-related protein, *Cartpt1*/2= cocaine-amphetamine-regulated transcript variants 1 and 2 combined, *Mc3r* and *Mc4r* = melanocortin 3 and 4 receptor.

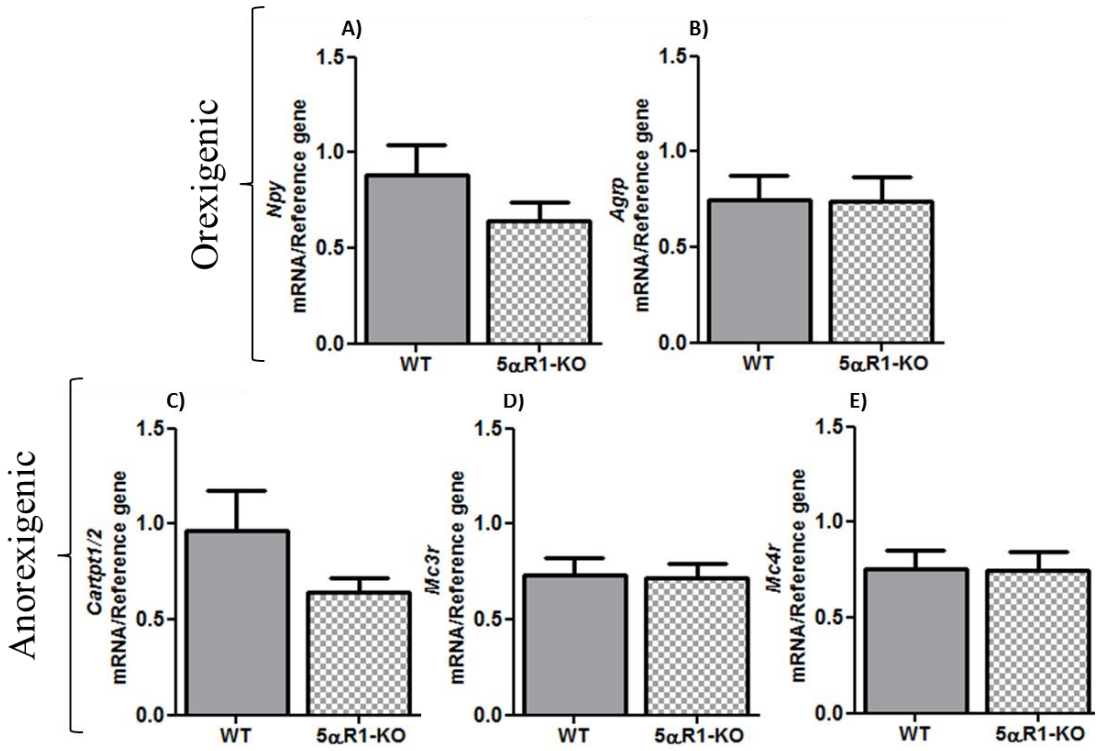

Figure 3.8 Expression of transcripts for hypothalamic neuropeptides involved in appetite regulation (Females)

In female mice, abundance of transcripts for hypothalamic feeding-regulatory peptides was not different between WT and 5αR1-KO mice. Data are mean ± SEM for n=11/group. Comparisons were made by Student's unpaired t-test. Mice were aged 7 months. WT = wild-type, 5αR1-KO = 5α-reductase type 1 knock-out, *Npy* = neuropeptide-Y, *Agrp* = agouti-related protein, *Cartpt1*/2= cocaine-amphetamine-regulated transcript variants 1 and 2 combined, *Mc3r* and *Mc4r* = melanocortin 3 and 4 receptor.

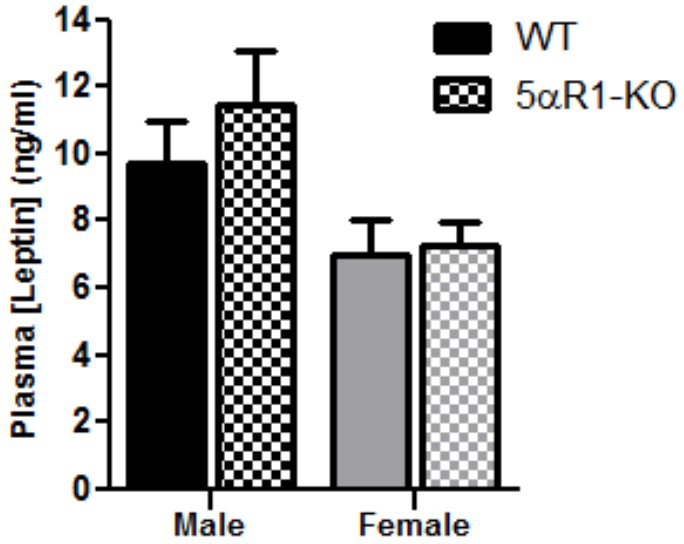

Figure 3.9 Plasma leptin levels

There was no difference between plasma leptin levels in WT and 5αR1-KO mice of either sex. However, female mice overall had lower circulating leptin in comparison to male mice ($p<0.01$). Bars represent mean measurements ± SEM for n = 18-20/group. Comparisons were made by 2-way ANOVA with Bonferroni post-hoc testing. Mice were aged 7 months. WT = wild-type, 5αR1-KO = 5α-reductase type 1 knock-out.

3.4 Results 2 – Behavioural profiling

3.4.1 Free ambulatory, nocturnal activity

Frames with infrared detectors in the X and Y plane were used to measure free ambulatory activity overnight. Figure 3.10 shows total activity (A), distance travelled (B), active time (C) and inactive time (B) over a 14.5 hour period. No differences were found in nocturnal activity between WT and 5αR1-KO mice, neither males nor females. However, it does seem that male mice overall are more active than females with males having a trend to increased total activity levels (Figure 3.10, A, $p = 0.07$) and time spent active (Figure 3.10, C, $p<0.05$) whilst females spent more time in an inactive state (Figure 3.10, D, $p<0.05$). There was no difference in distance travelled between the sexes (Figure 3.10, B).

3.4.2 Assessing anxiety using the elevated plus maze (EPM)

Mice were tested in the EPM to determine any differences in anxiety levels between genotypes. Across all experimental groups, mice spent most time in the closed zone, less in the centre and less still in the open zone, which is the most anxiogenic area of the maze (Figure 3.11, males A, females, D, both $p<0.001$). In both sexes the overall distribution of time spent between the three zones differed by between WT and 5αR1-KO mice (Figure 3.11, A for males, D for females, both $p<0.01$). In males, there were no differences in % time spent in the open or centre zones but 5αR1-KO mice did spend less time in the closed zone vs. WT controls (Figure 3.11, A, $p<0.05$). Moreover, male 5αR1-KO mice made significantly more entries into the open, most anxiogenic zone compared to WT (Figure 3.11, B, $p<0.05$). Similar to males, female 5αR1-KO mice spent less time in the closed zone of the EPM vs. WT (Figure 3.11, D, $p<0.05$) but the % time spent in each of the other two zones did not differ between genotypes. The number of entries into the open, anxiogenic zone was comparable between female WT and 5αR1-KO mice (Figure 3.11, E). The distance travelled was the same between sex and genotype (Figure 3.11, C and F).

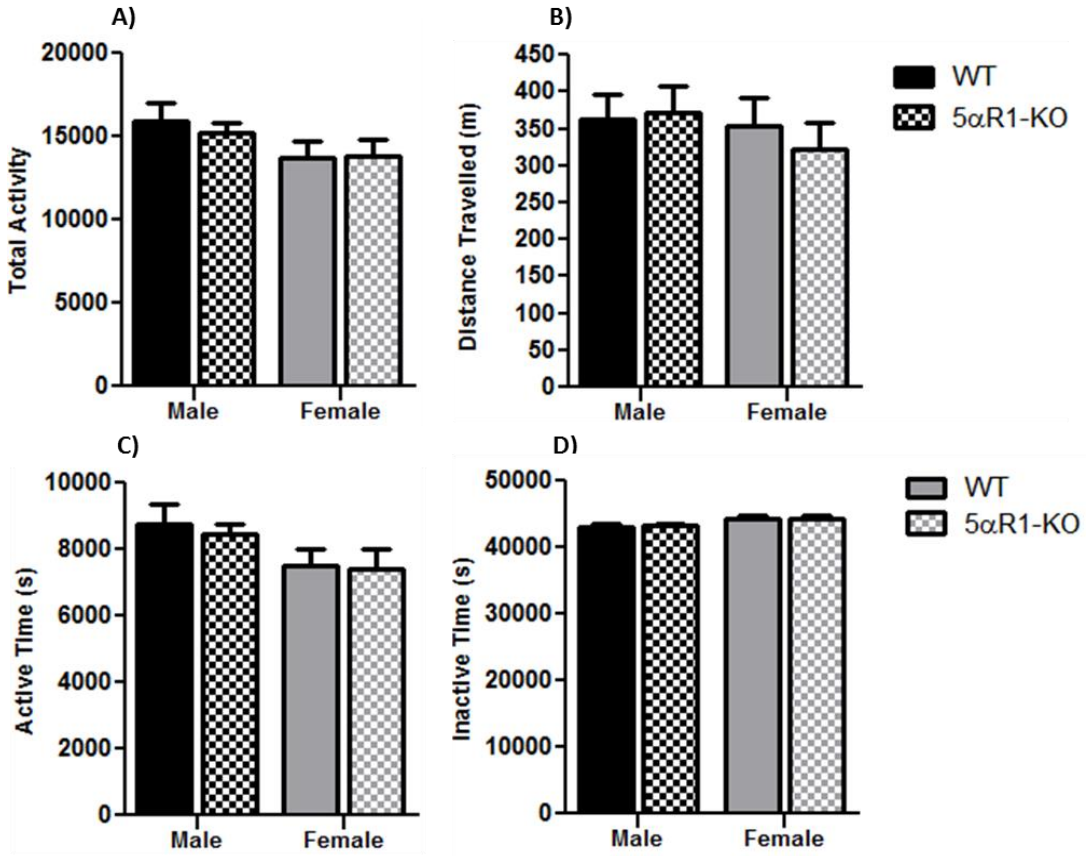

Figure 3.10 Nocturnal locomotor activity

No differences were seen in the total locomotor activity, distance travelled or active and inactive times of WT and 5αR1-KO mice (A-D, respectively). Irrespective of genotype however, it was apparent that male mice overall were significantly more nocturnally active than females ($p=0.07$ for total activity, A; $p<0.05$ for active time, C; $p<0.05$ for inactive time, D) although there was no difference in distance travelled (B). Bars are mean ± SEM for n=19-20 mice/group. Comparisons were made by 2-way ANOVA with Bonferroni post-hoc tests. Mice were aged ~ 6 months at the time of testing. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

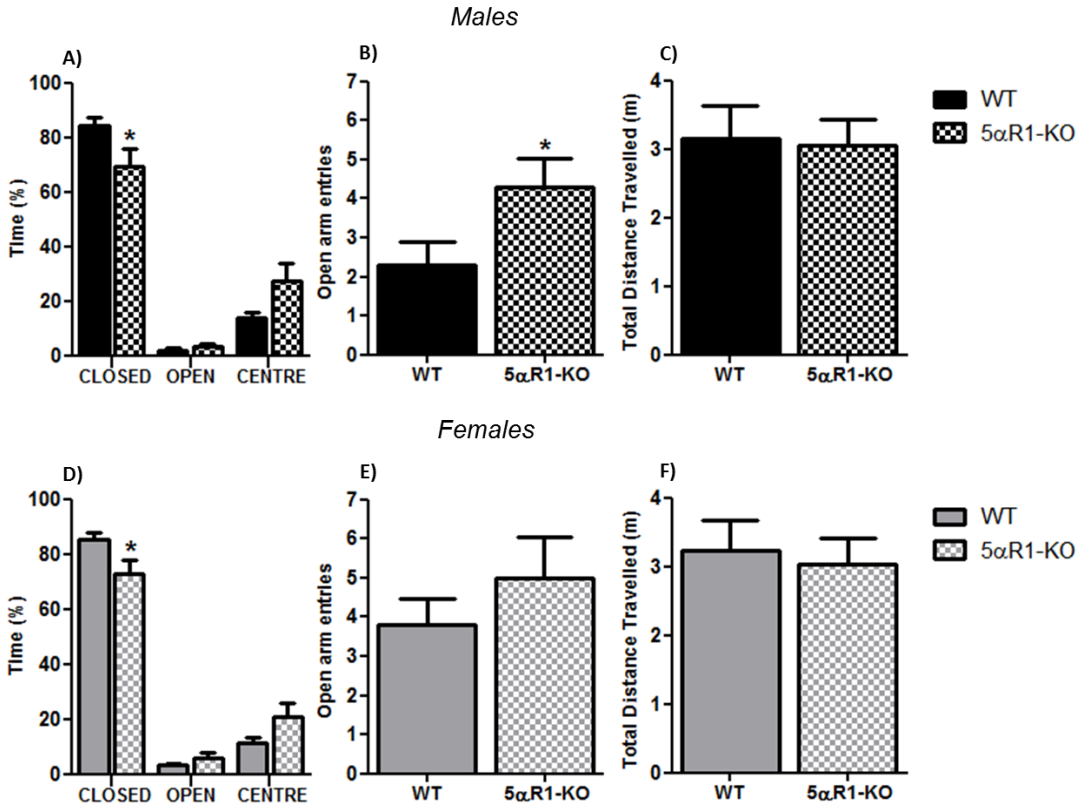

Figure 3.11 Anxiety-like behaviour in the elevated plus maze (EPM)
As expected, mice spent most time in the closed zones, less in the centre and least in the open, anxiogenic area (A and D, $p<0.001$). Both male (black, A) and female (grey, D) 5αR1-KO mice (chequered) spent significantly less time in the closed zone vs. WT (solid, $p<0.05$). There was no genotype difference in the time spent in each of the other zones (A and D). Male 5αR1-KO mice made more entries into the most anxiogenic zone when compared to WT littermate controls (B, $p<0.05$) but there was no such effect in female animals (E). There was no genotype difference in distance travelled during testing for either males (C) or females (F). Bars are mean ± SEM for n=19-20 mice/group. Comparisons were made by 2-way ANOVA with Bonferroni post-hoc testing where appropriate or Student's unpaired t-test where * = $p<0.05$. Mice were aged 6 months at the time of testing. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

3.5 Discussion

In this study and in keeping with previous cohorts, female, but not male, 5αR1-KO mice were heavier than their WT controls. 5αR1-KO mice of both sexes were less glucose tolerant, but retained similar sensitivity to an insulin challenge. These changes were associated with hyperphagia in males, but not reduced locomotion in either sex.

Male 5αR1-KO mice were hyperinsulinaemic and hyperglycaemic in the fasting state i.e. demonstrating progression towards type II diabetes. In the early development of type II diabetes mellitus, insulin secretion from pancreatic β-cells is increased leading to hyperinsulinaemia as a compensatory reaction to reduced insulin sensitivity in tissues (Carlsson *et al.* 1996; Pettersson *et al.* 2012), but here this was insufficient to control fasting glucose. Male 5αR1-KO mice were also intolerant of exogenous glucose with a trend towards exaggerated endogenous insulin response in order to maintain the same plasma glucose concentrations as those seen in WT controls. However, male 5αR1-KO mice were tolerant of an exogenous insulin challenge and glucose levels were decreased similar to WT across the time-course indicating insulin sensitivity was intact. Female 5αR1-KO mice demonstrated features of the Metabolic Syndrome, namely obesity, fasting hyperinsulinaemia and hepatic steatosis. Also indicative of insulin insensitivity was the markedly increased plasma concentrations of insulin released by female 5αR1-KO mice to counteract a single fixed dose of glucose i.e. following glucose injection mice lacking 5αR1 elicited an exaggerated and prolonged insulin response to counteract hyperglycaemia. However, although plasma insulin was raised in 5αR1-KO mice, glucose levels over the time course of the test were maintained similar to WT in 5αR1-KO mice (both sexes).

It is possible that a lack of glucocorticoid inactivation in 5αR1-KO mice could promote insulin resistance. ADX in mice genetically predisposed to obesity (*ob/ob* mice) improves hyperinsulinaemia (Naeser 1974; Turkenkopf *et al.* 1991), reduces blood glucose levels (Bray *et al.* 1979) and also abolishes hyperphagia (Vander Tuig *et al.* 1984; Freedman *et al.* 1985). Moreover, 11βHSD1-KO mice, with suppressed

glucocorticoid signalling, have an improved metabolic profile with improved glucose tolerance (Morton *et al.* 2001) and are resistant to the hyperglycaemic effects of chronic high-fat feeding (Kotelevtsev *et al.* 1997). Conversely, and similar to our findings, at least in male 5αR1-KO mice, dexamethasone-treated mice have impaired glucose tolerance combined with hyperphagia and weight gain (Gounarides *et al.* 2008). However, it is possible that there may be a significant contribution towards the above phenotypes from altered androgen metabolism (most notably in males) in 5αR1-KO mice.

Male animals overall presented with higher insulin levels, both fasting and stimulated, compared to females. Females (both pre-menopausal humans and animals) are known to be somewhat protected from metabolic disease. For example, female DIO mice are protected from features of the metabolic syndrome such as hyperinsulinaemia and islet hypertrophy (Pettersson *et al.* 2012). Also reported in the same study, male DIO mice had low-grade systemic inflammation. Again this phenotype was absent in female mice possibly due to increased anti-inflammatory mediators such as Foxp3+ T lymphocytes (T_{reg}) in adipose tissue in response to obesity (Pettersson *et al.* 2012).

Androgen deprivation therapy in men with prostate cancer very often results in the development of metabolic syndrome (Braga-Basaria *et al.* 2006; Smith *et al.* 2008) with the risk of new onset type II diabetes mellitus being greatly increased due to hyperinsulinaemia (Dockery *et al.* 2003; Basaria *et al.* 2006; Smith *et al.* 2006; Kintzel *et al.* 2008; Smith *et al.* 2008; Alibhai *et al.* 2009; Keating *et al.* 2010). A lack of contribution from 5αR1 in 5αDHT formation may lead to reduced concentrations of this most potent androgen in male 5αR1-KO mice which may also predispose male mice to a poorer metabolic outlook, albeit in the absence of obesity, compared to females.

Despite not gaining extra weight, hyperphagia was observed in male 5αR1-KO mice whilst calorific consumption of the obesity-prone female 5αR1-KO mice was comparable to that of WT controls.

Secretion of glucocorticoids from the adrenal cortex is under tight control of the HPA axis via the release of neuropeptides including CRH which acts upon CRHR1 in the pituitary. Based on preliminary data from previous studies (Livingstone *et al.* 2014b) which showed attenuated HPA axis stress responses, it is proposed that key mediators which drive the HPA axis such as CRH and its main receptor CRHR-1 may be downregulated as a result of greater glucocorticoid negative feedback in 5αR1-KO mice (HPA axis regulation explored in greater detail in Chapter 4). Indeed, alterations in *Crh* and *Crhr-1* expression within selected brain areas have been shown to affect appetite, feeding behaviours and food intake (Krahn *et al.* 1986; Menzaghi *et al.* 1994; Contarino *et al.* 2000; Challis *et al.* 2004). CRH has anorexigenic properties implicated in the regulation of feeding indices by mediating suppression of appetite and thus food intake (Krahn *et al.* 1986). Therefore we may expect the attenuation of anorexigenic influences such as CRH to alter feeding circuits in the brain resulting in increased food intake and an accompanying weight gain in 5αR1-KO mice.

The hypothalamus is a key regulator of food intake and its neurones express orexigenic and anorexigenic peptides which can either promote or repress feeding respectively, in conjunction with signals from other peptides including leptin and insulin. Important orexigenic peptides of interest include NPY and AgRP which are expressed in the arcuate nucleus of the hypothalamus (Gehlert 1999; Smith *et al.* 2008; Valassi *et al.* 2008) whilst anorexigenic peptides such as CART1 and 2 and CRH are expressed in the PVN (Schwartz *et al.* 2000; Valassi *et al.* 2008). The abundance of transcripts for *Cart* is reduced in mice genetically predisposed to developing obesity (*ob*/*ob* mice; Vettor *et al.* 2002). Moreover, in rats, central administration of CART reduces food intake in a dose-dependent manner (Vettor *et al.* 2002). Glucocorticoids usually stimulate NPY release together with AgRP i.e. *Npy* and *AgRP* mRNA is reduced by ADX but stimulated by corticosterone replacement (Akabayashi *et al.* 1994; Savontaus *et al.* 2002).

However, in the male 5αR1-KO mice, hyperphagia was not driven by increased expression of orexigenic peptides. Indeed, transcripts for *Npy* and *AgRP* in the

hypothalamus are less abundant in male 5αR1-KO mice vs. WT whilst transcripts for *Mc4r* were upregulated. Thus altered balance i.e. a net decrease and increase in orexigenic and anorexigenic drive respectively, may be a compensatory change in hypothalamic neuropeptide expression to maintain body weight in spite of hyperphagia, thus allowing weight maintenance. Moreover, insulin receptors are found in the arcuate nucleus of the hypothalamus and central administration of insulin decreases *Npy* expression (Marks *et al.* 1990; Sipols *et al.* 1995; Benoit *et al.* 2002) but increases the hyperpolarisation of AgRP neurones in the arcuate nucleus (Claret *et al.* 2007; Konner *et al.* 2007). Therefore in male 5αR1-KO mice, higher fasting insulin may have a modulatory effect on appetite and neuropeptide expression in the hypothalamus over and above that of glucocorticoids.

Circulating leptin did not differ between genotypes, although it may have been anticipated to rise with increased body weight and fat mass in female 5αR1-KO mice. Glucocorticoids up-regulate leptin signalling (Cavagnini *et al.* 2000); dexamethasone treatment leads to hyperleptinaemia in both lean and obese states (Miell *et al.* 1996; Dagogo-Jack *et al.* 1997). Furthermore, androgens have a suppressive effect on leptin (Wabitsch *et al.* 1997; Machinal *et al.* 1999). Female mice overall had reduced plasma leptin levels compared to males. This may have been due to reduced 5αDHT production and removal of the androgen-induced leptin suppressive effects thus causing leptin to rise, more so in male animals. However, since this effect was seen in both WT and 5αR1-KO animals, this theory does not explain the sex-differences seen in plasma leptin concentrations. Moreover, increased adipose mass in female 5αR1-KO mice may have been expected to result in increased leptin levels compared to WT, which was not seen here.

In normophagic female 5αR1-KO mice, the abundance of mRNA coding for hypothalamic neuropeptides key in the regulation of food intake was not different compared to controls. Thus, excess weight gain in the female 5αR1-KO mice could not be explained by increased feeding drive or calorific consumption. Therefore it was proposed that locomotor activity may be reduced in female 5αR1-KO mice, and

increased in male 5αR1-KO mice who were hyperphagic whilst maintaining a normal body weight.

Locomotor activity was tested in two settings; once in the home cage overnight and the other during moderately stressful behavioural testing. No differences were seen in the nocturnal locomotor activity between genotypes. There was however, a sex difference whereby, although both sexes covered a similar distance in their home cage over the 14 hr test period, male animals overall spent more time "active" than females. There are very few publications which outline the normal nocturnal activities of male and female mice whilst undisturbed in their home cage. Chen *et al.* showed that male mice had reduced locomotor activity compared to female mice overall (Chen *et al.* 2000) which is the opposite of our data. There are also numerous reports of increased activity in female over male animals during stressful or anxiety-inducing test scenarios such as the open field test (Archer 1975; Norton 1977; Dulawa *et al.* 1999) which are also usually performed during the day. However, in the setting of behavioural testing in the EPM, physical activity (i.e. distance travelled) did not differ by either genotype or sex in the study here presented. This also highlights the differences between testing in the basal non-stressed state vs. testing under mild-moderate stress since a sex difference was found under basal testing only.

There are reports of glucocorticoid-mediated effects on physical activity such as locomotion. 11βHSD1-KO mice showed 38% greater locomotor activity compared to WT controls (Yau *et al.* 2003). Following, knock down of 11βHSD1, mice fed a high-fat diet increased their energy expenditure (determined by both respiratory quotients and locomotor activity) which protected them from obesity as well as insulin resistance (Li *et al.* 2012). It was proposed therefore that mice with reduced tissue glucocorticoid clearance may gain more weight due to increased sedentariness and this may be more marked in female mice, rendering them susceptible to weight gain. However, there were no differences in the physical activity of WT and 5αR1-KO mice in either sex suggesting increased or decreased locomotion (for males and

females respectively) is not the underlying mechanism of weight gain (females) or weight maintenance (males) in 5αR1-KO mice

Specifically to address why female mice were observed to lose weight upon social isolation, behaviour of these mice was first studied in the basal "unstressed" state i.e. in group housing. Propensity to anxiety was assessed in the EPM; a well-regarded testing system designed for the assessment of relative anxiety levels. This test works by exploiting the rodents naturally curiosity and exploratory behaviour in a novel environment. However, since the testing apparatus is raised high off the ground and comprises two opposing arms which have no side walls and thus represent a rather vulnerable space for the mouse, the time the mouse spends in or how often it enters this "open zone", can be regarded as a reflection of how anxious (or "un-anxious") a given animal is.

Where the glucocorticoid load is increased within tissues (be it from endogenous or exogenous sources) or in the case of syndromes related to metabolic disturbances such as Cushing's and its associated conditions (obesity, type II diabetes mellitus etc.), there are often correlations with CNS disorders (Haskett 1985; Hudson *et al.* 1987; Dorn *et al.* 1995; Kelly 1996). Moreover, prolonged elevation of endogenous glucocorticoids resulting from HPA axis dysregulation can be psychologically detrimental (Raber 1998; Raber *et al.* 2000). Anxiety is one affective disorder which is highly documented to occur in instances where there is concomitant glucocorticoid excess.

Here, the time spent in each of the three maze zones (closed, open and centre) followed an expected pattern such that across all experimental groups the closed zone was most occupied during testing and the least time was spent exploring the open, most anxiogenic zone. A moderate amount of time was spent in the centre decision-making area where the animal is generally still (due to the size of the area) or can be seen stretching its head curiously into the open zone. The time spent in the closed zone was reduced in both male and female 5αR1-KO mice but the time in each of the two other zones did not differ. Moreover, male 5αR1-KO mice made

more entries into the open zone during testing; an observation which was not significant in females. These data argue against our hypothesis that 5αR1-KO mice, and in particular female mice, would be more anxious; the 5αR1-KO mice are if anything subtly less anxious, at least when group-housed. However, this original hypothesis was based on observations made during single-housing so it is still possible that an increased tendency towards anxiety may be revealed upon social isolation (explored further in Chapter 5).

The anxiolytic effect in males is somewhat more profound than in females, since 5αR1-KO mice did make more entries into the open zone. A potential explanation may have existed in the reduced generation of 5αDHT, but this is unlikely given literature supporting the anxiolytic properties of 5αDHT (Edinger *et al.* 2005). Men are less likely to develop mood disorders and depression compared to women (Earls 1987; Kessler 2003). Moreover, male, testosterone-treated rats displayed signs of reduced anxiety vs. vehicle-treated animals when tested in the EPM (Bitran *et al.* 1993). In females, 5α-reduced progesterone metabolites (ALLO) may serve as a natural anxiolytic steroid, which would be deficient in the 5αR1-KOs, in particular the female mice. However, this scenario would predict greater anxiety in female 5αR1-KOs, again contrary to our findings. It would indeed be insightful to measure not only basal corticosterone, testosterone, 5αDHT and ALLO levels in plasma but also to measure the concentrations of these steroid hormones within the brain. This can be done via microdialysis which allows the sampling of substances from the interstitial fluid in live, freely-moving animals at any time of the day (for review see (Chefer *et al.* 2009)). The microdialysis technique has been used to sample a wide number of substances (Chefer *et al.* 2009) and its effectiveness has been demonstrated in the brain (Hallstrom *et al.* 1989) and in the mouse (Rollema *et al.* 1989). It would be valuable both to future studies and to the interpretation of the existing study presented here to establish whether 5αR1-KO mice have significant accumulation of glucocorticoids specifically within the brain and whether the concentration of other 5αR1 substrates such as testosterone and progesterone and their 5α-reduced metabolites 5αDHT and ALLO are significantly affected.

In this chapter, sex has been shown to have a modulatory effect on the presentation of the 5αR1-KO phenotype. Metabolically, male 5αR1-KO mice were less well-off and although there was no considerable body weight difference they were hyperphagic, hyperglycaemic and hyperinsulinaemic. Male 5αR1-KO mice also displayed reduced anxiety-like behaviour in the EPM. Conversely, although female mice lacking 5αR1 were normophagic, they still gained more weight than WT controls on a normal chow diet suggesting the weight gain phenotype of 5αR1-KO mice is governed by something other than calorific consumption. Nocturnal activity was not affected by genotype, although it would be useful to evaluate the basal metabolic rates of these animals in future studies by measuring their respiratory quotients and core body temperatures especially since 11βHSD1-KO mice have increased core temperatures (Morton *et al.* 2004).

Some of the metabolic and behavioural changes described here commonly occur in instances where the concentration of glucocorticoids is raised excessively high and often for prolonged periods of time. This can either be caused by or be a causal factor of HPA axis dysfunction where feedback on the axis is disrupted. 5αR1-KO mice were previously shown to have a dampened HPA axis response to acute stress possibly due to exaggerated negative feedback in the brain resulting from 5αR1 loss. The main aim of the following chapter was to elucidate the mechanisms which underlie this shift in HPA axis function.

Chapter 4: The effects of 5αR1 disruption on pathways which regulate the HPA axis

4.1 Introduction

Glucocorticoids play a regulatory role in many metabolic and homeostatic processes whilst also mediating the stress response (Munck *et al.* 1984). The HPA axis is the main mechanism through which normal physiological concentrations of glucocorticoid hormones are maintained. Activated by diurnal cues or stressors, the HPA axis mediates a cascade of neuroendocrine signals, ultimately leading to glucocorticoid release from the adrenals into the circulation. Although beneficial in coping with stress short-term, the HPA axis response must be counter-regulated to prevent the deleterious effects of glucocorticoid excess. This is achieved through negative feedback loops exerted by glucocorticoids themselves and mediated through GR and MR at various levels of the axis (hypothalamus [PVN] and anterior pituitary) but also in the hippocampus (Jacobson *et al.* 1991). These feedback loops regulate glucocorticoid concentrations in the periphery and may also influence intracellular steroid concentrations. In addition to central control of glucocorticoid concentrations by the HPA axis, availability of glucocorticoids to bind to GR can be influenced at a local level and in a tissue specific manner. Within tissues such as the liver, adipose and brain, metabolic enzymes including 5αRs and 11βHSDs also influence local glucocorticoid levels.

Disruption of enzymes such as 11βHSD1 (which activates the inert 11-dehydrocorticosterone into corticosterone) by genetic mutation (Kotelevtsev *et al.* 1997; Harris *et al.* 2001) can critically influence GR signalling within tissues. Abnormal HPA axis control has been reported in 11βHSD1-KO mice with elevated plasma corticosterone and ACTH levels basally and a prolonged corticosterone peak post-stress (Harris *et al.* 2001) implying increased HPA axis activity in response to enhanced glucocorticoid clearance within tissues. 11βHSD1-KO mice also had hyperplasia of the adrenal glands which would facilitate increased glucocorticoid production in the absence of reduced active steroid exerting negative feedback upon the axis (Kotelevtsev *et al.* 1997). These findings are clear evidence demonstrating that metabolic enzymes play an important role in regulating not only glucocorticoid levels within tissues but also in regulating the activity of the HPA axis.

Broadly, reduced 5αR1 activity *in vivo* may be expected to induce effects on HPA axis function which are opposite to those seen in mice with 11βHSD1deficiency. 5αR1-KO mice, with lesser ability to metabolise glucocorticoids and therefore potentially elevated glucocorticoid levels within tissues which express 5αR1, may therefore have increased negative feedback upon corticosteroid receptors involved in HPA axis regulation, facilitating reduced axial drive. Indeed, our group has shown previously that 5αR1-KO mice (both male and female) have normal circulating basal corticosterone levels and normal adrenal gland size and morphology but reduced HPA axis responsiveness (corticosterone) to acute stress (Figure 4.1; Livingstone *et al.* 2014b). Furthermore, the 5αR isozymes show tissue-specific distribution patterns. 5αR1 predominates in the brain (Poletti *et al.* 1998) and is expressed at sites where it could influence the activity of the HPA axis including the anterior pituitary (Massa *et al.* 1972), hypothalamus and hippocampus in rats (Pelletier *et al.* 1994) and the hypothalamus and pituitary in humans (Thigpen *et al.* 1993; Pelletier *et al.* 1994; Yokoi *et al.* 1996). On the basis that 5αR1 is expressed in these brain areas (which also show high expression of corticosteroid receptors) and in higher abundance than 5αR2 (Lephart 1993; 1993; Thigpen *et al.* 1993; Lund *et al.* 2006), disruption of the axis in 5αR1-KOs is not surprising, with anticipated local increases in corticosterone, increasing negative HPA feedback mediated by GR and MR.

4.1.1 Hypothesis

Disruption of 5αR1 will result in altered expression of genes related to regulation of the HPA axis with a view to increased negative feedback.

4.1.2 Aims

- To investigate and determine the central mechanisms which underpin attenuated HPA axial responses following acute restraint stress in 5αR1-KO mice.

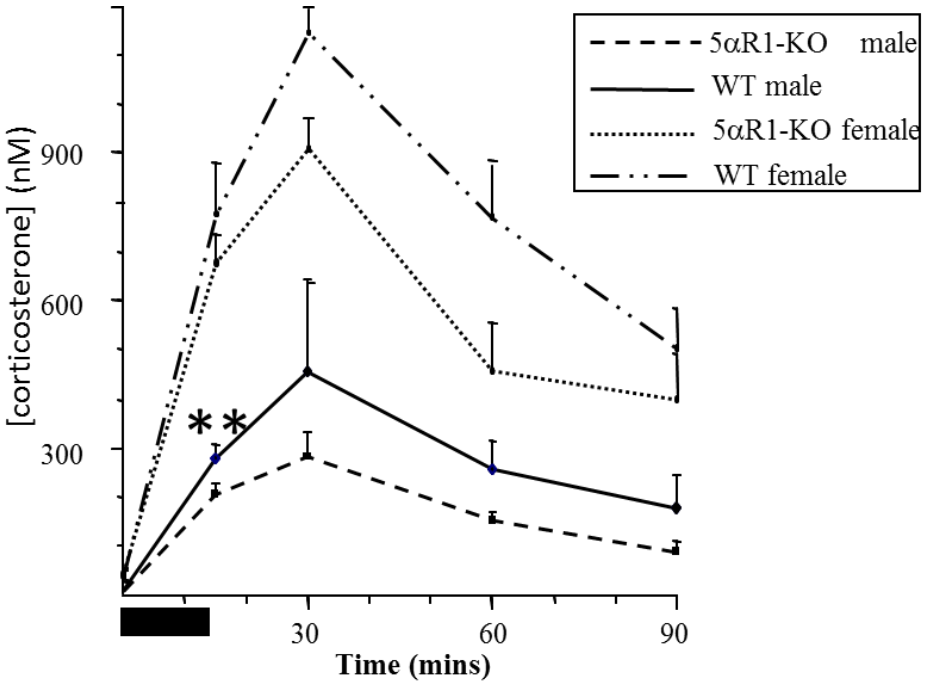

Figure 4.1 Attenuated acute HPA axis responses in 5αR1-KO mice.
Lines show both male and female 5αR1-KO mice (dashed and dotted lines respectively) reach a significantly lower plasma [corticosterone] peak following a 15 min restraint stress compared to WT male and female mice (solid and dashed/dotted lines respectively). Values are mean ± SEM for n = 6/group. Data were compared by 2-way repeated measures ANOVA where ** = $p<0.01$. Mice used were of the mixed genetic strain and were 6 months old. WT = wild type and 5αR1-KO = 5α-reductase type 1 knock-out.

4.2 Methods

4.2.1 HPA axial control and the mechanism underlying reduced stress responses in 5αR1-KO mice

4.2.1.1 Experimental outline

Some of the mice studied here were previously used in the acute restraint experiments (cohort 1, Figure 4.1) and the second cohort was that studied in Chapter 3 (cohort 2). All animals were 6 month old WT and 5αR1-KO mice (male and female). Archived tissues from cohort 1 were used to quantify the abundance of transcripts for *GR* and *MR* in the hippocampus, *Crh* in the PVN and *GR*, *MR* and *Crhr-1* in the pituitary. Tissues from cohort 2 were used to quantify the abundance of transcripts for *GR* and *Avp* in the hypothalamus.

4.2.1.2 Animal maintenance

All mice were obtained from an in-house breeding colony (2.6.1) and maintained under group-housing as described (2.6.3). The mice studied were as follows:

- 6-month old, male, WT and 5αR1-KO mice (cohort 1, n = 9; cohort 2, n = 13)
- 6-month old, female WT and 5αR1-KO mice (cohort 1, n = 9; cohort 2, n = 13)

4.2.1.3 Terminal procedures and tissue collection

Section 2.6.10 described the method used for tissue collection following cull by decapitation (between 0800 and 1000 hrs). Whole brains were collected and snap frozen on dry ice.

4.2.1.4 *In situ* hybridisation

The abundance of *GR* and *MR* transcripts in hippocampal regions (DG and CA1-4) and *Crh* mRNA in the PVN of the hypothalamus from WT and 5αR1-KO mice were quantified by *in situ* hybridisation as described (2.3.5).

4.2.1.5 Quantification of mRNA expression by qPCR

Total RNAs were extracted from the hypothalami and pituitaries (2.3.1.4) of WT and 5αR1-KO mice and used to synthesise first strand cDNA by RT (2.3.2). qPCR was performed (2.3.4) to quantify the expression of transcripts for *GR* and *Avp* in the hypothalamus and *GR*, *MR* and *Crhr-1* in the pituitary of which the abundance was expressed as a ratio to appropriate reference genes which are highlighted in Table 4.1 below. These reference genes or combinations of genes were selected due to their lack of variation between groups (male and female, WT and 5αR1-KO mice).

4.2.2 Statistical analyses

All data are presented as mean ± SEM and were compared using either Student's unpaired t-test or 2-way ANOVA with Fisher's LSD post-hoc tests where appropriate.

Tissue	Reference gene(s)	p Value	
Pituitary	TATA box-binding protein (*Tbp)*	G	S
		0.311	*0.293*
Male Hypothalamus	TATAbox-binding protein (*Tbp*)/ Glyceraldehyde-3-phosphate dehydrogenase *(Gapdh)*	*0.285*	
Female Hypothalamus	Glyceraldehyde-3-phosphate dehydrogenase *(Gapdh)*	*0.314*	

Table 4.1 Reference genes used for qPCR in pituitary and hypothalamus
P value indicates the results of a 2-way ANOVA between male and female, WT and 5αR1-KO mice or Student's unpaired t-test between WT and 5αR1-KO mice within each of the two sexes. The reference gene (or combination or reference genes) with the highest p value and thus least variation between experimental groups was selected. G = genotype and S = sex.

4.3 Results

4.3.1 Body and tissue weights

Body and tissue weights for male and female, 5αR1-KO mice (cohort 1) are shown in Table 4.2 below. There were no significant differences in either body, adrenal or thymus weights between male WT and 5αR1-KO mice. In female animals, body weight was significantly increased in 5αR1-KO mice vs. WT ($p<0.05$) but there was no difference in either adrenal or thymus weight (Table 4.2).

For cohort 2 (as shown in Chapter 3 section 3.3.1) there were no body weight differences between male WT and 5αR1-KO mice at cull (Table 3.2). In female mice from cohort 2, 5αR1-KO mice were consistently heavier over time ($p<0.01$) and also at cull compared to WT littermates (Figure 3.1, $p<0.01$).

4.3.2 Alterations in pathways which regulate the HPA axis

4.3.2.1 Corticosteroid receptors

GR and *MR* mRNA expression was quantified in hippocampal and pituitary tissue of both male and female, WT and 5αR1-KO mice. *GR* and *MR* transcripts were present across all hippocampal regions in both male and female mice (Figure 4.2, panels A-D). The abundance of *MR* but not *GR* transcripts in the brains of male 5αR1-KO mice was significantly reduced overall ($p<0.01$) reaching significance through post-hoc testing in the CA2 region ($p<0.05$) compared to WT (Figure 4.2; B). There was no difference in either *GR* or *MR* expression in hippocampus from female 5αR1-KO mice (Figure 4.2, C and D respectively). In the pituitary, *GR* expression levels were upregulated in male but not female 5αR1-KO mice vs. WT (Figure 4.2; E; $p<0.05$). *GR* mRNA was more abundant in female vs. male pituitary in WT mice only (Figure 4.2; E; $p<0.01$). The opposite was true for *MR* transcripts, which were significantly more abundant in male vs. female mice overall, irrespective of genotype (Figure 4.2; F; $p<0.05$). The abundance of transcripts for *GR* was quantified in the hypothalami of male and female, WT and 5αR1-KO mice. In male mice, transcripts for *GR* were upregulated in 5αR1-KO mice vs. WT (Figure 4.2; G, $p<0.05$). The opposite was seen in females where there was a trend towards reduced hypothalamic *GR* transcript abundance in 5αR1-KO animals compared to control (Figure 4.2, H, $p = 0.064$).

Weight		WT	5αR1-KO	p Value
Male	Body (g)	26.3 ± 0.85	27.0 ± 0.76	>0.05
	Adrenal (mg)	2.2 ± 0.08	2.1 ± 0.09	>0.05
	Thymus (mg)	40.4 ± 3.3	39.2 ± 3.5	>0.05
Female	Body (g)	28.91 ± 0.99	**32.27 ± 4.42**	***<0.05***
	Adrenal (mg)	2.26 ± 0.25	2.32 ± 0.24	>0.05
	Thymus (mg)	34.07 ± 2.26	34.87 ± 2.91	>0.05

Table 4.2 Body and tissue weights

Body, adrenal and thymus weights were not different in male 5αR1-KO mice compared to WT. Female 5αR1-KO mice were significantly heavier than WT ($p<0.05$) but adrenal and thymus weights were not different between genotypes. Data are mean ± SEM for n = 12-16/group and comparisons were made by unpaired Student's t-test. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out. (Livingstone *et al.* 2014b for male mice and Livingstone *et al.* unpublished for female mice).

4.3.2.2 *Crh* and *Avp* neuropeptides and the CRH receptor (*Crhr-1*)

The abundances of transcripts for *Crh* and *Crhr-1* were quantified in the PVN of the hypothalamus, and the pituitary gland respectively, of WT and 5αR1-KO mice. Figure 4.3 A shows representative microscopy images for *Crh* expression in the PVN of female WT (A i) and 5αR1-KO (A ii) mice. Figure 4.3, A iii demonstrates the lack of silver grains in a PVN section from a WT mouse using the sense probe which eliminates the possibility of non-specific binding. The abundance of transcripts for *Crh* was reduced in 5αR1-KO mice vs. WT overall (Figure 4.3; B; $p<0.01$) with significance from post-hoc testing in both male ($p<0.05$) and female ($p<0.01$) mice. Sexually dimorphic alterations (effect of sex = $p<0.05$) in the abundance of *Crhr-1* transcripts between genotypes were found whereby, in females only, global KO of 5αR1 reduced *Crh*r-*1* mRNA abundance in the pituitary compared to WT (Figure 4.3; C; $p<0.05$).

The abundance of *Avp* transcripts was quantified in the hypothalami of male and female, WT and 5αR1-KO mice. In male mice, there was a trend for reduced levels of *Avp* mRNA in 5αR1-KO mice vs. WT (Figure 4.3; D; $p = 0.063$) but *Avp* mRNA abundance in female 5αR1-KO mice was comparable to WT controls (Figure 4.3, E).

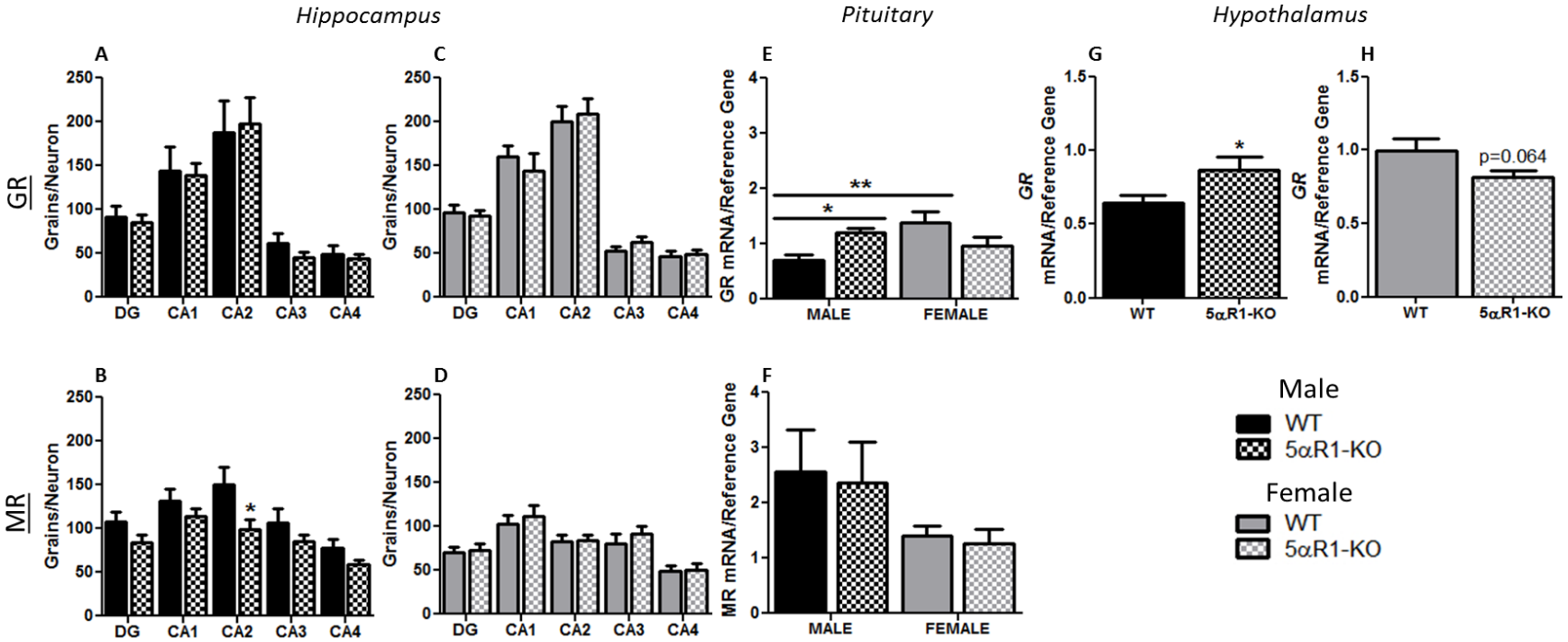

Figure 4.2 *GR* and *MR* mRNA expression profile in brain regions contributing to feedback control of the hypothalamic-pituitary adrenal axis

GR and *MR* mRNA abundance was quantified in pyramidal neurons of the DG, CA1, CA2, CA3 and CA4 regions of the hippocampus. Both *GR* (A, C) and *MR* (B, D) were expressed across all hippocampal regions in both male (A, B, black) and female (C, D, grey) mice. *MR* mRNA transcripts were less abundant in male 5αR1-KO mice (chequered) vs. WT (solid) overall ($p<0.01$), reaching statistical significance in the CA2 region (5αR1-KO; B; $p<0.05$) compared to WT. In the pituitary (E-F), *GR* expression was upregulated in male 5αR1-KO mice compared to WT, whilst there was a 2 fold increase in *GR* mRNA abundance in female WT mice compared to WT males (E). *MR* transcripts were more abundant in male pituitary than female (F, $p<0.05$), without any difference between genotypes (F). In the hypothalamus, the abundance of *GR* transcripts was increased in male 5αR1-KO mice (G, $p<0.05$) but decreased in female 5αR1-KOs (H, trend, $p=0.064$) vs. WT controls of the same sex. Bars are mean ± SEM for n=9-13/group. *=$p<0.05$ and **=$p<0.01$ determined by 2-way ANOVA with Fisher's LSD post-hoc tests where appropriate or Student's unpaired t-test. GR = glucocorticoid receptor, MR = mineralocorticoid receptor, DG = dentate gyrus, CA1-CA4 = cornu ammonis regions 1-4, WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

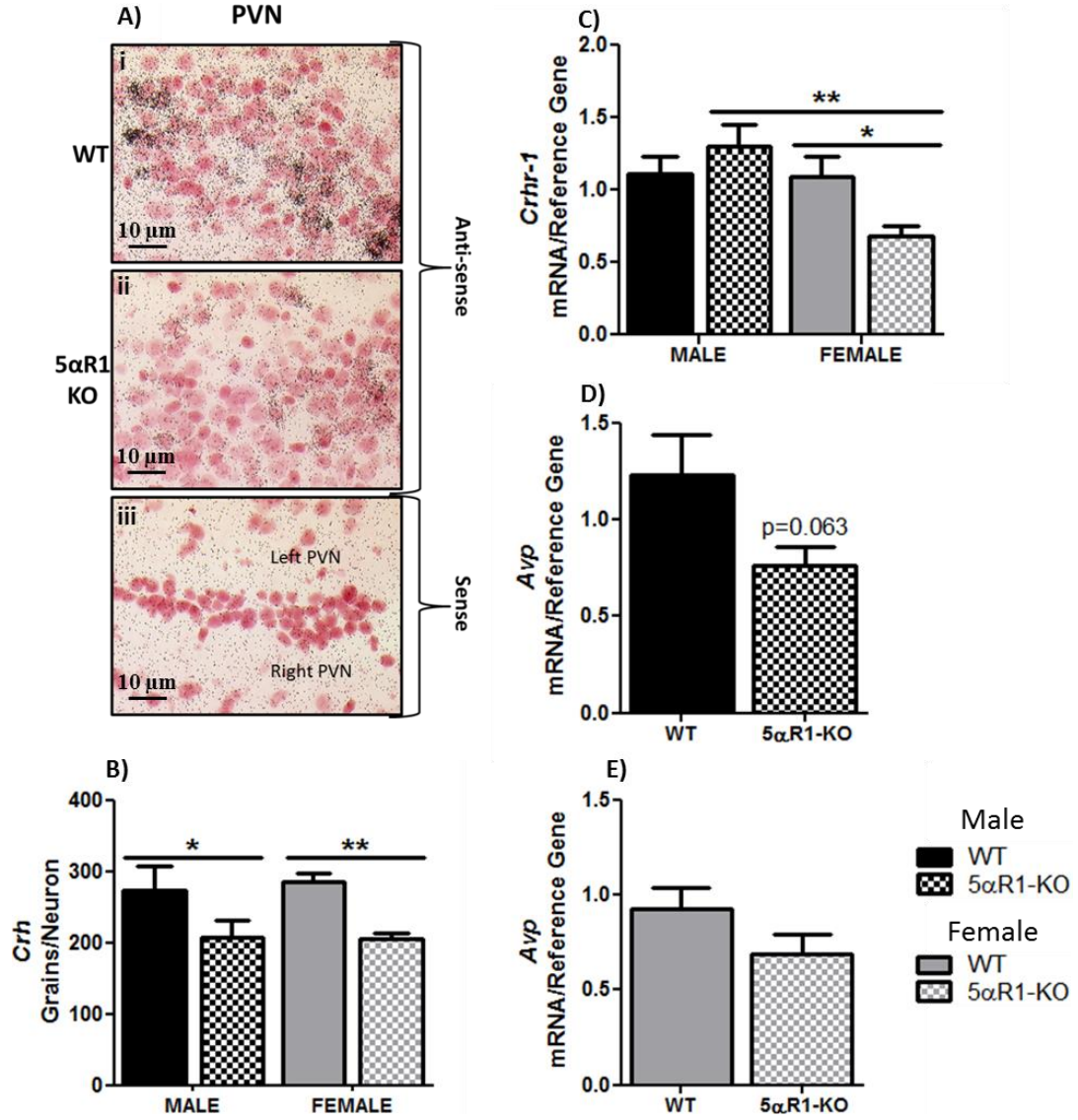

Figure 4.3 *Crh*, *Crhr-1*and *Avp* mRNA expression in brain regions involved in regulating the hypothalamic pituitary adrenal axis

A shows representative images of *Crh* transcript abundance in the PVN of the hypothalamus measured as silver grains for WT (i) and 5αR1-KO (ii) mice using the anti-sense probe and lack of silver grains in a WT animal using the control sense probe (iii). The abundance of *Crh* mRNA in the PVN (B) was reduced overall in mice with disruption of 5αR1 (chequered, $p<0.01$), and reached significance in both male (black, $p<0.05$) and female (grey, $p<0.01$) mice compared to WT (solid). There was an overall effect of sex on *Crhr-1* expression in the pituitary (C, $p<0.05$) but transcripts were only downregulated in female 5αR1-KO mice vs. WT (C, $p<0.05$) with no significant genotype differences in males. Hypothalamic *Avp* mRNA expression levels were downregulated in male mice lacking 5αR1 vs. WT (D; $p=0.063$) but there was no such difference in female animals (E). Values are mean ± SEM for n=9-13/group. Significance was determined by 2-way ANOVA with Fisher's LSD post-hoc tests where appropriate or Student's unpaired t-test. *=$p<0.05$, **=$p<0.01$. . WT = wild-type, 5αR1-KO = 5α-reductase type 1 knock-out, *Crh* = corticotrophin-releasing hormone, *Crhr-1* = corticotrophin-releasing hormone receptor type 1, PVN = paraventricular nucleus and *Avp* = arginine vasopressin.

4.4 Discussion

We hypothesised that global knock-out of 5αR1 would impair corticosteroid metabolism thereby leading to increased glucocorticoid levels at sites of the expression of the enzyme. Previously in our group, it has been shown that basal corticosterone levels at the diurnal nadir were comparable between WT and 5αR1-KO mice (Livingstone *et al.* 2014b). This is not surprising as the HPA axis readily balances production with clearance to maintain a basal set-point. However, genotype differences were revealed upon manipulation of the system, with 5αR1-KO mice having a dampened HPA axis response to acute restraint stress compared to WT controls (Livingstone *et al.* 2014b). One explanation is that corticosteroid levels locally within brain structures known to mediate negative feedback on the axis, such as the hippocampus, hypothalamus and pituitary, may be increased and responsible for this dampened stress response. Impaired clearance of glucocorticoids in the periphery would also be expected to exert an additional level of negative feedback on the axis.

In the brain, glucocorticoids mediate their effects via both GR (low affinity) and MR (high affinity) which are expressed abundantly in the limbic system (such as the hippocampus) linking with their role in HPA axis regulation as well as in emotion and cognition (Ahima *et al.* 1990); discussed further in Chapter 5). Glucocorticoids themselves are able to influence and directly alter corticosteroid receptor expression in the brain and other target tissues (Sapolsky *et al.* 1984; McEwan *et al.* 1986). Normal function of the HPA axis is maintained by the activity of GR and MR via negative feedback loops which can increase or decrease glucocorticoid release from the adrenals to maintain levels at an optimum physiological set-point. Generally speaking, MR is less abundant than GR in the CNS (Keller-Wood *et al.* 2006) but also has a 10-fold higher affinity for glucocorticoids; for this reason it is thought that MR is mostly occupied at basal glucocorticoid levels during the circadian nadir (Reul *et al.* 1985; Oitzl *et al.* 1995). The interpretation of this is that MR is responsible for the maintenance of basal HPA activity as well as the initiation of the stress response, and that GR modulates responses to stress-induced increases in [corticosterone] to return levels to the basal set-point (de Kloet *et al.* 1987; de Kloet *et al.* 1998; Karst *et*

al. 2005). However, more recently it has been proposed that GR and MR work synergistically during the circadian peak (and post-stress) to shut off corticosterone release, or further still that MR alone can mediate negative feedback on the axis during periods when corticosterone concentrations are high (Bradbury *et al.* 1994; Reul *et al.* 1997; Arvat *et al.* 2001; Wellhoener *et al.* 2004). Given that the majority of authors believe both receptors play a part in HPA axis regulation, it has been proposed that changes in the equilibrium between GR- and MR-mediated responses in the CNS may underlie dysregulation of the axis (de Kloet 1991). Altered MR:GR balance could either exacerbate or inhibit an appropriate HPA axial response to stress as well as delayed recovery, predicting vulnerability to stress (Sapolsky 2000), as well as rendering individuals susceptible to metabolic disease and affective disorders (de Kloet 1991).

In vivo, daily infusion of exogenous glucocorticoids over several days results in reduced function of the HPA axis at a basal level (Akana *et al.* 1997). We proposed that attenuated HPA axis responsiveness to stress in the 5αR1-KO model may be due to glucocorticoid excess altering the balance of corticosteroid receptor expression as has been discussed before for similar models (de Kloet 1991; Sapolsky 2000).

In the current study, corticosteroid receptor expression followed an expected pattern across all hippocampal regions whereby expression of both *GR* and *MR* increased from the DG across to CA1 and CA2, and was lower in CA3 and CA4 (Sousa *et al.* 1989; Morimoto *et al.* 1996; Usuku *et al.* 2005; Nishi *et al.* 2007). To first address the male phenotype, transcripts for *GR* were unchanged between genotypes in the hippocampus but were more abundant in the hypothalamus and pituitary of 5αR1-KO mice vs. WT. Deletion of forebrain *GR* has been shown to induce an exaggerated corticosterone efflux under stressful conditions (Boyle *et al.* 2005; Boyle *et al.* 2006) suggesting *GR* is crucial in eliciting negative feedback to control corticosterone levels both during and post-stress. Increased *GR* in male 5αR1-KO mice is therefore in keeping with attenuated corticosterone release upon stress. Furthermore, it is encouraging to also compare these data with that from mice with genetic modifications in the GR gene, resulting in reduced GR signalling, which

have increased HPA activation due to loss of inhibition (Ridder *et al.* 2005; Harris *et al.* 2013). However, whether changes in *GR* in 5αR1-KO mice are due to locally elevated glucocorticoid levels is less clear, given that many reports suggest ADX results in GR upregulation (which is reversible upon glucocorticoid replacement), whilst high glucocorticoids downregulate the receptor (Burnstein *et al.* 1992; Joels *et al.* 1994). Moreover increased central glucocorticoid levels might be anticipated to invoke suppression of basal circulating corticosterone; a phenotype which was not seen in 5αR1-KO mice.

5αR1 is abundant in the hypothalamus (Campbell *et al.* 1989; Pelletier *et al.* 1994) and pituitary (Massa *et al.* 1972; Yokoi *et al.* 1996) and although the hippocampus is key in HPA axial control (Fendler *et al.* 1961; Knigge 1961; Feldman *et al.* 1980; Wilson *et al.* 1980) it is possible that 5αR1 expression in the hippocampus is low (Pelletier *et al.* 1994), however, the expression pattern for 5αR1 in the brain (particularly in mice) is not well documented. If 5αR1 is indeed underrepresented in the hippocampus compared to the hypothalamus and pituitary, 5αR1-KO may be expected to induce less of an effect upon corticosteroid receptor mRNA expression in this brain region compared to others. This may explain the region-specific upregulation of *GR* mRNA abundance in male 5αR1-KO mice, especially since the lower affinity GR is only occupied when corticosterone levels are high. Moreover, circulating glucocorticoids have been shown to have more potent regulatory effects upon hippocampal *MR* expression compared to that of *GR* (Herman *et al.* 1993).

MR over-expression studies have also shown an effect on HPA axis activity whereby increased MR specifically in the forebrain resulted in reduced corticosterone responses to acute restraint stress (Rozeboom *et al.* 2007). ADX animals (whose circulating corticosterone is essentially removed), show hippocampal upregulation of *MR* mRNA (Vazquez *et al.* 1993). In male mice lacking 5αR1, *MR* transcripts were downregulated in the hippocampus (possibly reflecting auto-regulation) but were unchanged in the pituitary. MR binds corticosterone with a much greater affinity than GR and therefore may be more sensitive to smaller changes in clearance of the steroid. In brain regions such as the hippocampus where *MR* is highly expressed,

corticosterone may be elevated due to local loss of 5αR1, thus leading to overstimulation and desensitisation of MR ultimately resulting in downregulation of the receptor. Reports from Karst *et al.* suggest MR is primarily involved in initiating the HPA axis cascade whilst GR is responsible for mediating negative feedback upon the axis (Karst *et al.* 2005). Thus by physiologically lowering *MR*- and increasing *GR* expression within brain regions, the net effect would be reduced HPA axis activity to prevent further glucocorticoid production in 5αR1-KO mice. These mechanisms would apply both basally to maintain physiologically optimal corticosterone levels in spite of reduced clearance and also during acute periods of stress where animals would be unable to reach corticosterone peaks comparable to WT and would dampen down their response more rapidly.

The literature surrounding auto-regulation of GR and MR by ligand concentration is conflicting. Some reports suggest reduced hippocampal *GR* following ADX (Sheng *et al.* 2003) whilst others found no evidence at all for ADX-induced changes in corticosteroid receptor expression in the brain (Chao *et al.* 1989). Although, in agreement with reduced GR after ADX, *GR* is downregulated in the hypothalamus in 11βHSD1-KO mice (Harris *et al.* 2001). Models of glucocorticoid excess such as obese Zucker rats with elevated corticosterone levels, had normal *GR* but decreased *MR* expression in the hippocampus (Mattsson *et al.* 2003) as was seen in the current study in 5αR1-KO mice. There are also regional variations in GR and MR autoregulation for example, ADX has no effect on *GR* mRNA content in the hypothalamus (Peiffer *et al.* 1991) or *MR* expression in the neocortex (Herman *et al.* 1993); conflicting with the upregulation seen in the hippocampus. Indeed our data goes against the classical concept that ligand excess locally leads to desensitisation and downregulation of its receptor. Instead we propose that increased GR-mediated negative feedback in the hypothalamus and pituitary could be an attempt to counter reduced peripheral glucocorticoid clearance and maintain normal corticosterone levels basally in plasma. Additionally it could be that glucocorticoids are not sufficiently increased centrally in 5αR1-KO mice to induce receptor downregulation, with mice lacking 5αR1 being a somewhat modest model of glucocorticoid excess.

Corticosterone concentrations in the brain have been quantified in a separate cohort of animals, the results of which are discussed in Chapter 5.

As well as corticosteroid receptors, neuropeptides such as CRH, described as "the link between hormonal-, metabolic- and behavioural responses to stress" (Kovács 2013) play an important role in maintaining adequate HPA axis function. CRH is synthesised and secreted into the hypothalamic-pituitary portal system by parvocellular neurons in the PVN (Owens *et al.* 1991). This process is necessary for normal activation of the HPA axis to elicit an hormonal response to stress (Owens *et al.* 1991). Several days of corticosterone treatment inhibited HPA responsiveness to acute stress (Wilkinson *et al.* 1981; Scribner *et al.* 1993) possibly due to increased negative feedback upon the axis; a process which most likely occurs at the level of the pituitary (ACTH) and the hypothalamus (CRH; Dallman *et al.* 1985). The attenuated stress response in 5αR1-KO mice is consistent with impaired central CRH signalling. Indeed, male 5αR1-KO mice have reduced mRNA for *Crh* in the PVN but *Crhr-1* is unchanged in abundance. Reduced stimulation of the anterior pituitary by *Crh* may therefore result in reduced forward drive towards corticosterone production by the adrenals, protecting from excessive glucocorticoid production.

The CRH-KO model has provided a useful tool with which to investigate the involvement of CRH in HPA axis responses to stress. Several authors have demonstrated attenuated stress responses in these animals. For example, Makino *et al.* report blunted pituitary-adrenocortical responses via reduced plasma [corticosterone] vs. controls following restraint stress (Makino *et al.* 2005). Other authors also report limited increases in corticosterone secretion in response to both psychological (restraint) as well as physiological (insulin-induced hypoglycaemia) stressors in CRH-KO mice (Muglia *et al.* 1995; Jeong *et al.* 1999; Jacobson *et al.* 2000). Similarly, CRHR1-KO mice had severely blunted corticosterone responses although female mutant mice maintained a rudimentary corticosterone response to stress in the absence of CRHR1 (Smith *et al.* 1998).

AVP alone has a weak effect on ACTH secretion but released concomitantly with CRH from the PVN of the hypothalamus, AVP interacts with CRH to potentiate ACTH secretion from the anterior pituitary; promoting glucocorticoid release from the adrenals (Lamberts *et al.* 1984). CRH-producing neurons in the PVN co-express AVP, however, AVP levels are usually low basally and are dependent on the relative stress levels experienced by an organism (Williamson *et al.* 2005). For example, AVP synthesis is upregulated in response to chronic stress and also following ADX (Whitnall 1988; 1993). This is suggestive of a modulatory role for AVP whereby glucocorticoid levels are sustained by prolonged ACTH release which would otherwise be inhibited by negative feedback loops (Williamson *et al.* 2005). Here *Avp* mRNA was reduced similarly to *Crh* which, along with altered corticosteroid receptor expression, may be a driver in reduced HPA axis responses to acute stress in at least in male 5αR1-KO mice.

In support of the data here presented, endogenous glucocorticoids, dexamethasone treatment and stress are all known to reduce *Crh* expression *in vivo*; likely mediated by GR (Jingami *et al.* 1985; Beyer *et al.* 1988; Boyle *et al.* 2006; Sharma *et al.* 2013). Glucocorticoids (and stress) also attenuate *Crhr-1* expression (Makino *et al.* 1995) and activation of corticosteroid receptors suppresses both the synthesis and release of AVP as well as CRH from the PVN (de Kloet *et al.* 1998). Dexamethasone treatment reduces AVP concentrations in hypophyseal portal plasma (Fink *et al.* 1988). Conversely, and in support of our findings, hypophyseal portal plasma AVP and CRH as well as transcripts for *Crh* in the PVN are increased following ADX (Fink *et al.* 1988; Luo *et al.* 1995). Taken together, reduced *Crh, Crhr-1* and *Avp* in the HPA axis system supports the model of glucocorticoid excess within tissues resulting in enhanced negative feedback mediated mainly by GR in male 5αR1-KO mice.

5αR1 metabolises not only glucocorticoids but also other A-ring steroids including sex hormones such as testosterone and progesterone and unsurprisingly we have found strong sexual dimorphism in gene expression phenotypes throughout this study even though HPA axis responses were similarly reduced in male and female 5αR1-

KO mice. In females, no genotype difference was found in corticosteroid receptor expression in the hippocampus. However, female mice expressed transcripts for *MR* at lower levels than males in the pituitary, whilst *GR* mRNA transcripts were more abundant in the female pituitary vs. the male in WT animals only. This sexually dimorphic pattern of reduced *MR* and increased *GR* in females was reminiscent of the differences between the 5αR1-KO males vs. WT mice. Therefore it is possible that the pattern of changes in *GR* and *MR* are related to the low androgen state, either demonstrated by female mice or by mice lacking 5αR1 who are potentially deficient in 5αDHT in the brain. Possibly the smaller change in 5αDHT concentrations in female 5αR1-KO mice rather than male 5αR1-KOs (due to lower testosterone in females) may explain the lack of alteration in receptor levels.

Despite similar changes in *MR* and *GR* transcripts, the gender differences in other aspects of the HPA axis were opposite in female mice vs. males when compared with 5αR1-KO males vs. WT. For example, in the hypothalamus *GR* was upregulated and down regulated in male and female 5αR1-KO mice respectively vs. WT mice of the same gender. Female mice have higher [corticosterone] than males, both basally and post-stress, as demonstrated by a number of authors (Critchlow *et al.* 1963; Dallman *et al.* 1987; Vamvakopoulos *et al.* 1994), and as has been seen previously in our lab when quantifying basal and stressed corticosterone (Livingstone *et al.* unpublished). The low MR, high GR theory proposed by Karst *et al.* in male mice (Karst *et al.* 2005) for maintaining HPA activity in spite of 5αR1 loss does not fit with corticosteroid expression patterns and physiology in females. However, downregulation of *MR* in the female hypothalamus suggests female 5αR1-KO mice may be more sensitive to reduced glucocorticoid clearance given their basal set-point is already increased relative to male animals. The female 5αR1-KO data does fit with the classical receptor downregulation theory in response to high ligand concentrations, suggesting local glucocorticoids are increased at least in the female 5αR1-KO hypothalamus.

Close inter-relationships exist between the HPA and the hypothalamic-pituitary-gonadal (HPG) axes. In mammals, stress and glucocorticoid excess causes

disruption of HPG function including the synthesis and release of sex steroids (Rivier *et al.* 1991; Tilbrook *et al.* 2000) but sex steroids induce reciprocal actions back upon the HPA axis. Oestrogens and androgens (both substrates for 5αR1) act upon several levels of the HPA axis including CRH and AVP synthesis in the PVN, stress-induced ACTH release and ultimately corticosteroid synthesis (Dallman *et al.* 2002). In addition to their effects on feed-forward components of the HPA axis, sex steroids are also implicated in the regulation of the negative feedback loops mediated by glucocorticoids (Young 1996). The majority of studies which investigate HPA function are performed using male animals with the assumption that their sex steroid profile provides greater stability compared to females. However, testosterone levels (also metabolised by 5αR1) vary with the circadian rhythm and are also dependent upon age, social status and reproductive experience (Bartke *et al.* 1973; Sencar-Cupovic *et al.* 1976; Kalra *et al.* 1977; Simpkins *et al.* 1981; Winters *et al.* 1991; Seeman *et al.* 2001; Viau 2002). *In vivo,* testosterone has been shown to attenuate the HPA axis stress response (Williamson *et al.* 2005) whilst in females ovariectomy leads to attenuated HPA axis responses but oestradiol replacement may induce HPA axis stimulation (Lesniewska *et al.* 1990; Norman *et al.* 1992). Since 5αR1-KO mice may have low 5αDHT and higher oestradiol levels (Mahendroo *et al.* 1999), it is unlikely that changes in sex steroids underpin the attenuated HPA axis.

This thesis demonstrates that both male and female WT animals show equivalent abundance of transcripts for *Crh* and *Crhr-1*. Tissues from female animals lacking 5αR1 have a down-regulation of mRNA for *Crh* and a corresponding decrease in transcripts for its main receptor (*Crhr-1*) in the brain (PVN of the hypothalamus). Similar to males, it is thought this reduced *Crh* and *Crhr-1* in females may result in response to raised availability of corticosterone and therefore more negative feedback on axial drive. Thus less *Crh* and reduced availability of its primary receptor would further reduce drive upon the axis and therefore glucocorticoid production by the adrenals. Here, observations that transcripts for *Crh* (both males and females) and *Crhr-1* (females only) are reduced, align well with a dampening of the HPA axis response. However, although there were trends for reduced *Avp* in the male 5αR1-KO hypothalamus there was no change in female 5αR1-KO mice. This

again is evidence that there are profound differences between HPA axis function and regulation between male and female animals.

To conclude, the results presented here are consistent with glucocorticoid excess within the brain. 5αR1-KO mice show altered corticosteroid receptor expression balance in brain areas involved in HPA axis regulation. It may be that an imbalance between *GR* and *MR* along with loss of the potentiating effects of *Avp* in males and reduced *Crh* and *Crhr-1* in females are the underlying mechanisms responsible for dampened responses to acute stress in 5αR1-KO mice.

Changes in neuropeptide levels are not only associated with HPA axis dysfunction but also with the aetiology of anxiety and feeding disorders. In light of reduced expression of neuropeptides such as *Crh* in 5αR1-KO mice, and previous observations of maladaptation to social isolation, the behaviour of 5αR1-KO mice was assessed under both basal (group-housing) and mildly stressful (single-housing) conditions.

Chapter 5: Behavioural alterations in 5αR1-KO mice housed under the stress of social isolation

5.1 Introduction

In previous experiments performed by other research group members, obesity in female 5αR1-KO mice was modulated by environmental factors. Specifically, female 5αR1-KO mice only developed obesity and fatty liver when housed together in groups. This phenotype was reversible within 4 weeks of separation and single housing during which the extra weight was lost and 5αR1-KO mice became metabolically comparable to WT controls i.e. weighed the same and were no longer hyperinsulinaemic. It was therefore hypothesised that female 5αR1-KO mice were predisposed to anxiety and susceptible to environmental stress induced by social isolation, although the mechanism of weight loss was unknown. Certainly, a large number of studies have shown social isolation of rodents to be detrimental, modifying locomotor activity, thermoregulation, learning, memory and anxiety (Juraska *et al.* 1984; Bates *et al.* 1985; Jones *et al.* 1991; Hellemans *et al.* 2004; Võikar *et al.* 2005).

Social isolation-induced changes involving peripheral, behavioural and neurochemical functions, have been identified leading to the theory of "isolation syndrome" in mice which is most-likely related to chronic stress (Valzelli 1973). Moreover, individual housing of rodents brings about pathophysiological changes such as impaired HPA axis and sympathetic nervous system responses (Pashko *et al.* 1980; Kim *et al.* 1996) and altered emotional and cognitive behaviours (Valzelli 1973; Guidotti *et al.* 2001; Võikar *et al.* 2005). Given the metabolic phenotype is attenuated during isolation, the female 5αR1-KO mice may cope differently with chronically stressful conditions and adjust their food intake accordingly. Importantly, chow intake is usually decreased in rodents during periods of chronic stress (Dallman *et al.* 2001), which could lead to weight loss, and this can be modulated by glucocorticoids (Pralong *et al.* 1993; Alfarez *et al.* 2008). Both female and male 5αR1-KO mice showed reduced peak plasma corticosterone levels following restraint compared to WT littermates (Livingstone *et al.* 2014b for male mice and Livingstone *et al.* unpublished for female mice, as shown in Chapter 4), potentially rendering them less able to initiate an acute stress response. In Chapter 3, female 5αR1-KO mice did not demonstrate anxiety-like behaviour whilst male

5αR1-KO mice actually had reduced anxiety compared to controls. However these experiments were performed under group-housing conditions and anxiety may be revealed upon social isolation.

Deviations away from the optimal physiological levels of glucocorticoid hormones (be this stress-induced or as a result of ageing) are also believed to exacerbate or be causal of the reduced cognitive ability seen with increasing age (Kaye *et al.* 1987; Rybakowski *et al.* 1992; Yau *et al.* 1995; Gregus *et al.* 2005; Lai *et al.* 2007; Rozeboom *et al.* 2007; Holsboer *et al.* 2008). Indeed any type of stress and prolonged exposure to stress-related hormones can induce neuronal atrophy and death implicated in stress-induced neuronal degeneration and cognitive impairments (Alleva *et al.* 1996; Aloe *et al.* 2002). Dysfunction of the HPA axis has been reported in association with several mental disorders which are also characterised by cognitive disturbances e.g. major depressive disorder (Deuschle *et al.* 1997; Strickland *et al.* 2002; Bhagwagar *et al.* 2005; Burke *et al.* 2005; Vreeburg *et al.* 2009). In preclinical models, stressed, middle-aged mice with memory-impairments have a corresponding and dramatic increase in corticosterone levels within the hippocampus (Beracochea *et al.* 2011) whilst in humans, several investigations have shown that acute administration of glucocorticoids impairs long-term memory retrieval in healthy volunteers (Roozendaal 2002; Het *et al.* 2005; Buchanan 2007). However, a key feature of acute glucocorticoid effects on physiological functions (and importantly on cognition), is that there is a fine line between what constitutes beneficial or "normal" and what constitutes excessive and detrimental, with glucocorticoids both facilitating and impairing memory and its consolidation dependent on their physiological concentration.

The hippocampus; a key locus for cognition and neuroendocrine control, is especially sensitive to even small changes (both increases and decreases) in local glucocorticoid concentrations (McEwen 1999). In aged humans and rodents, an association exists between chronic corticosterone excess, hippocampal atrophy and resulting cognitive decline (Kizaki *et al.* 1998; Lupien *et al.* 1998) and ADX with low-dose corticosterone replacement at mid-life substantially attenuates cognitive

impairments (Landfield *et al.* 1978). Altered glucocorticoid metabolism in the brain is sufficient to modify behaviour and cognitive ageing which is mediated mainly by the hippocampus. Examples include work by Yau and colleagues showing mice null for 11βHSD1 are protected from age-related cognitive deficits mediated by the hippocampus via a switch from GR- to MR-mediated cognitive control (Yau *et al.* 2001; Yau *et al.* 2007; Sooy *et al.* 2010; Yau *et al.* 2011). Despite aged 11βHSD1-KO mice having raised plasma corticosterone, intra-hippocampal corticosterone levels were markedly reduced (Yau *et al.* 2001). This shows that not only are glucocorticoids mediating their effects on cognitive ability via the hippocampus itself, but also that local glucocorticoid levels within the brain (as determined by the activity of metabolic enzymes) can act independently from those circulating in the periphery. In addition, the selective 11βHSD1 inhibitor UE1961 significantly improves spatial memory in aged mice (Sooy *et al.* 2010). Work in humans has mirrored the cognitive effects of glucocorticoid excess seen in rodents (Keenan *et al.* 1995; Kirschbaum *et al.* 1996; Naber *et al.* 1996; Comijs *et al.* 2010; Potvin *et al.* 2013).

In this study, loss of 5αR1 *in vivo,* potentially causing local glucocorticoid excess, may be expected to induce an effect on cognition opposite to that shown in 11βHSD1-KO mice and mice treated with an 11βHSD1 inhibitor whereby learning and memory capabilities would be attenuated, especially in aged mice. Outcomes of studying cognition in 5αR1-KO mice may be predicted to correspond to premature age-associated cognitive impairments shown in transgenic mice with forebrain-specific 11βHSD1 overexpression, even though glucocorticoid levels in the circulation were unchanged (Holmes *et al.* 2010).

5.1.1 Hypothesis

Impaired glucocorticoid metabolism in the brain of 5αR1-KO mice leads to glucocorticoid excess which may be associated with anxiety and cognitive deficiencies; exaggerated by the stress of social isolation as well as ageing.

5.1.2 Aims:

- To determine if 5αR1-KO mice are predisposed to anxiety under the chronic stress of social isolation.
- To investigate if 5αR1-KO mice are more susceptible to cognitive impairment and if this is exacerbated by ageing.

5.2 Methods

5.2.1 Genetic background

Studies described thus far have used 5αR1-KO mice on a mixed genetic background. Behavioral experiments can generate data which is highly variable between individual animals, therefore the existing mixed strain mice were back-crossed onto a C57Bl/6j background to reduced variance and therefore increase the power of the experiment. Mice were backcrossed for 10 generations (i.e. until >99% congenic) before breeding the experimental animals studied here.

5.2.2 Experimental outline

Female, WT and 5αR1-KO mice were housed in groups from weaning until the age of 5 months when they were split into experimental housing (single cages or retained in groups of 4 animals/cage for 4 wks prior to initiation of a battery of behavioural investigations. All mice were subject to anxiety testing in both the EPM (2.6.8.1.1) and OFT (2.6.8.1.2) and were further tested in the Y-maze (2.6.8.1.3) and Morris water-maze (2.6.8.2) which assesses learning and spatial memory abilities. Mice were rehoused in groups at age 7.5 months, remaining so until aged (13-14 months) when they were again subjected to experimental housing in identical groups as before. The behavioural experiments were repeated after 4 wks of experimental housing (14-15 months old). Additionally, prior to cull, morning blood samples (0730 – 0830 hrs) were taken by tail nick to determine basal plasma corticosterone concentrations. Mice were culled 2 wks after the last behavioural experiment took place. Culling and tissue collection was carried out as described (2.6.10).

5.2.3 Experimental animals

Mice were obtained from an in-house breeding colony (2.6.1) and maintained as described (2.6.3). Body weight and food consumption was measured weekly (2.6.4) encompassing the social isolation period or equivalent time for group-housed animals. The four cohorts of mice studied were:

- WT mice housed in groups of 4 animals/cage.
- WT mice housed singly.
- 5αR1-KO mice housed in groups of 4 animals/cage.
- 5αR1-KO mice housed singly.

5.2.4 Assessment of HPA axis function

5.2.4.1 Basal corticosterone levels

Basal tail-blood samples were collected in the morning (2.6.5) from young mice pre- and post-behavioural testing (3 days before testing began and 3 days after testing was complete). Corticosterone was quantified in plasma using a radioimmuno assay (2.4.5). Blood was also collected (2.6.5) from aged mice after further behavioural testing (3 days after the last behaviour test only). Corticosterone was quantified in these plasma samples by ELISA (2.4.4.2); the change in assay type was due to loss of sensitivity of the RIA.

5.2.4.2 Abundance of transcripts of interest by qPCR

Snap frozen hypothalami and pituitaries from WT and 5αR1-KO mice were used to extract total RNAs (2.3.1.4) which were used as a template to synthesise first strand cDNA by RT (2.3.2). qPCR was performed (2.3.4) to quantify the expression of transcripts for *GR*, *MR* and *Crh* in the hypothalamus and *GR*, *MR* and *Crhr-1* in the pituitary, of WT and 5αR1-KO mice. Abundance was expressed as a ratio to appropriate reference genes which are highlighted in Table 5.1 below. Reference genes were selected due to lack of difference between experimental groups.

5.2.5 Terminal procedures and tissue collection

Section 2.6.10 described the method of tissue collection following cull by decapitation (between 0800 and 1000 hrs). Tissues including brain, hypothalamus, pituitary, liver and gonadal fat were collected and snap frozen on dry ice.

Tissue	Reference Gene	p Value	
Hypothalamus	Cyclophilin A (*Ppia)*	G	H
		0.40	*0.92*
Pituitary	Cyclophilin A (*Ppia)*	G	H
		0.68	*0.79*

Table 5.1 Reference genes used for qPCR analysis.
Ppia was used as a reference for gene expression studies in both the hypothalamus and the pituitary. P value indicates the results of a 2-way ANOVA between WT and 5αR1-KO mice housed both singly and in groups for the specified reference gene, demonstrating no statistical differences with housing or between genotypes.
G = genotype and H = housing.

5.2.6 Quantification of brain corticosterone levels

Steroids were extracted from half brains (halved in the sagittal plane) of aged WT and 5αR1-KO mice as described (2.5.1.2). Corticosterone was quantified by LC-MS/MS as described previously (2.5.2).

5.2.7 Statistical analyses

All data are presented as mean ± SEM and were compared using Student's unpaired t-test, 2-way ANOVA or 2-way repeated measures ANOVA with either Bonferroni (GraphPad Prism) or Fisher's LSD (Statistica) post-hoc tests where appropriate.

5.3 Results 1 – metabolic profiling

5.3.1 Body weight gain

Figure 5.1 shows the weight gain for mice of both genotypes, without subdivision by housing. Over the period of wks 4-20 when housed in groups, 5αR1-KO mice weighed more than WT mice (Figure 5.1, $p<0.0001$). Figure 5.2 (A-C) shows excess weight gain was attenuated during the period of experimental housing but more so in the singly-housed animals, which actually lost weight (more significant weight loss was seen in 5αRI-KO mice; see Figure 5.3 for Δ weight) thus there were no genotype differences in body weight between 20-30wks. Once re-housed in groups at wk 30, overall 5αR1-KO mice again became heavier until wk 53 (Figure 5.1, $p<0.05$). Again during the second period of experimental housing additional weight gain was attenuated in 5αR1-KO mice who again became comparable to WT but under single-housing only (Figure 5.2, C).

Figure 5.3 highlights changes in weight at defined time-points with regards to the effects of single housing and other experimental interventions. The data was sectioned by housing to assess the effects of each intervention individually on weight gain. At 4-20 wks, all animals were group-housed and 5αR1-KO mice gained more weight that WT (Figure 5.3, A, $p = 0.051$). At the 20 wk point and for 4 wks thereafter, when half the cohort was separated into single-housing, group-housed mice continued to gain weight whilst singly-housed mice did not (Figure 5.3, A, $p<0.001$). Moreover, between 20-24 wks there was a significant effect of genotype whereby singly-housed, 5αR1-KO mice lost more weight than WT (Figure 5.3, A, $p<0.05$). Behavioral testing was carried out between 24-30 wks without any further effects on body weight gain. Following this, all animals were returned to group-housing from 30-53 wks old and gained weight but previously singly-housed animals gained significantly more (Figure 5.3, A, $p<0.001$); compensating for previous weight loss during social isolation. Overall 5αR1-KO mice gained more weight than WT mice during this period (Figure 5.1, $p<0.05$). During further social isolation (53-57 wks) all singly-housed mice lost weight whilst group-housed mice continued to gain weight (Figure 5.3, A, $p<0.001$) but there was no effect of genotype.

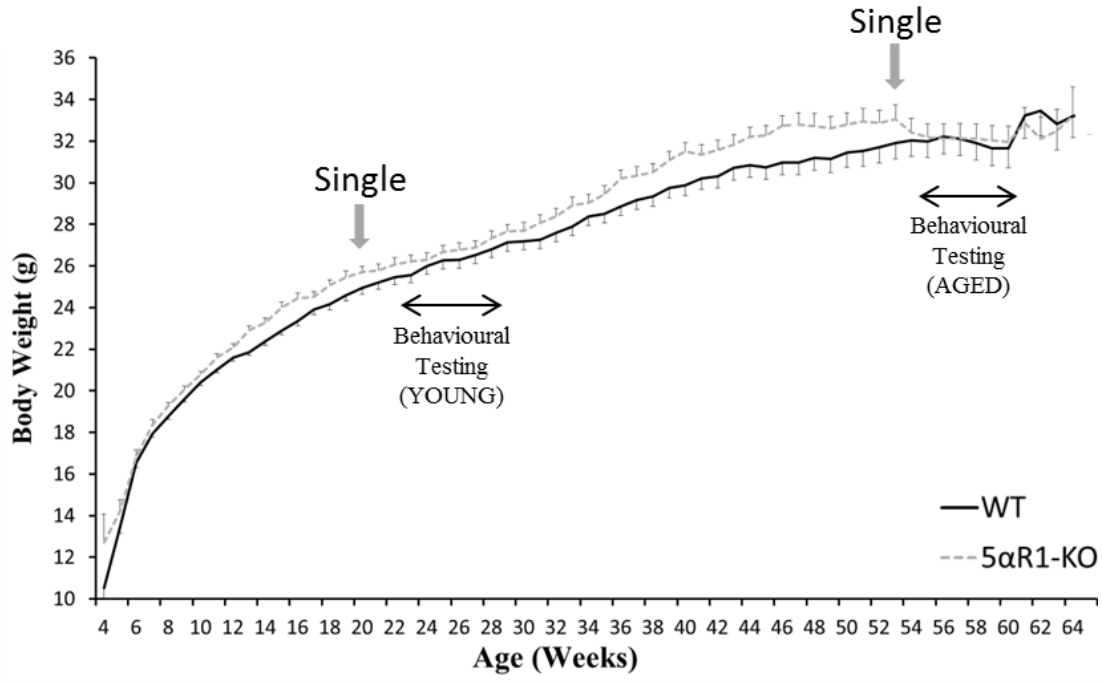

Figure 5.1 Cumulative weight gain of ageing mice

Graph shows weight gain over the 64 wk period for WT (black) and 5αR1-KO (grey) mice. Grey arrows represent the point at which half the animals were separated into single cages. During periods of group housing, 5αR1-KO mice gained more weight than WT littermates ($p<0.0001$ for 4-20 wks and $p<0.05$ for 31-53 wks). Each point represents a mean measurement ± SEM for n = 10-52/group. Statistical differences were determined by 2-way ANOVA for repeated measures with Fisher's LSD post-hoc testing. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

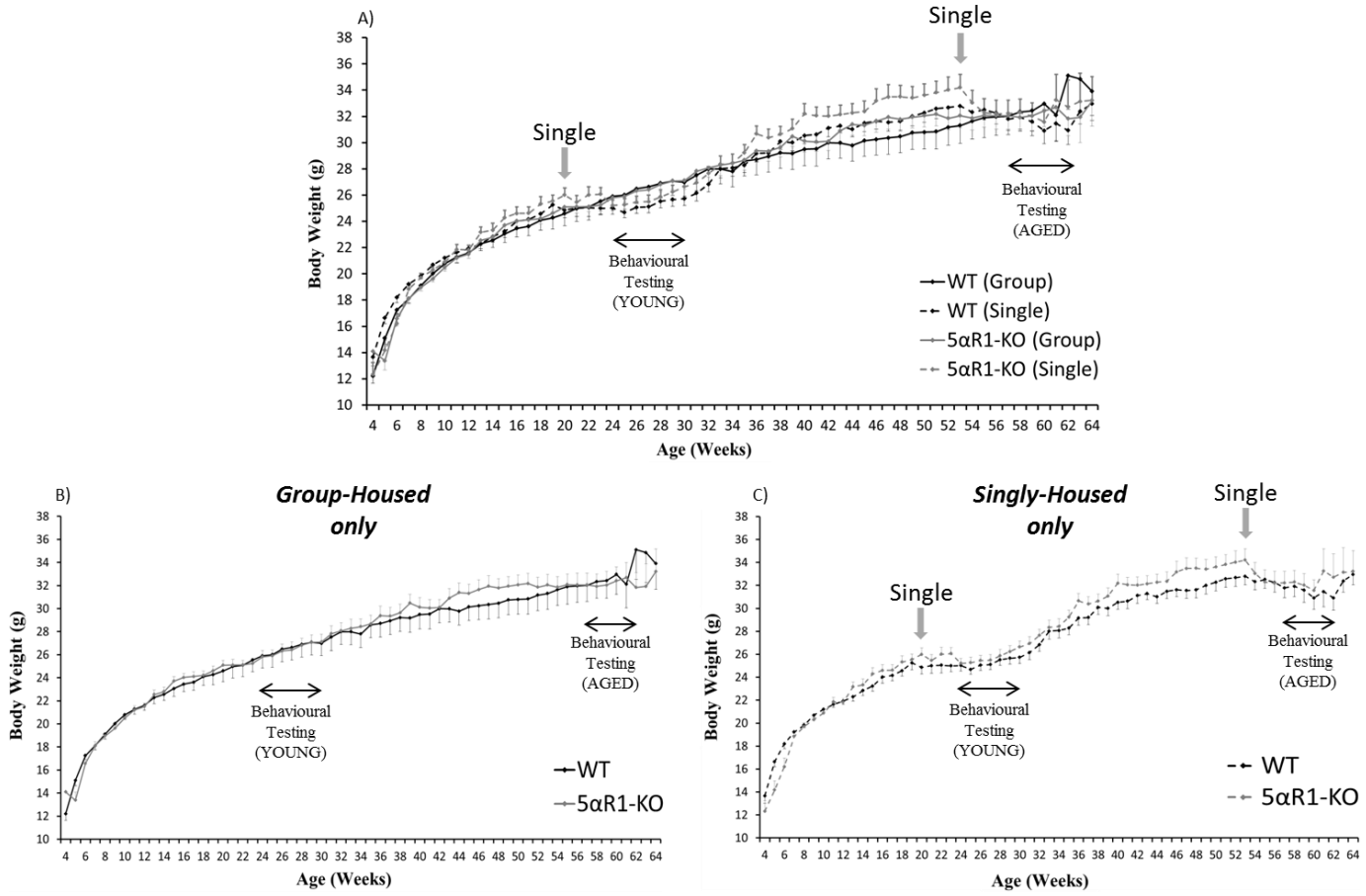

Figure 5.2 Cumulative weight gain under group- and single-housing

A) shows weight gain over the 64 wk period for WT (black) and 5αR1-KO (grey) mice both group- (solid) and singly- (dashed) housed. For clarity B) and C) show weight gain for group- and singly-housed mice alone, respectively. Exaggerated weight gain in 5αR1-KO mice was attenuated by single housing. Grey arrows represent the point at which half the animals were separated out into single cages and the periods where behavioural testing took place are indicated on the graphs. Each point represents a mean measurement ± SEM for n = 15-17/group. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

Behavioral testing of aged mice induced weight loss in all experimental groups. Finally, from wks 62-64 when still half of the cohort remained in single-housing, there was a genotype trend for weight gain in previously socially isolated WT mice but 5αR1-KO mice previously housed singly did not put on weight (Figure 5.3, A, $p = 0.09$, int = $p<0.05$).

5.3.2 Food intake

In young animals, there were no genotype differences in calorific consumption in young animals (Figure 5.4, A), however, all animals housed in social isolation consumed significantly more calories compared to group-housed controls (Figure 5.4, A, $p<0.001$). Moreover, after ageing, 5αR1-KO mice consumed significantly less calories vs. WT (Figure 5.4, B, $p<0.001$); these measurements were only recorded for singly-housed mice.

5.3.3 Tissue weights at cull

Singly-housed mice had significantly heavier livers than those housed in groups (Table 5.2, $p<0.05$). This group also had a marked decrease in the weight of the gonadal adipose pad compared to all group-housed mice (Table 5.2, $p<0.05$ and $p<0.001$ for absolute and body weight-corrected weight respectively). There were no genotype differences.

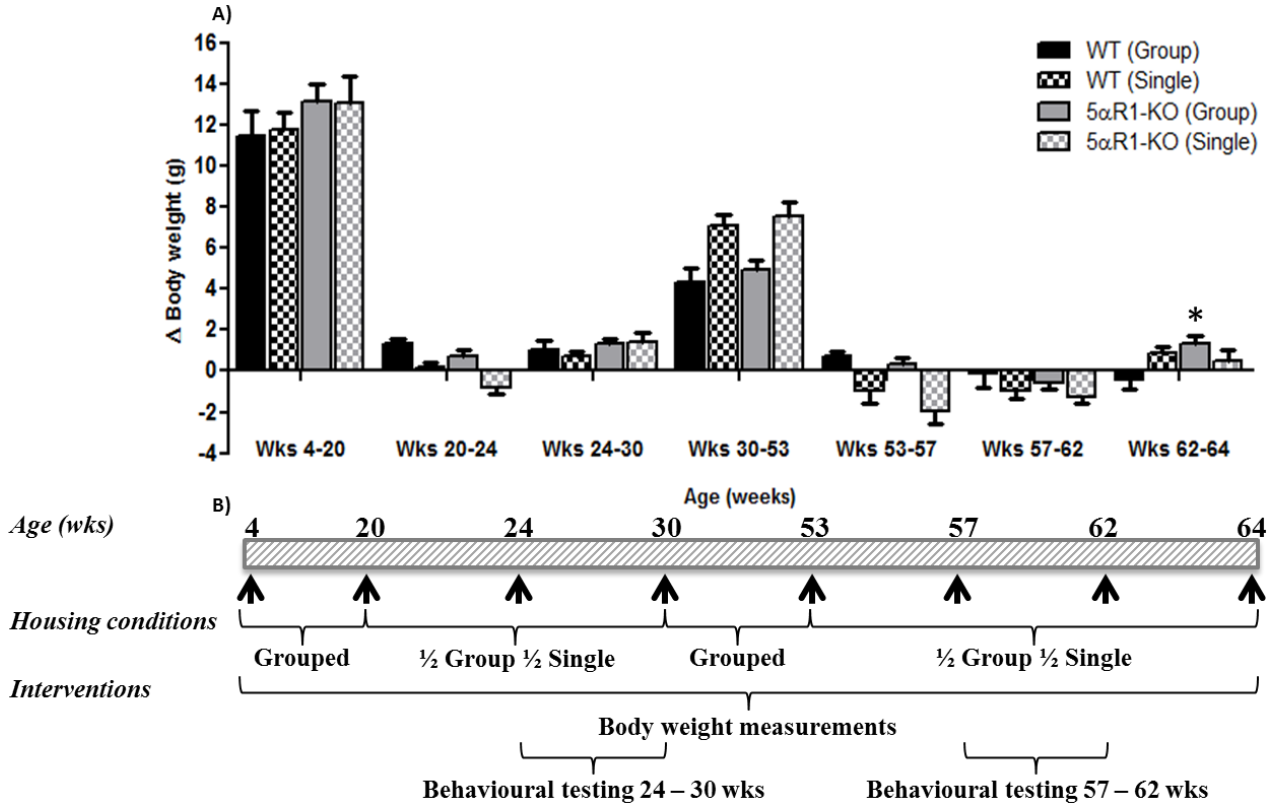

Figure 5.3 Effect of housing and behavioural testing on body weight
At 4-20 wks, 5αR1-KO mice gained more weight vs. WT ($p=0.051$). At 20-24 wks, group-housed mice (solid) gained weight whilst singly-housed mice (chequered) did not ($p<0.001$) and singly-housed, 5αR1-KO mice (grey) lost weight vs. WT (black, $p<0.05$). When re-grouped (30-53 wks) all mice gained weight but previously singly-housed mice gained significantly more ($p<0.001$). During further social isolation (53-57 wks), all singly-housed mice lost weight whilst group-housed mice continued to gain weight ($p<0.001$). From wks 62-64 during experimental housing, there was a genotype trend and an interaction whereby socially isolated, WT mice gained weight and 5αR1-KO mice tended to gain less weight compared to genotype-matched, group-housed mice ($p=0.09$, int = $p<0.05$). Behavioural testing had no effect on weight when young but induced weight loss in all groups when mice were aged. Data are mean ± SEM for n = 15-17/group. Comparisons were made by Student's unpaired t-test or 2-way ANOVA with Bonferroni testing where appropriate and* = $p<0.05$ (housing). WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

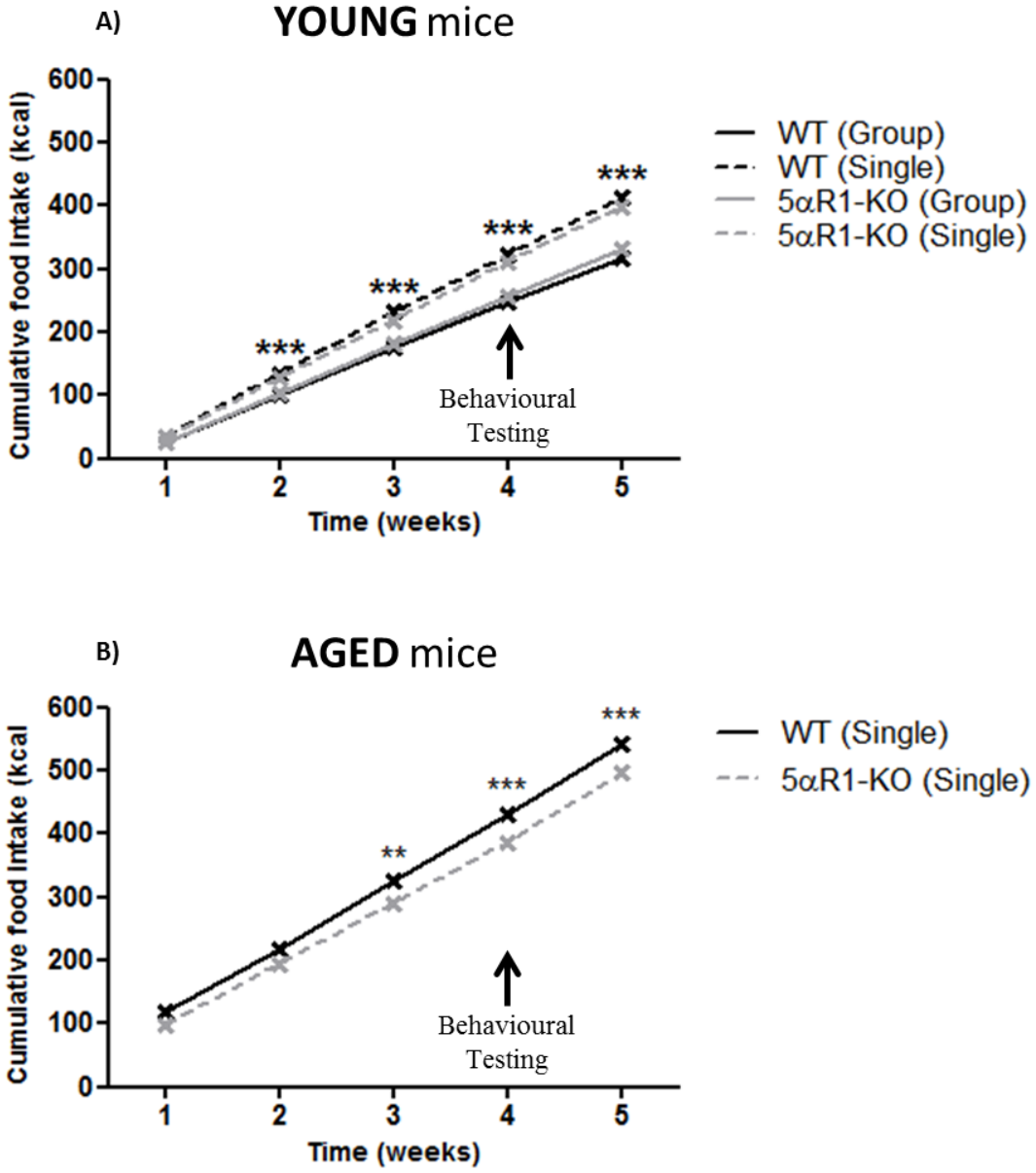

Figure 5.4 Cumulative food intake for young and aged mice

Panel A shows hyperphagia in young, singly-housed mice (both WT and 5αR1-KO; black dashed and grey dashed respectively), compared to all group-housed controls (solid black and solid grey for WT and 5αR1-KO respectively, $p<0.001$). There was no difference between genotypes. When aged, singly-housed 5αR1-KO mice consumed significantly less food vs. WT controls (B, $p<0.001$). Each point represents a mean measurement ± SEM for n = 8-17/group. Comparisons were made by 2-way repeated measures ANOVA with Bonferroni post-hoc tests where appropriate. **=$p<0.01$ and ***=$p<0.001$ for housing effects in panel A and genotype effects in panel B. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

Body and Tissue Weights						
	Group-Housed		Singly-Housed		p Value	
	WT	5αR1-KO	WT	5αR1-KO	G	H
Body Weight (g)	31.86±1.36	31.64±1.11	31.45±0.78	31.77±0.91	0.967	0.895
Absolute Tissue Weight (mg)						
Liver	1194.56±54.57	1217.64±51.11	**1358.48±44.21**	**1286.81±42.89**	0.621	**0.020**
Gonadal adipose	1642.21±180.54	1753.54±164.92	**1196.89±98.98**	**1325.26±134.51**	0.425	**0.005**
Tissue Weight/Body Weight (mg/g body weight)						
Liver	38.08±1.85	38.61±1.27	**43.28±1.10**	**40.64±1.18**	0.452	**0.012**
Gonadal adipose	49.42±3.45	54.51±3.87	**37.20±2.46**	**40.70±3.14**	0.196	**0.0002**

Table 5.2 Body and tissue weights at cull

Body weight was not different between groups at the time of cull. Both liver and gonadal adipose weights were affected by housing but not genotype, whereby all singly-housed mice had significantly larger livers ($p<0.05$) and significantly less gonadal fat vs. group-housed animals ($p<0.05$ and $p<0.001$ for absolute and body weight-corrected weight respectively). Data are mean ± SEM and comparisons were made by 2-way ANOVA with Bonferroni post-hoc testing for n=15-17/group. Animals were aged 15 months. G = genotype, H = housing, WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

5.4 Results 2 – Behavioural profiling

5.4.1 Anxiety testing in YOUNG mice

5.4.1.1 Elevated Plus Maze (EPM)

5.4.1.1.1 Anxiogenic zone exploration

An expected exploratory pattern was observed in the EPM whereby all mice spent the majority of the test time in the closed zone (~60%), roughly 25% of the time in the decision-making centre zone and only occasionally entered the open zone (~15% of the total test time; Figure 5.5). However, both 5αR1-KO and WT littermates under each housing condition, spent an equal % of the total test time exploring the open, most-anxiogenic zone of the maze (Figure 5.5, A & B) and made a comparable number of entries into this zone (Figure 5.5, C & D).

5.4.1.1.2 Locomotor activity

There were no effects of either genotype or housing upon average speed, maximum speed or distance travelled during the EPM test time (Table 5.3).

5.4.1.2 Open Field Test (OFT)

5.4.1.2.1 Anxiogenic zone exploration

In another common behavioural test designed for investigating anxiety, a similar pattern was seen. Across all groups, animals spent the majority of the test time exploring the outer zone of the box (~80%) staying close to the walls for shelter, but would occasionally traverse the inner most anxiogenic zone to explore (~20% of the total test time). WT and 5αR1-KO spent equal times exploring the inner zone whether housed in groups or in social isolation (Figure 5.5, E & F) and made a similar number of entries into this zone during the test (Figure 5.5, G & H).

5.4.1.2.2 Locomotor activity

There was a trend for 5αR1-KO mice to move with a greater maximum speed during testing compared to WT animals overall (Table 5.3, $p = 0.065$) but there was no effect of housing. There were no differences in average speed or distance travelled.

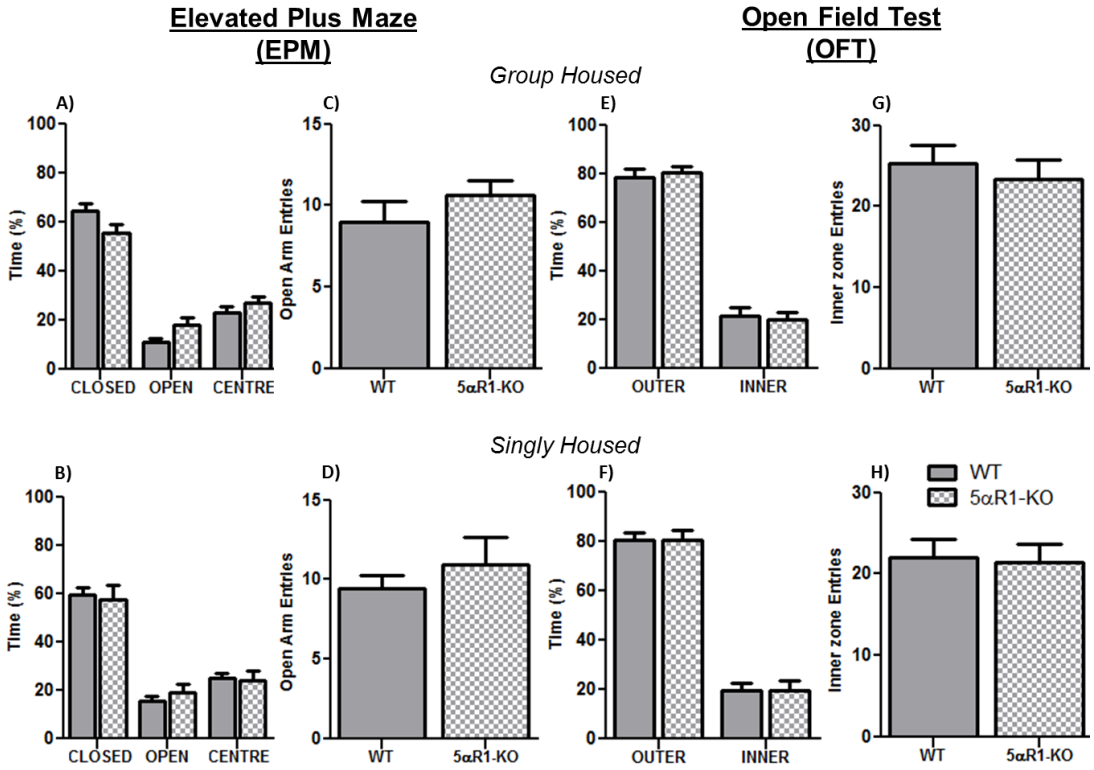

Figure 5.5 Anxiety testing in young mice

Graphs shown under EPM are representative of time spent in the closed, open and centre zones expressed as % of total test time for group- and singly-housed animals (A & B respectively). C & D show the number of entries into the open (anxiogenic) zone. There were no differences between WT (solid) and 5αR1-KO (chequered) mice tested in the EPM under either housing condition. Graphs under OFT represent the time (%) spent in each of the two zones (outer and inner) for group- and singly-housed mice, and the number of entries into the most anxiogenic, inner zone (E, F, G and H respectively). Data are mean measurements ± SEM for mice aged 6 months. n = 16-17/group and comparisons were made by 2-way ANOVA or Student's unpaired t-test. EPM = elevated plus maze, OFT = open field test, WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

Locomotor Activity Data for Young Mice						
Elevated Plus Maze (EPM)						
	Group-Housed		*Singly-Housed*		*p Value*	
	WT	*5αR1-KO*	*WT*	*5αR1-KO*	*G*	*H*
Distance (m)	7.231±0.408	7.250±0.467	7.182±0.628	7.046±0.633	0.914	0.816
Ave speed (m/s)	0.024±0.001	0.024±0.002	0.024±0.002	0.023±0.002	0.914	0.762
Max speed (m/s)	0.217±0.020	0.271±0.035	0.210±0.019	0.259±0.059	0.159	0.802
Open Field Test (OFT)						
	Group-Housed		*Singly-Housed*		*p Value*	
	WT	*5αR1-KO*	*WT*	*5αR1-KO*	*G*	*H*
Distance (m)	17.979±1.528	17.372±1.703	15.703±0.971	17.286±1.341	0.731	0.407
Ave speed (m/s)	0.060±0.005	0.058±0.006	0.052±0.003	0.058±0.004	0.721	0.407
Max speed (m/s)	0.258±0.009	0.283±0.016	0.262±0.009	0.278±0.009	**0.065**	0.967

Table 5.3 Locomotor activity during anxiety testing in young mice
During the EPM all mice covered an equal distance and at comparable speeds. During the OFT, there was no effect of genotype or experimental housing on distance travelled or average speed. However, under both housing conditions there was a trend for 5αR1-KO mice to reach a greater maximum speed during the OFT compared to WT animals overall ($p = 0.065$). Data are mean ± SEM for mice aged 6 months. n = 16-17/group and comparisons were made by 2-way ANOVA with Bonferroni post-hoc tests where appropriate. EPM = elevated plus maze, OFT = open field test, WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out, G = effect of genotype, H = effect of housing.

5.4.2 Anxiety testing in AGED mice

5.4.2.1 Elevated Plus Maze (EPM)

5.4.2.1.1 Anxiogenic zone exploration

As was seen when the animals were tested at a young age, an expected pattern of exploration was observed where all mice spent the majority of the test time in the closed zone, less time in the centre and less still in the open zone. However, this pattern was more marked in 5αR1-KO mice that were singly-housed. Under single housing, 5αR1-KO mice spent significantly more time in the closed zone vs. WT (Figure 5.6, B, $p<0.05$). This shift was accompanied by a corresponding decrease in the time spent in both the open and centre zones (Figure 5.6, B, both $p<0.01$). Moreover, singly-housed 5αR1-KO mice made less entries into the open zone compared to WT controls (Figure 5.6, D, $p<0.05$).

5.4.2.1.2 Locomotor activity

There were no differences in average speed, maximum speed or distance travelled between groups (Table 5.4).

5.4.2.2 Open Field Test (OFT)

5.4.2.2.1 Anxiogenic zone exploration

After the period of ageing, animals re-tested in the OFT displayed a similar exploratory pattern whereby most time was spent in the outer zone of the box (~80-90%) and less time in the inner, anxiogenic zone (~10-20% of the total test time; Figure 5.6, E & F). There were no genotype differences and no effect of housing whereby all mice spent the same amount of time in the inner zone, and made the same number of entries into that zone (Figure 5.6, G & H).

5.4.2.2.2 Locomotor activity

Furthermore, although there were no effects of either experimental housing or genotype on average speed or distance travelled, there were trends ($p = 0.0936$ for genotype, $p = 0.0621$ for housing) for group-housed 5αR1-KO mice to reach a higher maximum speed than WT controls (Table 5.4, $p<0.05$).

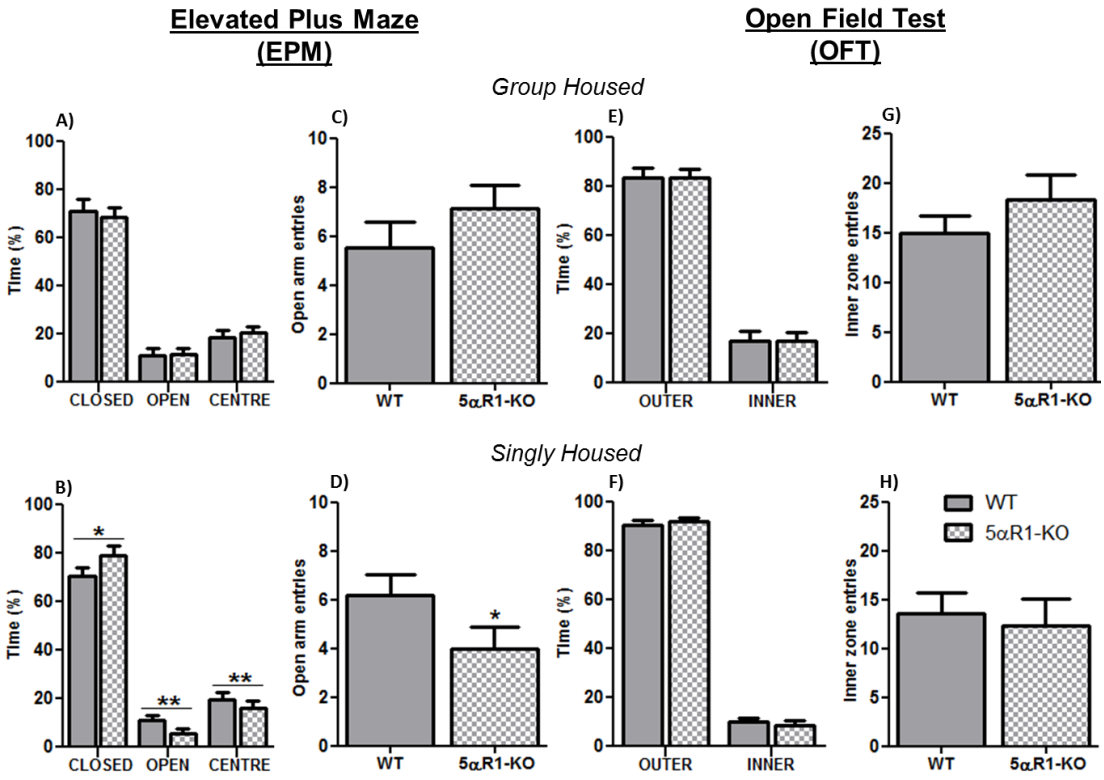

Figure 5.6 Anxiety testing in aged mice.

Graphs shown represent time spent in the closed, open and centre zones for EPM (A & B) and in the inner and outer zones for OFT (E & F) for group- and singly-housed mice (upper & lower panels respectively). In the EPM, 5αR1-KO mice (chequered) spent most time in the closed (safe) zone vs. WT littermates (solid) suggesting a more anxious phenotype, but only when singly-housed (B). 5αR1-KO animals also made fewer entries into the anxiogenic (open) zone when singly- but not when group-housed (D & C respectively). There were no differences between genotypes in either time spent in each zone (E & F) or entries to the inner zone (G & H) for group- and singly-housed mice tested in the OFT. Bars represent mean measurements ± SEM for mice aged 6 months. n = 16-17/group and comparisons were made by 2-way ANOVA with Fisher's LSD post-hoc tests where appropriate or Student's unpaired t-test. * = $p<0.05$ and ** = $p<0.01$. EPM = elevated plus maze, OFT = open field test, WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

Locomotor Activity Data for Aged Mice						
Elevated Plus Maze (EPM)						
	Group-Housed		*Singly-Housed*		*p Value*	
	WT	*5αR1-KO*	*WT*	*5αR1-KO*	*G*	*H*
Distance (m)	4.392±0.375	4.711±0.593	4.001±0.421	3.682±0.482	0.9996	0.1404
Ave Speed (m/s)	0.015±0.001	0.016±0.002	0.013±0.001	0.012±0.002	0.9785	0.1383
Max Speed (m/s)	0.171±0.009	0.166±0.006	0.177±0.006	0.161±0.006	0.1260	0.9500
Open Field Test (OFT)						
	Group-Housed		*Singly-Housed*		*p Value*	
	WT	*5αR1-KO*	*WT*	*5αR1-KO*	*G*	*H*
Distance (m)	10.833±0.860	13.045±1.029	11.751±1.108	11.724±1.759	0.3667	0.8677
Ave Speed (m/s)	0.036±0.003	0.044±0.003	0.039±0.004	0.039±0.006	0.3644	0.8670
Max Speed (m/s)	0.200±0.005	0.225±0.009*****	0.227±0.007	0.226±0.007	**0.0936**	**0.0621**

Table 5.4 Locomotor activity during anxiety testing in aged mice

During the EPM all mice covered an equal distance and at comparable speeds when aged. During the OFT, there was no effect of genotype or experimental housing on distance travelled or average speed of travel. However, there were trends for an effect of both genotype and housing on maximum speed of travel and post-hoc tests revealed that under group-housing, 5αR1-KO mice reached a higher maximum speed than WT controls ($p<0.05$). Values are mean measurements ± SEM for mice aged 14-15 months. n = 16-17/group and comparisons were made by 2-way ANOVA with Bonferroni post-hoc tests. EPM = elevated plus maze, OFT = open field test, WT = wild-type, 5αR1-KO = 5α-reductase type 1 knock-out, G = effect of genotype, H = effect of housing.

5.4.2.3 Effect of ageing on anxiety and locomotion

There was an overall effect (trend) of genotype on Δ open arm entries between aged and young mice (Figure 5.7, A, $p = 0.09$). Furthermore, singly-housed 5αR1-KO mice had a more pronounced decrease in open arm exploration (EPM) following ageing vs. group-housed animals of the same genotype (Figure 5.7, A, $p<0.05$). There was no effect of genotype or housing in the change in inner zone entries between aged and young mice tested in the OFT (Figure 5.7).

Similarly, reduced locomotor activity in the EPM and OFT was observed when mice were tested after ageing (Figure 5.8; Δ distance travelled, Δ average speed and Δ maximum speed), with the exception of Δ maximum speed in the OFT for singly-housed 5αR1-KO mice (Figure 5.8, F). However, there were no statistical differences between any of the experimental groups.

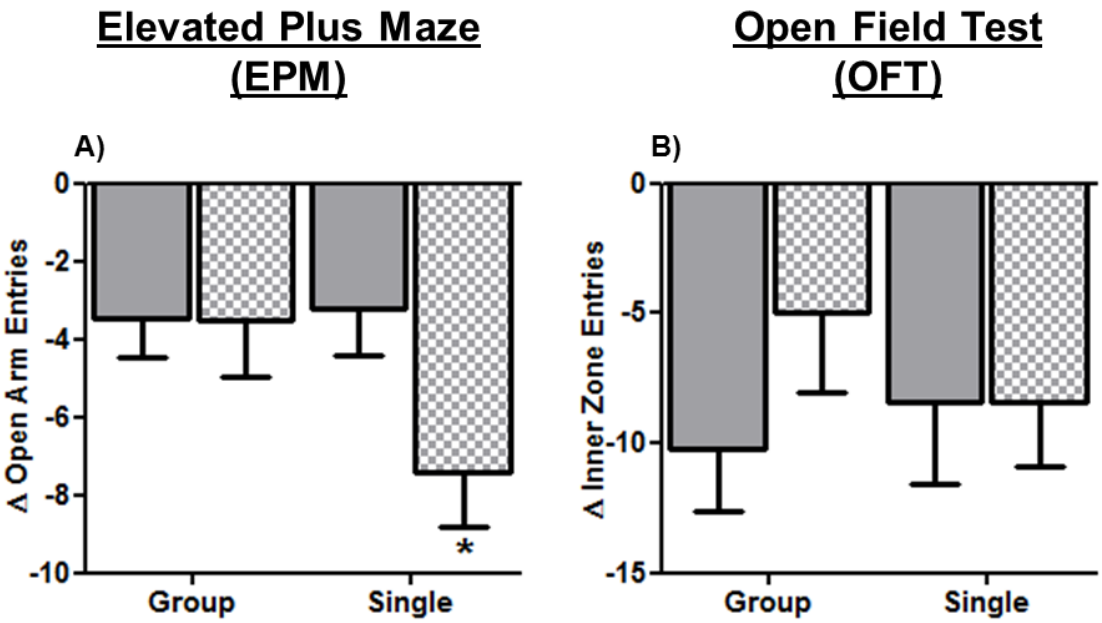

Figure 5.7 Change in anxiogenic behaviour between aged and young mice
In the elevated plus maze, there was a trend towards an effect of genotype ($p=0.09$) and post=hoc testing showed singly-housed 5αR1-KO mice (chequered) had a significantly greater decline in open arm entries after ageing compared to group-housed genotype-matched mice (A, $p<0.05$). There were no differences in Δ inner zone entries for the open field test with ageing between any of the experimental groups. Data are mean ± SEM for n = 16-17/group. Comparisons were made by 2-way ANOVA with Bonferroni post-hoc tests where appropriate. * = $p<0.05$. EPM = elevated plus maze, OFT = open field test, WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

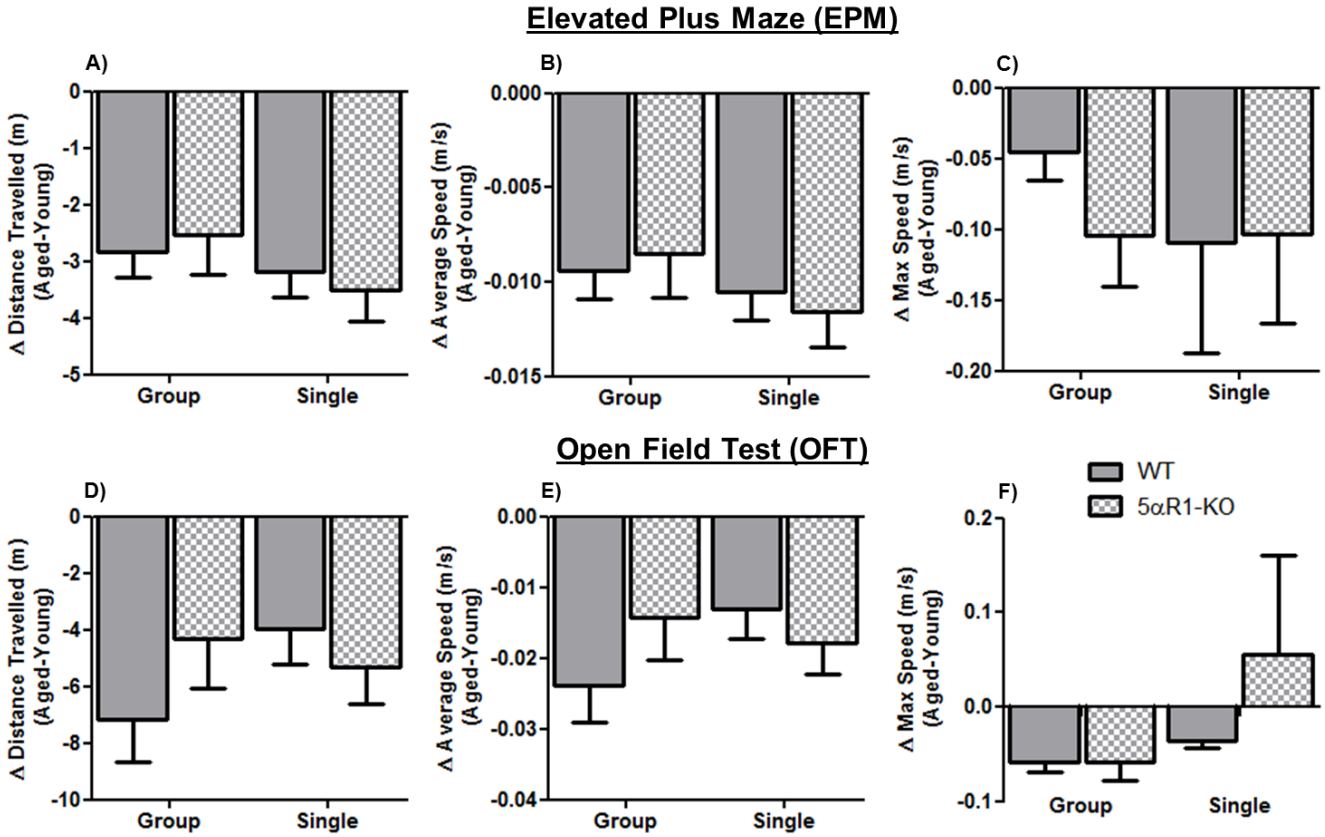

Figure 5.8 Change in locomotor activity during anxiety testing, calculated between aged and young mice

In all groups, the negative values for Δ distance (A & D), Δ average speed (B & E) and Δ maximum speed (C & F) infer reduced locomotion between aged and young mice in terms of both distance and speed, with the exception of the Δ maximum speed for singly-housed 5αR1-KO mice in the OFT. There were no effects of genotype or housing. Data are mean ± SEM for n = 16-17/group and comparisons were made by 2-way ANOVA. EPM = elevated plus maze, OFT = open field test, WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

5.4.3 Spatial learning and memory in YOUNG mice

5.4.3.1 Y-maze

5.4.3.1.1 Memory

In the Y-maze, time spent in the novel arm during the retention phase was used as a measure of working memory. When group-housed, there were no differences in novel arm exploration time between WT and 5αR1-KO mice after either the 1 min or 2 hour ITI (Figure 5.9, A & B respectively; ITI = inter-trial interval). All group-housed mice explored the novel arm significantly more than chance ($p<0.05$). Under single housing, 5αR1-KO mice had reduced novel arm exploration vs. WT following both the 1 min and 2 hour ITI (Figure 5.9, C & D, $p<0.05$). However, singly-housed mice explored the novel arm significantly more than chance) only after the 1 min ITI (Figure 5.9, C, $p<0.05$). Group- and singly-housed WT and 5αR1-KO mice made a similar number of entries into the novel arm following the 2 hour ITI, however, singly-housed mice made fewer entries vs. group-housed mice overall (Figure 5.9, E, $p = 0.058$).

5.4.3.1.2 Locomotor activity

During both the acquisition and retention phases of the 1 min ITI test, singly-housed mice travelled further (Figure 5.10, A and C respectively) and faster (Figure 5.10, B and D respectively) than group-housed mice overall ($p<0.01$). There were no effects of genotype or housing during the acquisition or retention phases of the 2 hour ITI test (Figure 5.10, E-H).

5.4.3.2 Water-maze

Preparatory visual testing established that all animals were able to visually locate the platform in the absence of spatial cues. In the water-maze spatial learning task, all animals were able to learn the escape task and find the location of the submerged platform during testing. Daily training reduced the escape latencies of all mice over 5 consecutive days (Figure 5.9, F) but there were no effects of genotype or housing on mean escape latencies of experimental groups (Figure 5.9, F). There was also no difference in target quadrant swimming time during probe testing at 1 and 24 hours between any of the groups (Figure 5.9, G).

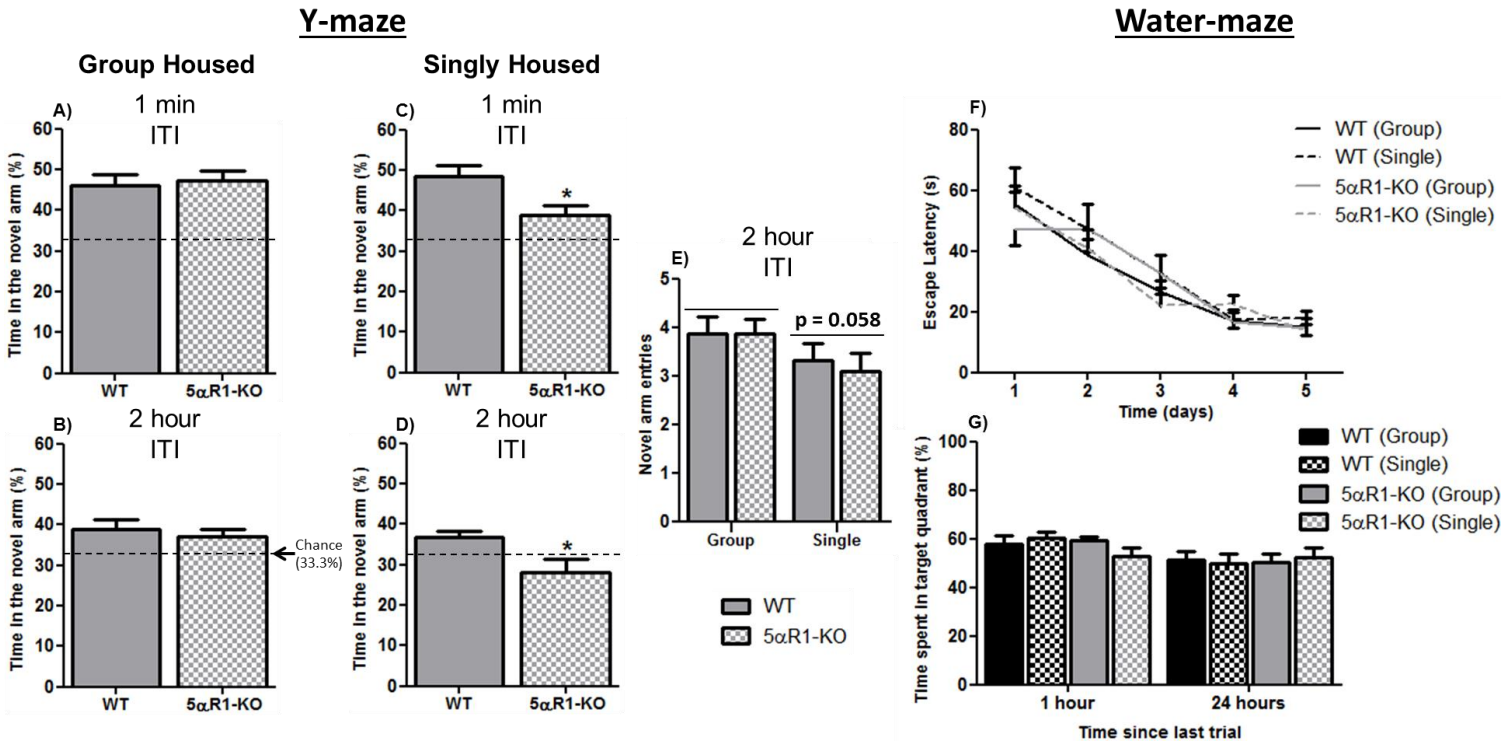

Figure 5.9 Y-maze and Morris water maze assessment of memory and learning capacity in young mice

For Y-maze, histograms illustrate time spent in the novel arm as a measure of memory (A-D). There were no differences in novel arm exploration between WT (solid) and 5αR1-KO (chequered) mice housed in groups during either ITI test (A & B). Singly-housed, 5αR1-KO mice spent less time in the novel arm vs. WT controls, during both the 1 min (C) and 2 hour (D) ITI but the latter did not differ significantly from chance (33%). All animals made a comparable number of entries into the novel arm following the 2 hour ITI (E). In the water-maze, no statistical genotype or housing differences were found in either escape latencies (F) or in target quadrant swimming time (G). Data are mean ± SEM for 6 month old mice. n = 16-17/group and comparisons were made by Student's unpaired t-test, 2-way repeated measures ANOVA or 2-way ANOVA with Bonferroni post-hoc tests when appropriate and *=$p<0.05$. WT = wild-type, 5αR1-KO = 5α-reductase type 1 knock-out and ITI=Inter-trial-interval.

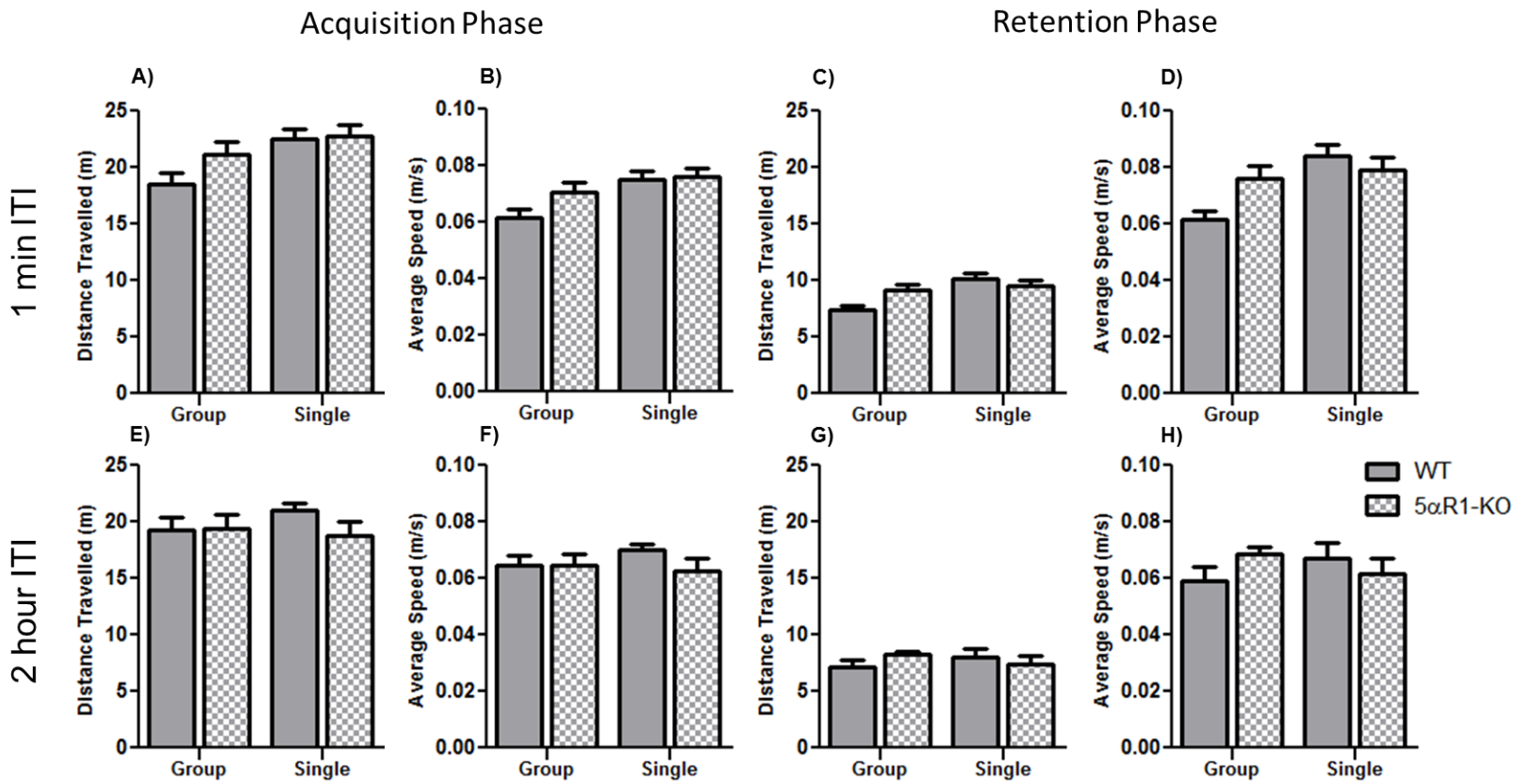

Figure 5.10 Speed and distance travelled during the Y-maze in young mice

During the acquisition and retention phases of the 1 min ITI, singly-housed mice travelled further (A & C respectively) and faster (B & D respectively) than group-housed mice overall ($p<0.01$). During the 2 hour ITI there was no effect of either genotype or housing in both the acquisition and the retention phases (E-H). Data are mean ± SEM for n = 16-17/group when mice were aged 6 months. Comparisons were made by 2-way ANOVA with Bonferroni post-hoc tests where appropriate. WT = wild-type, 5αR1-KO = 5α-reductase type 1 knock-out and ITI=Inter-trial-interval.

5.4.4 Spatial learning and memory in AGED mice

5.4.4.1 Y-maze

5.4.4.1.1 Memory

Following the 1 min ITI, novel arm exploration was significantly greater than chance only in group-housed mice (Figure 5.11, A, $p<0.05$) and WT mice housed singly (Figure 5.11, B, $p<0.05$). There were no differences in novel arm exploration time between WT and 5αR1-KO mice housed in groups following either the 1 min or 2 hour ITI (Figure 5.11, A & C). However, 5αR1-KO mice housed singly spent less time exploring the novel arm compared to WT littermates, but this was only seen during the 1 min ITI which did not differ from chance (Figure 5.11, B, $p<0.05$). WT and 5αR1KO mice housed singly did not differ in their novel arm exploration times during the 2 hour ITI (Figure 5.11, D). However, overall 5αR1-KO mice made significantly fewer entries into the novel arm vs. WT following the 2 hour ITI (Figure 5.11, E, $p<0.05$); a phenotype which was more marked in singly-housed mice.

5.4.4.1.2 Locomotor activity

An overall housing effect showed all singly-housed mice travelled further (Figure 5.12, A and C) and faster (Figure 5.12, B and D) than group-housed mice during both 1 min ITI phases ($p<0.001$). During the retention phase there was an overall genotype effect on distance (C) and speed (D, Figure 5.12, $p<0.05$) and post-hoc tests revealed that 5αR1-KO mice travelled less distance (Figure 5.12, C, $p<0.01$) and slower (Figure 5.12, D, $p<0.01$) than WT mice when housed singly. During the 2 hour ITI acquisition phase, singly-housed mice covered less distance (E) and moved slower (F) compared to group-housed mice (Figure 5.12, E and F, $p = 0.055$). During the 2 hour ITI retention phase, there were trends towards a genotype effect for reduced distance and speed in 5αR1-KO mice vs. WT (Figure 5.12, G and H, $p = 0.07$) and post-hoc testing showed reduced distance (Figure 5.12, G, $p<0.01$) and speed (Figure 5.12, H, $p<0.01$) in 5αR1-KO vs. WT mice under single housing.

5.4.4.2 Water maze

As with young mice, preparatory visual tests were done which ruled out visual deterioration with ageing. All aged WT and 5αR1-KO mice were able to learn the location of the hidden platform equally well during training, as shown by a decrease in mean escape latencies for all groups over the 5 day training period (Figure 5.11, F). However, on training day 1 5αR1-KO mice were slower to locate and escape onto the platform than WT overall (Figure 5.11, F, $p<0.05$) with no effect of housing. On all other training days, neither genotype nor housing had any effect on escape latency of aged mice. Probe testing showed that all groups retained the platform location and spent equal time swimming in the target quadrant at both 1 and 24 hours since the last training trial (Figure 5.11, G).

5.4.4.2.1 Effect of ageing

Comparisons were then made between escape latencies of these animals when young and when they were re-tested at 14-15 months of age. On day 1 of training, there was a trend towards a larger cognitive decline with ageing in 5αR1-KO mice overall (Figure 5.13, $p = 0.078$) with significance for WT vs. 5αR1-KO in group-housed mice (Figure 5.13, $p<0.05$) and WT vs. 5αR1-KO also for singly-housed mice (Figure 5.13, $p<0.05$). After 5 days of training in the water maze, there was no difference in Δ escape latency from young to aged between any of the four groups (Figure 5.13). These changes were not due to visual deterioration in the aged animals at 14-15 months of age.

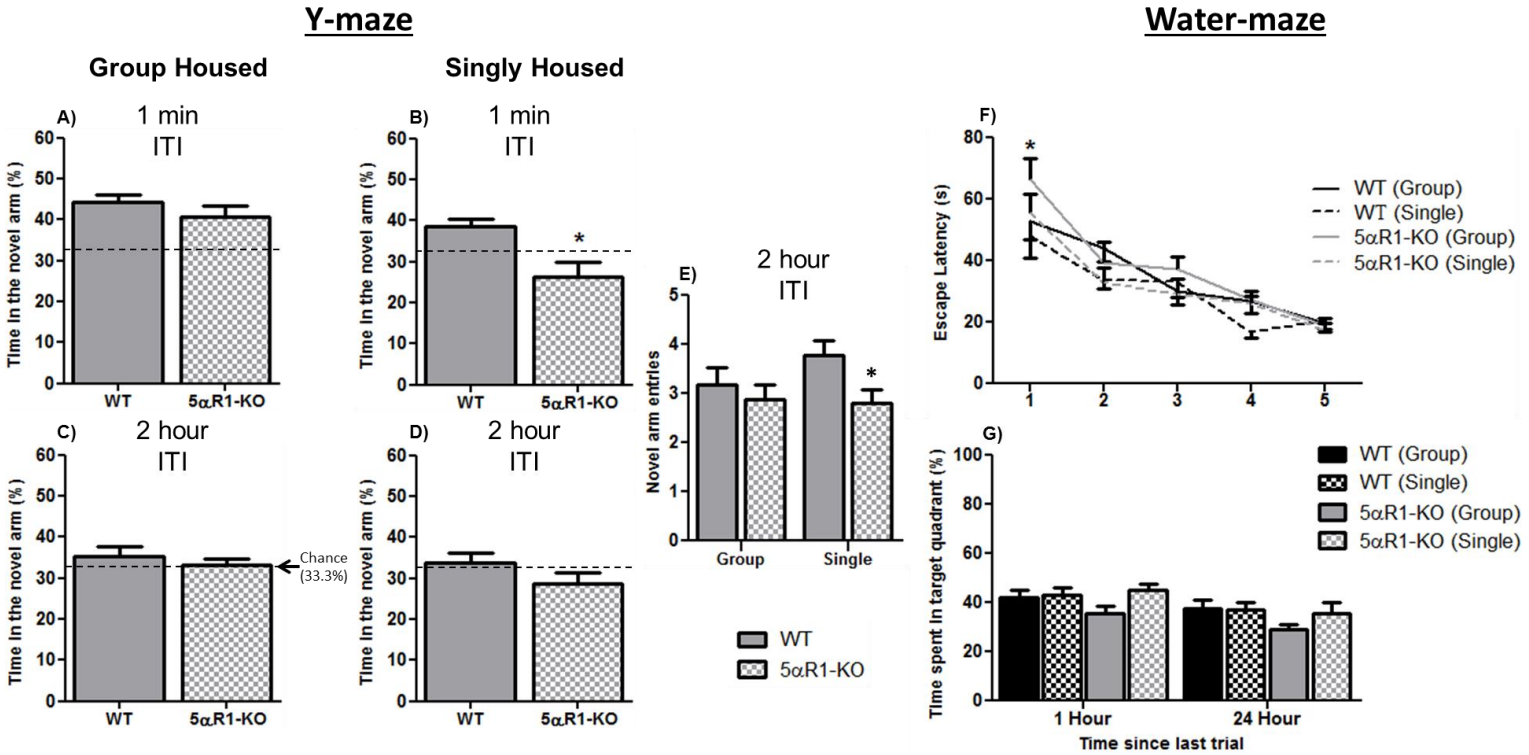

Figure 5.11 Y-maze and Morris water maze assessment of memory and learning capacity in aged mice

Histograms show time spent in the Y-maze novel arm as a measure of memory (A-D). There were no differences in novel arm exploration between WT (solid) and 5αR1-KO (chequered) mice housed in groups during either ITI test (A & B). 5αR1-KO mice housed singly spent less time exploring the novel arm vs. WT, but only during the 1 min and not the 2 hour ITI (C & D respectively). In E, all 5αR1-KO mice made significantly fewer entries into the novel arm vs. WT during the 2 hour ITI ($p<0.05$). In the water-maze, 5αR1-KO mice had longer escape latencies overall vs. WT on Day 1 of training (F) but no statistical genotype or housing differences were found in target quadrant swimming time (G). Data are mean ± SEM for aged mice (14-15 months old) of n = 16-17/group. *=$p<0.05$ and comparisons were made by Student's unpaired t-test, 2-way repeated measures ANOVA or 2-way ANOVA with Bonferroni post-hoc tests where appropriate. WT = wild-type, 5αR1-KO = 5α-reductase type 1 knock-out and ITI=Inter-trial-interval.

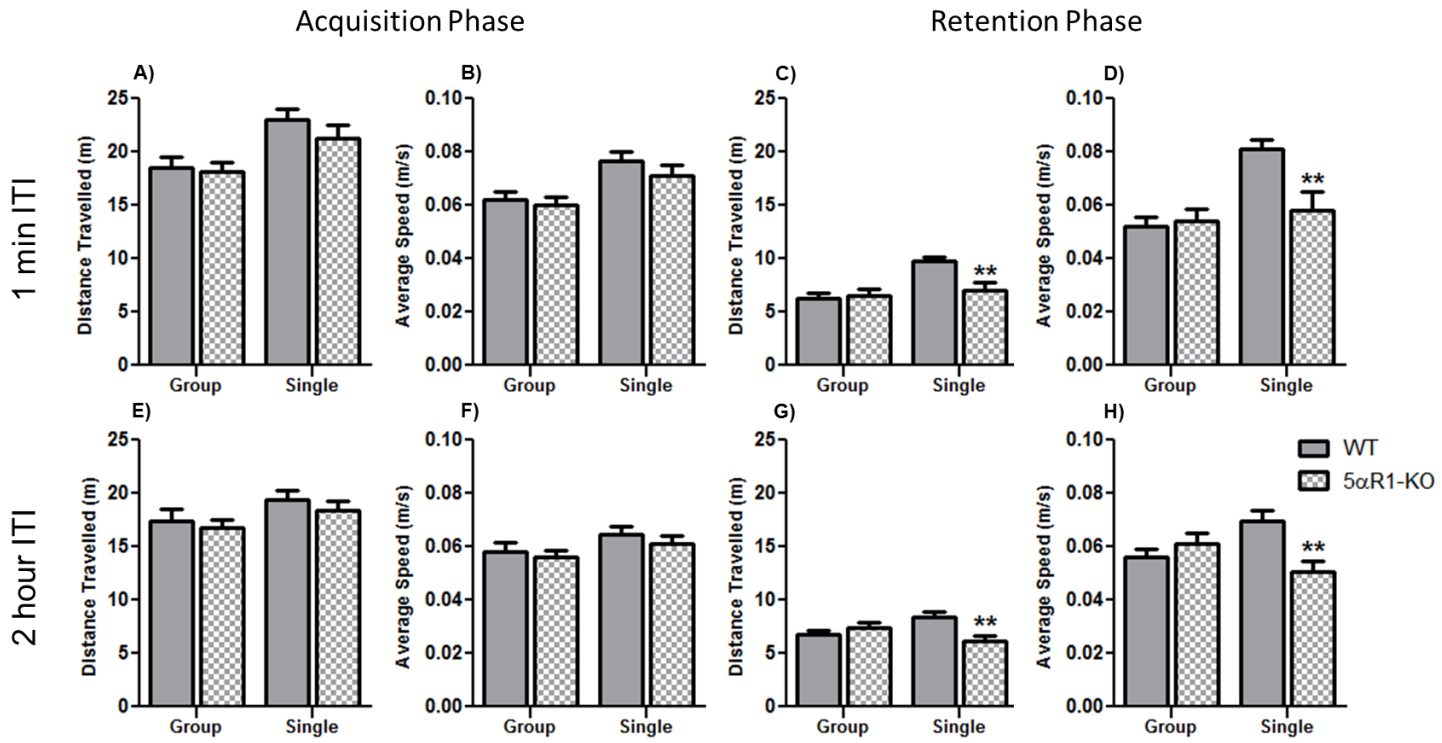

Figure 5.12 Speed and distance travelled during the Y-maze for aged mice

During the 1 min ITI, there was an overall effect of housing ($p<0.001$) such that all singly-housed mice travelled further (A & C) and faster (B & D) than group-housed mice. Moreover, during the retention phase (C & D) there was an effect of genotype on both distance and speed ($p<0.05$) and singly-housed 5αR1-KO mice travelled less distance (C, $p<0.01$) and slower (D, $p<0.01$) that singly-housed WT mice. During the 2 hour ITI, there was a similar overall effect of housing during the acquisition phase only (E & F, $p = 0.055$) but no genotype effect. During the 2 hour ITI retention phase, there were trends towards a genotype effect (G & H, $p = 0.07$) and post-hoc testing showed reduced distance (G, $p<0.01$) and speed (H, $p<0.01$) in 5αR1-KO vs. WT mice under single housing. Animals were 14-15 months old. Data are mean ± SEM for n = 16-17/group. Comparisons were made by 2-way ANOVA with Bonferroni post-hoc tests where **=$p<0.01$. WT = wild-type, 5αR1-KO = 5α-reductase type 1 knock-out and ITI=Inter-trial-interval.

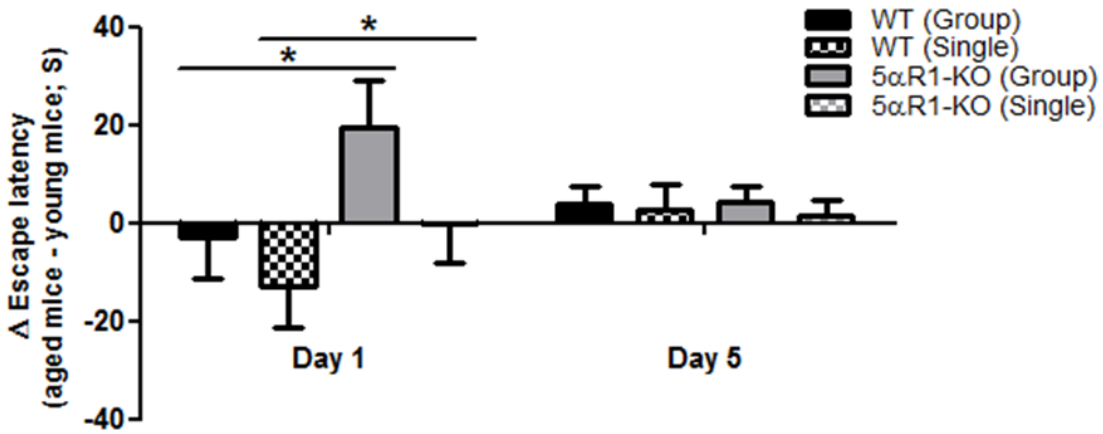

Figure 5.13 Change in water-maze escape latency between aged and young mice
Histograms represent the change in escape latencies between paired young and aged mice. On training day 1, there was a trend towards increased cognitive decline in 5αR1-KO mice overall (grey; $p=0.078$) with significance for WT vs. 5αR1-KO in group-housed mice (solid black and grey respectively, $p<0.05$) and WT vs. 5αR1-KO also for singly-housed mice (chequered black and grey respectively, $p<0.05$). The change in escape latencies between young and aged mice on day 5 of training did not vary by either genotype or housing. Bars represent mean measurements ± SEM for n = 16-17/group. Statistical differences were determined by 2-way ANOVA followed by Bonferroni post-hoc tests where appropriate where *=$p<0.05$. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

5.5 Results 3 – Characterisation of the HPA axis

5.5.1 Basal plasma corticosterone

5.5.1.1 YOUNG mice

There was no difference in basal (AM) corticosterone levels between young WT and 5αR1-KO female mice prior to experimental behavioural investigations (Figure 5.14, A). Immediately following a battery of behavioural testing using 4 different experiments and 10 wks of experimental housing conditions, plasma corticosterone was measured once again. There were no effects of either single-housing or behavioural testing on plasma corticosterone levels in young mice (Figure 5.14, B).

5.5.1.2 AGED mice

Post-behavioural testing and just prior to cull, 5αR1-KO mice overall showed a trend towards reduced plasma corticosterone levels compared to WT littermate controls (Figure 5.15, $p = 0.06$). There was no effect of housing.

5.5.2 Alterations in HPA regulatory pathways (AGED mice)

5.5.2.1 Corticosteroid receptor expression

In the hypothalamus, the abundance of transcripts for *GR* was dowregulated in 5αR1-KO mice vs. WT under both housing conditions (Figure 5.16, A, $p<0.05$). Hypothalamic *GR* expression was upregulated in singly- vs. group-housed mice overall (Figure 5.16, A, $p<0.001$). Similarly, the abundance of transcripts for *MR* in the hypothalamus was upregulated in singly- vs. group-housed mice overall (Figure 5.16, B, $p<0.01$) with no additional effects of genotype.

5.5.2.2 *Crh* and *Crhr-1* in the hypothalamus and pituitary respectively

There was no effect of genotype or housing on the abundance of transcripts for *Crh* in the hypothalamus (Figure 5.16, C). However, *Crhr*-1 mRNA abundance in the pituitary was significantly upregulated in 5αR1-KO mice vs. WT overall (Figure 5.16, F, $p<0.05$) with no additional effect of housing.

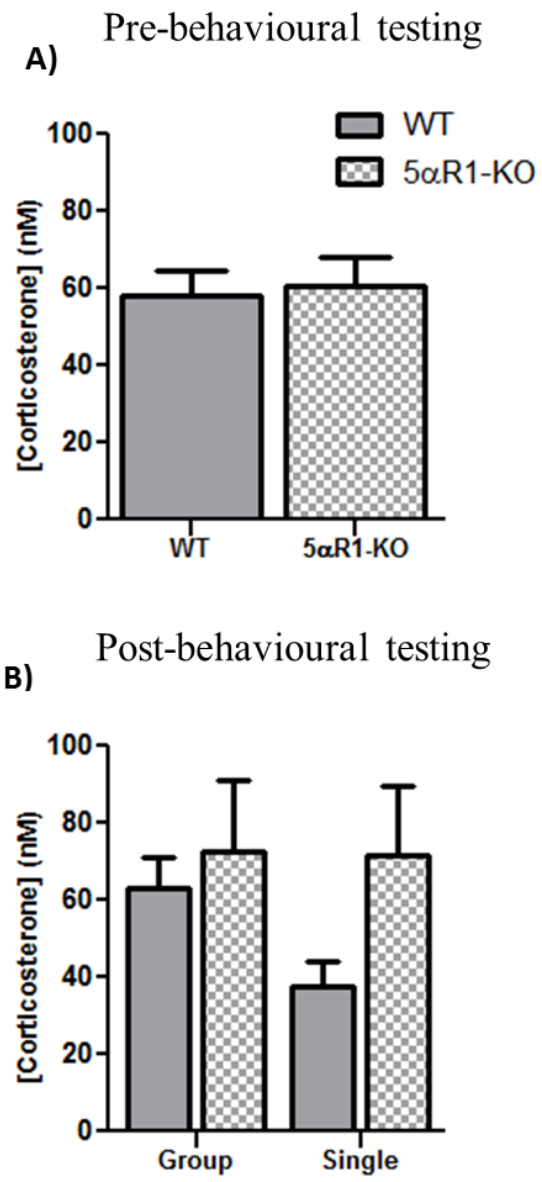

Figure 5.14 Plasma corticosterone levels at the diurnal nadir in young animals pre- and post-behavioural testing

Basal plasma corticosterone levels in WT (solid fill) and 5αR1-KO (chequered) littermates did not differ prior to experimentation when all animals were group-housed (A). Experimental housing and behavioural testing had no effect on plasma corticosterone levels in any of the groups (B). Animals were 5-7 months old and values are mean ± SEM where n=16-34/group. Comparisons were made by Student's unpaired t-test or 2-way ANOVA. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

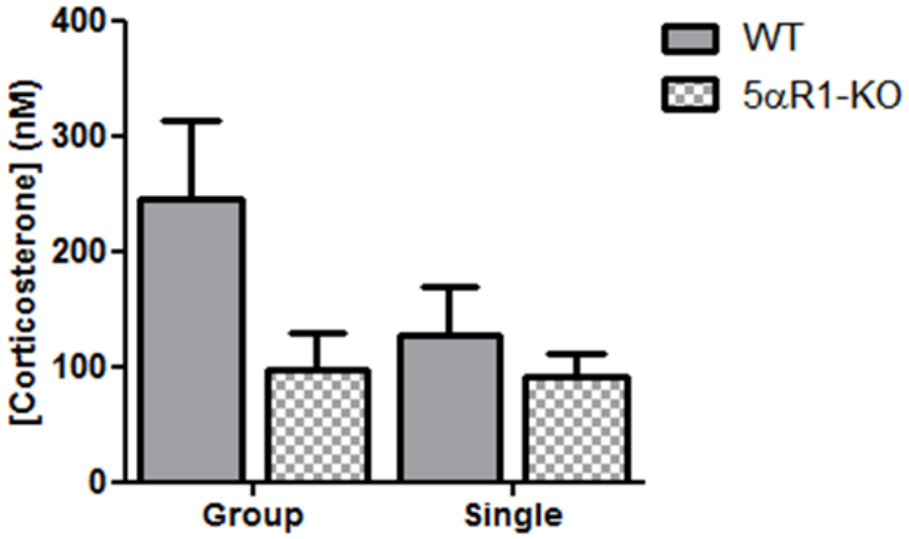

Figure 5.15 Plasma corticosterone levels at the diurnal nadir in aged mice post-behavioural testing

Independent from housing, 5αR1-KO mice overall showed a trend towards reduced plasma corticosterone concentrations basally compared to WT controls ($p = 0.06$). Animals were aged ~15 months. Data are mean ± SEM for n = 16-17/group. Comparisons were made by 2-way ANOVA with Bonferroni post-hoc testing. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

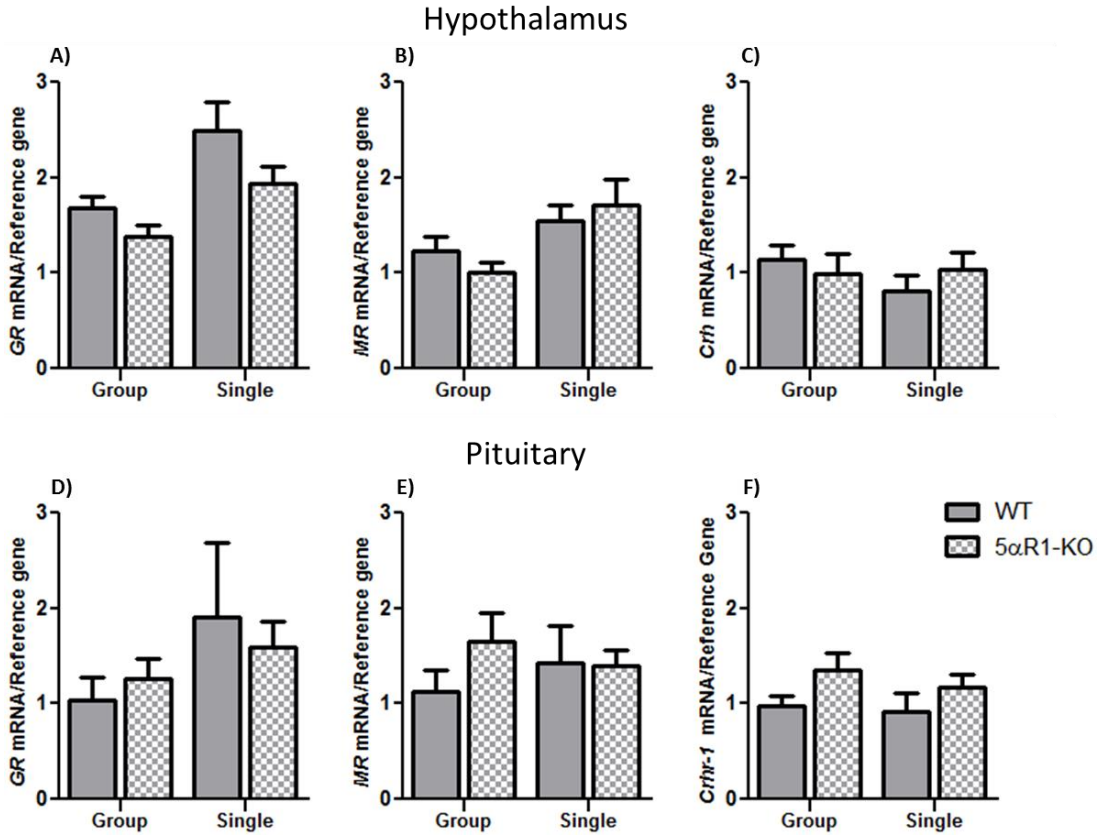

Figure 5.16 Gene expression profile in the hypothalamus and pituitary of aged mice
In the hypothalamus (A-C), transcripts for *GR* were downregulated in 5αR1-KO mice (chequered) housed both singly and in groups (A, $p<0.05$). Moreover, transcripts for *GR* were more abundant in singly- vs. group-housed mice overall (A, $p<0.001$). *MR* mRNA was upregulated in singly- vs. group-housed mice in the hypothalamus, independent of genotype (B, $p<0.01$). Transcripts for *Crh* in hypothalamus and for *GR* and *MR* in the pituitary were not different between any of the experimental groups (C-E respectively). Transcripts for *Crhr-1* were more abundant in 5αR1-KO mice vs. WT controls under both housing conditions (F, $p<0.05$). Bars represent mean ± SEM for mice aged ~15 months where n = 16-17/group. Comparisons were made by 2-way ANOVA with Bonferroni post-hoc tests where appropriate. WT = wild-type, 5αR1-KO = 5α-reductase type 1 knock-out, GR = glucocorticoid receptor, MR = mineralocorticoid receptor , *Crh* = corticotrophin-releasing hormone and *Crhr-1* = corticotrophin-releasing hormone receptor type 1.

5.6 Discussion

The purpose of this set of experiments was to assess the behavioural effects of 5αR1 loss in the brain and if such phenotypes were modified by the stress of social isolation. 5αR1-KO mice displayed anxiety-like behaviours during single-housing. Age-associated cognitive impairments were exaggerated in 5αR1-KO mice, and to a greater degree in animals subjected to social isolation.

These experiments were performed using mice bred onto a C57Bl6/j background and initial confirmation was sought of continuity of the excess weight gain phenotype in 5αR1-KO mice and their exaggerated weight loss in response to social isolation. Across the experimental period, all animals gained weight over time but to which extent was modifiable by genotype, social isolation and behavioural testing. From weaning (4 wks) up to 20 wks old, the only experimental interventions were weekly weighing and all mice were housed in groups. During this time, 5αR1-KO mice were significantly heavier than WT controls. As with the previous mixed strain, single housing induced weight loss in all C57Bl/6j mice but to a greater extent in 5αR1-KOs. Following re-grouping, all singly-housed mice regained weight and 5αR1-KO mice were again significantly heavier than WT. Exaggerated weight loss upon social isolation in aged 5αR1-KO mice was also observed. These data suggest that obesity-prone 5αR1-KO mice are more susceptible to the effects of social isolation stress but that the resulting weight loss is reversible upon return to group-housing. Social isolation is a known inducer of weight loss and a model of chronic stress (Yamada *et al.* 2000). Chronic mild stress can lower body weight (Matthews *et al.* 1995; Forbes *et al.* 1996), due to loss of both lean and fat-tissue loss (Harris *et al.* 1998). Indeed, here we showed singly-housed mice had reduced gonadal fat compared to group-housed mice overall, suggesting loss of fat mass contributed to weight reduction during social isolation. Moreover, when corrected for total body weight, the significance of this result was enhanced meaning the contribution of fat to the total body weight was reduced in singly-housed mice.

There are conflicting reports of stress-induced hyperphagia in both rodents (Levine *et al.* 1981) and humans (Stone *et al.* 1994), but also hypophagia in rodents (Dallman *et*

al. 2001). In this study, weight loss or attenuated gain ensued in young animals of both genotypes upon single-housing, despite, paradoxical hyperphagia. As was also seen in prior investigations (see Chapter 3), food intake alone does not determine body weight and this phenotype is therefore suggestive of increased energy expenditure. In contrast however, upon ageing, greater weight loss with singly-housed in 5αR1-KO mice was associated with hypophagia.

Social isolation induces increased locomotor activity in both mice and rats (Hilakivi *et al.* 1989; Heidbreder *et al.* 2000; Hickey *et al.* 2012) as was observed here for both WT and 5αR1-KO mice when tested in the Y-maze. There was only limited evidence, in the OFT, that locomotor activity might be more vigorous in 5αR1-KO mice. Even though basal locomotion was not measured here, it is likely that increased physical activity is a main factor in weight loss during single housing. Another cause of weight loss on single housing is maladaptive thermogenesis. This was not assessed here, but singly-housed 5αR1-KO mice may utilise more energy to maintain their body temperature.

Stressful stimuli which induce weight loss in experimental animals are often linked to increased anxiety-like behaviours (Chourbaji *et al.* 2005; Kwak *et al.* 2009). Many authors have reported instances of glucocorticoid-induced anxiety, either through increased ligand or up-regulation of corticosteroid receptors (Seckl 2004; Kajiyama *et al.* 2010). For example, anxiety was reduced in a forebrain-specific GR-KO mouse model (Boyle *et al.* 2006) whilst prednisolone-treated mice displayed more anxiety-like behaviours in the EPM and OFT, possibly related to remodelling and damage to key brain structures such as the hippocampus (Kajiyama *et al.* 2010). If 5αR1 was preventing glucocorticoid inactivation, then this may underpin a propensity for anxiety; basally or in response to stress.

Behavioural phenotypes sensitive to social isolation can often be detected by assessing performance in mazes designed to test anxiety; the OFT and EPM involve conflicts between enclosed and open spaces. For example, anxiety is exacerbated by single housing with significant decreases in time spent in the open arms of the EPM

(Hellemans *et al.* 2004). When young, WT mice behaved as expected in tests designed to assess anxiety; spending the majority of the test time exploring designated "safe" zones such as the enclosed arms of the EPM apparatus and the peripheral areas of the OFT box. Moreover, there was no evidence for an anxious-like phenotype in 5αR1-KO mice compared to WT controls under either housing condition. However, following ageing, 5αR1-KO mice showed increased anxiety in the EPM, but only concomitantly with single housing. It is possible that the anxious phenotype is revealed upon the rise in glucocorticoid levels associated with the normal ageing process. Although differences were not detected in circulating corticosterone levels in singly-housed aged mice, plasma levels may not reflect those locally within the brain. Attempts to measure brain (and liver) corticosterone concentrations were unsuccessful, due to levels being below the limits of quantification of the assay.

The same aged animals were not more anxious compared to control when tested in the OFT. One reason why the data obtained from behavioural testing is not always easily reproducible is that even minor environmental changes can have a large impact on the resulting phenotype. A huge range of factors such as time of day and circadian rhythm, housing, temperature, noise and other external stressors, prior handling of the animals, gender, strain, environmental enrichment, olfactory cues and prior exposure to the experimenter can all alter behavioural phenotypes (Brett *et al.* 1990; McIlwain *et al.* 2001; Võikar *et al.* 2004; Võikar *et al.* 2005). Great care was taken to control for these variables wherever possible. For example, all animals in cages were left together in a quiet room during the testing period, tests were always carried out between 0800 hrs and 0100 hrs each day and all groups were handled with equal frequency and provided with the same environmental enrichment. Furthermore, the OFT is often suggested as less a test of anxiety and more a test of locomotion and physical activity under mild stress. Therefore, when interpreting data from the OFT, it is often difficult to separate measures of anxiety from other indices (File 2001). Both young and aged 5αR1-KO mice overall travelled a similar distance and at an average speed comparable to controls in the OFT, but their maximum speed of travel was increased. This may be interpreted as a measure of

anxiety or "panic" behaviour rather than locomotion *per se* in which case 5αR1-KO mice are suggested to have an exaggerated anxious-like response to the OFT, thus searching more urgently for a means to escape.

Glucocorticoids profoundly affect memory and other behaviours. Moreover, their elevated levels lead to impaired spatial learning mediated through the hippocampus (Stenzel-Poore *et al.* 1994; McEwen *et al.* 1995; Yau *et al.* 2001; Boyle *et al.* 2006; Rozeboom *et al.* 2007; Wei *et al.* 2007; Yau *et al.* 2007; Kolber *et al.* 2008; Kajiyama *et al.* 2010). In rodents, normal hippocampal function is required for adequate exploratory behaviour in any novel environment as well as for spatial memory (Britton *et al.* 1982; Gray 1982; Sutton *et al.* 1982; Lee *et al.* 1989). Glucocorticoid concentrations higher than normal biological levels may be neurotoxic and can negatively influence neuronal structure leading to its subsequent remodelling, whilst also interfering with electrophysiological processes such as LTP (Yau *et al.* 2007). Indeed, glucocorticoids in excess inhibit neurogenesis (Mirescu *et al.* 2006), implicated not only in cognitive decline but also in the pathogenesis of both anxiety and depression in the adult mouse (Snyder *et al.* 2011).

Many ageing studies have demonstrated that glucocorticoid exposure increases linearly from late middle age onwards in rodents (approximately 10 months and older) and in late old age in humans (Meaney *et al.* 1992; Sapolsky 1992; Lupien *et al.* 1994; Montaron *et al.* 2006). Disruptions in learning and memory are typical of the ageing hippocampus and numerous studies exploring the effects of glucocorticoid excess or chronic stress have replicated features of the aged hippocampus. For example cognitive impairments in aged rats (23-27 months old) and humans (60-80 years old) correlate with elevated glucocorticoid secretion both basally and post-stress (Issa *et al.* 1990; Lupien *et al.* 1994; Seeman *et al.* 1997). The contributory role of glucocorticoids is evident in studies of ADX at middle age, which reduces the neuronal loss commonly observed in aged rats (Landfield *et al.* 1981). Indeed ADX and GR blockade, reducing life-long glucocorticoid exposure, preclude cognitive abnormalities in the aged hippocampus (Landfield *et al.* 1981; Talmi *et al.* 1996). In the present study, a role for glucocorticoid metabolism by 5αR1 in the maintenance

of spatial memory was proposed. It was hypothesised that glucocorticoid excess within the brains of 5αR1-KO mice may lead to cognitive impairments possibly as a result of neuronal damage within the hippocampus; the opposite of what has been seen in the 11β-HSD1 KO mouse model. Aged, 11β-HSD1 deficient mice are protected from learning and memory impairments due to a lack of local glucocorticoid amplification in the brain which facilitates a switch from GR to MR-mediated cognitive control (Yau *et al.* 2011).

The Y-maze is a behavioural test used to determine memory capacity but is sensitive to stress. Whilst the test itself induces little or no intrinsic stress, the outcome is still sensitive to chronic changes in glucocorticoid levels (Coburn-Litvak *et al.* 2003). Young, WT animals housed in groups performed as expected in the Y-maze test of memory, showing good motivation and intact spatial recognition memory across all Y-maze phases. These mice spent more time exploring the novel arm than either of the other two arms. This indicates they identified this arm as novel and "remembered" previously exploring the other areas of the maze based on their spatial memory of the extra-maze queues. Single-housing alone did not affect memory assessed by Y-maze such that WT animals housed under social isolation performed equally well as group-housed, controls. However young 5αR1-KO mice housed in social isolation spent less time in the novel arm following the 1 min and 2 hour ITI however, the time in the novel arm after the 2 hour ITI fell below the level of chance (33%) and so cannot be taken as reduced memory capacity. The 1 min ITI is too short an interval for any difference to be attributable to memory loss alone. This immediate Y-maze memory task assesses the spontaneous novelty exploration behaviour of mice and so is not dependent on hippocampal function (Sarnyai *et al.* 2000). It is merely an indicator of adequate vision and motivation to participate in the test and changed in novel arm exploration between groups following the 1 min ITI may instead reflect altered locomotor activity. Paradoxically, this reduced novel arm exploration in singly-housed 5αR1-KO mice is not consistent with the increased locomotion normally seen under social isolation. Indeed, when young, there were no differences between WT and 5αR1-KO mice in locomotive indices in the Y-maze,

although all singly-housed mice travelled further and faster vs. group-housed animals.

Following ageing, there were some changes to the Y-maze phenotype. 5αR1-KO mice housed singly made fewer entries into the novel arm suggestive of reduced memory capacity. Furthermore, the same housing effect on locomotion was seen in aged animals with singly-housed mice travelling further and faster during testing. As seen in young animals, singly-housed 5αR1-KO mice spent less time in the novel arm following the 1 min ITI, possibly because they covered less distance and travelled at a reduced speed than matched WT mice. Reduced locomotion and exploratory behaviour has been attributed to other behavioural changes e.g. depression (Kalueff *et al.* 2004; Mineur *et al.* 2006). Although distinctions exist, there is broad overlap between the symptoms of depressive illnesses and anxiety disorders in humans and the two may be expected to coincide in animal models also. Therefore it would be useful to assess these animals for depression in further studies. Investigations could include the use of such tests including the forced swim test and/or the tail suspension test commonly used to determine the efficacy of anti-depressants in pre-clinical studies using animal models of depression.

As with the anxiety phenotype, it may be that the chronic stress effects of social isolation may be additive to deficient glucocorticoid clearance within the brains of 5αR1-KO mice leading to cognitive impairments possibly mediated through glucocorticoid-mediated hippocampal dysfunction. There is evidence to suggest that social isolation leads to memory impairment and that this phenotype is more pronounced in older mice (Hellemans *et al.* 2004). Further cognitive assessment was carried out using the Morris water-maze which is widely utilised to study spatial learning and memory, mostly facilitated by the hippocampus (Morris *et al.* 1982).

All mice performed as expected during water-maze testing in this study whereby escape latency i.e. elapsed time to locate the hidden platform submerged in opaque water, decreased across five consecutive training days. The data presented here do not show cognitive deficits in the learning abilities of 5αR1-KO mice when

compared to WT animals neither when young nor when aged. However, when looking at the decline in cognitive ability over time between paired animals when young and again when old, there is a greater cognitive decline associated with ageing in 5αR1-KO mice compared to WT controls. This phenotype reached significance in both group- and singly-housed mice. This is consistent with our hypothesis that a lack of adequate glucocorticoid metabolism has induced an exacerbated ageing phenotype in 5αR1-KO mice in relation to cognition, albeit subtle. Again, this contrasts as expected with enhanced spatial learning and memory mediated by the hippocampus seen in aged 11β-HSD1 null mice (Yau *et al.* 2007). The effect size seen was small but may have been greater if the mice had been aged for a further period; a typical life span may extend to 24 months.

An important consideration when assessing the effects of 5αR1-KO in the brain is that not only is the enzyme involved in metabolising active glucocorticoid molecules, it is also the vital rate limiting step in neurosteroidogenesis in the brain. The *de novo* synthesis of neurosteroids within the CNS was first demonstrated in the early 1990s by Baulieu *et al. (Baulieu et al. 1990).* By interacting with neurotransmitter-gated ion channels at neuronal synapses located throughout the central and peripheral nervous systems, neurosteroids can rapidly alter neuronal excitation or inhibition. Later in the mid-90s, it was discovered that 5αR1 plays an essential role in synthesising the neurosteroid ALLO from progesterone by pyramidal neurons in the brain (Cheney et al. 1995). The conversion is stimulated during acute periods of stress when ALLO levels increase significantly (Vallee *et al.* 2000); a process which can be inhibited dose-dependently by the use of finasteride (a 5αR inhibitor) in rats (Mukai *et al.* 2008). ALLO (as well as its precursor 5α-dihydroprogesterone; 5α-DHP) positively modulates the action of the major inhibitory neurotransmitter in the brain (γ-aminobutyric acid; GABA; Puia *et al.* 1990; Herd *et al.* 2007) in a direct, non-genomic manner by inducing allosteric changes in the ionotropic receptor $GABA_A$. Increased influx of negatively charged ions enhances GABA's inhibitory effects upon neuronal firing. These receptors are widely distributed in the glutamatergic neurons of the cortex and limbic areas, such as the hippocampus and the amygdala, two of the main brain structures involved in the regulation of

emotionality and behaviour. ALLO allosterically alters $GABA_A$. Through its modulation of local neuronal activity, ALLO has endogenous anxiolytic and sedative-like properties, and together with progesterone plays a modulatory role in the pathogenesis of psychotic disorders in rodents (Rupprecht *et al.* 1999; Khisti *et al.* 2002).

Indeed, associations have been made between impaired neurosteroid biosynthesis, and behavioural dysfunctions in rodents such as anxiety, depression and changes in contextual fear conditioning responses (Pinna *et al.* 2003; Uzunova *et al.* 2006; Kita *et al.* 2008; D'Aquila *et al.* 2010). Decreased ALLO is associated with emotional instability, aggression and increased locomotor activity in novel environments (Pibiri *et al.* 2008) which is also shown to be inducible by social isolation (Matsumoto *et al.* 1999; Dong *et al.* 2001). 5αR1 mRNA was markedly down-regulated (up to 37%) in the frontal cortex, hippocampus and the amygdala (areas know to be associated with behaviour and emotion) in mice socially isolated for 4 wks (Agis-Balboa *et al.* 2007). Therefore the release of this inhibitory brake within 5αR1-KO mice may cause an altered behavioural adaptation to stress by influencing neural circuitry in the CNS. Also, in clinical studies, decreases in ALLO concentrations in serum, plasma and cerebrospinal fluid are linked to anxiety spectrum disorders, depression and post-traumatic stress disorder (PTSD; Rapkin *et al.* 1997; Romeo *et al.* 1998; Uzunova *et al.* 1998; Nappi *et al.* 2001; Rasmusson *et al.* 2006).

Given the sexual dimorphism within the obesity phenotype and the propensity towards increased sensitivity to environmental stress in the female mice from our preliminary 5αR1-KO model, it is likely that further factors may play a modulatory role over and above glucocorticoids. Behavioural phenotypes may be more pronounced in female mice whose progesterone levels are endogenously much higher due to peripheral sources. Reduced ALLO in 5αR1-KO mice (particularly females) may render them more susceptible to anxiety. To fully elucidate the role of ALLO, its fluctuations would have had to have been more closely monitored in female mice since at least some of the substrate, progesterone, is peripherally derived and displays cyclical fluctuations which directly affect the concentrations of

neurosteroid (Rapkin *et al.* 1997). Future studies will be necessary to quantify ALLO in the brain, since from current data it cannot be concluded whether anxiety and cognitive deficits in aged, socially isolated 5αR1-KO mice are due to increased corticosterone or reduced ALLO in the brain. If 5αR1-KO mice had reduced neurosteroid biosynthesis within the brain, ALLO replacement studies may also be carried out to see if the social isolation phenotype can be reversed.

It is also noteworthy that neurosteroids such as pregnenolone (PREG) and ALLO are involved in HPA axis regulation. As an example, i.p. injection of PREG stimulated HPA activity via increased hypothalamic AVP resulting in raised ACTH and corticosterone levels in the circulation (Naert *et al.* 2007). The same authors also reported ALLO-induced activation of the axis where all of CRH, AVP, ACTH and corticosterone were increased following i.p. injection to rats. Again lack of ALLO may therefore account in part for the previously reported attenuated stress response in mixed strain 5αR1-KO mice.

Basal plasma corticosterone did not differ by genotype in young mice, but levels were reduced in aged 5αR1-KO mice overall, consistent with attenuated stress responses reported previously, but not glucocorticoid excess. Within the brain however, *GR* mRNA was downregulated in the hypothalamus of 5αR1-KO mice which is consistent with receptor downregulation in response to high ligand concentrations. This result is similar to findings in Chapter 4 in young mixed strain mice (females only). However, the abundance of transcripts for *Crh* was not affected by genotype, in contrast to prior findings which showed a significant downregulation of hypothalamic *Crh* in young 5αR1-KO mice of the mixed strain (Chapter 4). Also in contrast to findings in mixed strain animals, *Crhr-1* was increased in the pituitary in aged 5αR1-KO mice. Given these differences the question was posed whether these were effects of ageing or whether the HPA phenotype of the C57Bl/6j strain differed from the mixed background (explored in the following chapter).

As well as strain, housing also influenced mRNA abundance of transcripts which are key in HPA axis regulation. In the hypothalamus, social isolation induced

upregulation of transcripts for both *GR* and *MR*. Some reports suggest social isolation of rodents has no effect on *GR* or *MR* mRNA abundance in the CNS (Schrijver *et al.* 2002; Weiss *et al.* 2004) but hypothalamic *MR* and *GR* mRNA is upregulated in socially isolated pigs (Kanitz *et al.* 2009). Here, increased *GR* and *MR* expression is consistent with increased negative feedback on the HPA axis in light of the chronic stress of social isolation i.e. in an effort to limit or terminate the chronic stress response. Indeed, acute stress responses are usually downregulated during periods of chronic stress (Katz *et al.* 1981; Rich *et al.* 2005).

To summarise, in this chapter there is no evidence to suggest an anxious phenotype in 5αR1-KO mice, our proposed model of apparent glucocorticoid excess, when tested whilst young. Following considerable ageing, and a period of social isolation and at an age when glucocorticoid levels were higher still, 5αR1-KO mice were anxious compared to controls. Moreover, there is some evidence that 5αR1-KO mice have an exaggerated cognitive decline following ageing in spite of reduced plasma levels of corticosterone basally. Strain related differences in the effect of 5αR1 loss on key regulatory features of the HPA axis have been highlighted here. The true extent of this strain difference and the potential mechanism behind it is explored in Chapter 6 but due to time constraints this could only be studied in young mice.

Chapter 6: Influence of genetic background on the phenotype of 5αR1-KO mice

6.1 Introduction

Chapter 5 highlighted potential discrepancies between phenotypes seen in 5αR1-KO mice on a congenic C57Bl/6j background and those seen previously in mixed strain (C57Bl/6j/SvEv/129), 5αR1-KO mice. In previous studies, female, mixed strain, 5αR1-KO mice gained more weight when fed a normal chow diet; a phenotype which was sensitive to social isolation whereby animals lost weight and became comparable to WT (Livingstone *et al.* unpublished). These animals also had metabolic phenotypes including hyperphagia, insulin resistance (but stable plasma glucose) and increased adipose tissue (all fat pads) compared to WT controls. The data reported in this thesis using 5αR1-KO mice on a C57Bl/6j background, recapitulated the excess weight gain, which was again attenuated upon single housing. However, the magnitude of excess weight gain on the C57Bl/6j background seemed less than the mixed strain when both young and aged, and adipose tissue mass did not become statistically significantly heavier than controls. Moreover, on the C57Bl/6j background, 5αR1-KO mice were hypophagic. Although age is a potential confounding factor between experiments, it is also possible that strain is a phenotypic modulator in 5αR1-KO mice.

It is not uncommon for phenotypic differences (including different degrees of HPA axis activity) to exist between strains and many authors have reported such phenomena in both mice and rats. For example, comparisons between Fischer 344 and LOU/C rat strains revealed LOU/C rats had lower corticosterone across the circadian cycle overall, and that their stress responses were of a lesser magnitude (Marissal-Arvy *et al.* 2007). Strain differences can affect the responsiveness of the HPA axis to changes in glucocorticoid metabolism. 129/MF1 mice with transgenic disruption in the 11βHSD1 gene had elevated corticosterone levels both basally and post-stress and hypertrophied adrenals (Kotelevtsev *et al.* 1997; Harris *et al.* 2001) but 11βHSD1-KO mice on a C57Bl/6j background also had hypertrophied adrenals and elevated stressed corticosterone responses but in the absence of altered basal levels (Carter *et al.* 2009). The same authors also reported increased *GR* mRNA in the hippocampus and PVN of C57Bl/6j but not 129/MF1 mice indicating compensatory responses in C57Bl/6j mice to normalise feedback control of the HPA

axis. Therefore, this raises the question of whether mice with loss of 5αR1 bred onto a C56Bl/6j background may adapt differently from the original mixed strain mice. The following set of experiments was designed to directly compare the phenotype of 5αR1-KO mice on mixed vs. C57Bl/6j strain.

6.1.1 Hypothesis

It was hypothesised that glucocorticoid clearance would be attenuated to a lesser degree in 5αR1-KO mice of the C57Bl/6j strain compared to those of the mixed strain and that this would manifest as less disruption of metabolism and less suppression of HPA axis stress responses.

6.1.2 Aims

- To validate deficiency of 5αR1 in mice bred onto the C57Bl/6j strain.
- To determine if 5αR1-KO mice of the C57Bl/6j strain were glucose intolerant and hyperinsulinaemic similar to mixed strain mice.
- To determine if HPA axis responses to acute stress in 5αR1-KO mice were reduced similarly to mixed strain mice.
- To determine the efficiency of glucocorticoid clearance in WT and 5αR1-KO mice and to elucidate whether this differed between strains.

6.2 Methods

6.2.1 Validation of disruption of *Srd5a1* in 5αR1-KO backcrossed onto the C57Bl/6j background

6.2.1.1 Experimental outline

Whole brains from female 5αR1-KO mice (and WT controls) on each genetic background were obtained after cull. C57Bl/6j and mixed strain mice were 15 and 7 months old respectively.

6.2.1.2 PCR

RNA was extracted from liver, brain and prostate (positive control; sections 2.3.1.1 and 2.3.1.2) and reverse transcribed (2.3.2). Using the primers shown in Table 2.1 of the main methods in Chapter 2, PCR was performed according to 2.3.3 to determine the presence or absence of transcripts for *Srd5a1* and *Srd5a2* in brains and livers of WT and 5αR1-KO mice on both genetic backgrounds. Bands were visualised as described 2.3.3.1. PCR was also performed using primers for 11βHSD1 Table 2.1 as a positive control.

6.2.2 Assessing the phenotype of C57Bl/6j 5αR1-KO mice

6.2.2.1 Metabolic Biochemistry

Plasma was obtained from non-fasted trunk blood from female, WT and 5αR1-KO mice on a C57Bl/6j genetic background described in the previous chapter (aged 15 months at cull). Plasma glucose (hexokinase assay, 2.4.2) and insulin levels (ELISA, 2.4.1) were compared.

6.2.2.2 Assessment of acute HPA axis responses to stress

A further cohort of C57Bl/6j mice (male and female, WT and 5αR1-KO mice aged 6 months) were used to study responsiveness of the HPA axis. Following acute restraint stress (2.6.9.1), plasma corticosterone was quantified by ELISA (2.4.4).

6.2.3 Glucocorticoid clearance in mixed strain and C57Bl/6j mice

6.2.3.1 Experimental outline

Mice cohorts studied were as follows:

- **Mixed strain**
  - Male WT (n=8)
  - Male 5αR1-KO (n=9)
  - Female WT (n=7)
  - Female 5αR1-KO (n=8)
- **C57Bl/6j strain**
  - Male WT (n=5)
  - Male 5αR1-KO (n=8)
  - Female WT (n=9)
  - Female 5αR1-KO (n=8)

Mice (6 months old and obtained/maintained as described in 2.6.1 and 2.6.3) were used to determine the efficiency of glucocorticoid clearance both acutely and chronically. Following chronic glucocorticoid administration, the abundance of transcripts for enzymes (other than 5αR1) which determine glucocorticoid levels in tissues were quantified by qPCR.

6.2.3.2 Adrenalectomy

Prior to experimentation, all animals underwent adrenalectomy surgery to remove any natural disparities in endogenous corticosterone flux from the adrenal glands both basally and during acute corticosterone treatment. Bilateral adrenalectomy (2.6.9.2) was performed on weight- and aged-matched mice (approximately 27-30 g at 5 months old).

6.2.3.3 Assessment of acute glucocorticoid clearance

To assess clearance of acutely administered corticosterone, ADX mice were injected subcutaneously (s.c.) with corticosterone (2 μg in 10% ethanol with 0.025% β-cyclodextrin in 0.9% saline). Blood was collected by venesection at time 0 (immediately prior to injection) and at 15, 30, 60 and 90 mins following the injection. Plasma corticosterone levels were determined by ELISA (2.4.4).

6.2.3.4 Assessment of chronic glucocorticoid clearance

To assess clearance of chronically administered corticosterone, ADX mice were implanted with osmotic minipumps (2.6.9.2.1) loaded to deliver 100 μg of corticosterone per day in 1:1 DMSO:propylene glycol at a flow rate of 0.5 μL/hr. Mice were culled after 2 wks of infusion and tissues collected as in 2.6.10. Corticosterone was quantified from plasma by ELISA (2.4.4) and hepatic levels by LC-MS/MS (2.5.2) following steroid extraction from the liver (2.5.1).

6.2.3.5 qPCR to quantify mRNA abundance in liver

Following chronic corticosterone infusion, total RNAs were extracted from snap frozen livers (2.3.1.1) and used to synthesise first strand cDNA by RT (2.3.2). Using the primers and probes designated in Table 2.2, qPCR (2.3.4) was performed to

quantify the expression of transcripts for *Srd5a1* (5αR1), *Akr1d1* (5βR), *Akr1c6* (3αHSD) and *Hsd11b1* (11βHSD1). The abundance of transcripts of interest were expressed as a ratio to the mean of the transcript levels of a combination of reference genes as outlined in Table 4.1. The combination of reference genes was selected due to a lack of variation between experimental groups.

6.2.4 Terminal procedures

All mice were humanely culled and tissues collected onto dry ice as described in section 2.6.10.

6.2.5 Statistical analyses

Data are presented as mean ± SEM and were analysed by unpaired Student's t-tests, 2-way repeated measures ANOVA or 2-way ANOVA followed by Bonferroni post-hoc tests where appropriate. All statistical analyses were done using GraphPad Prism software.

Tissue	Reference Gene Combination	p Value			
Liver	Cyclophilin A (*Ppia)* TATA-box binding protein *(Tbp)* 18s ribosomal RNA *(Rn18s)*	Male		Female	
		G	S	G	S
		0.979	0.685	0.948	0.672

Table 6.1 Reference genes used for qPCR analysis.
The mRNA abundances of the above three genes were combined and used as the mean reference against which the abundance of transcripts of interest in the liver was normalised. P value indicates the results of a 2-way ANOVA for the combined reference genes, demonstrating lack of change between experimental groups. G = genotype, S = strain.

6.3 Results

6.3.1 Model validation

6.3.1.1 Validation of 5αR1-KO in tissues of interest

Genotyping from ear samples obtaining during breeding had confirmed global 5αR1-disruption in the DNA but in light of strain-related phenotypic differences, validation of complete 5αR1-KO at the level of the RNA was sought, in case of alternative transcripts. Transcripts for *Srd5a1* were not detected in brain or liver samples from 5αR1-KO mice of both the C57Bl/6j and mixed genetic strains (Figure 6.1, A) and, as a positive control for sample and process integrity, *Hsd11b1* mRNA was demonstrated to be expressed in all samples (Figure 6.1, B). Moreover, *Srd5a2* mRNA was not detected in either brain or liver samples from WT or 5αR1-KO mice of either strain (Figure 6.2), but was detected in prostate, the positive control.

6.3.1.2 Bioinformatics

There also remained the possibility of alternative splicing of the *Srd5a1* gene to produce multiple mRNA variants and thus potentially more than one 5αR1 protein. The *Srd5a1* gene was blasted on the Ensembl Genome Browser online and revealed there were two possible transcripts resulting from the gene (Table 6.2). The first was the conventional transcript known to produce the functional 5αR1 protein (*Srd5a1-001*) and the second (*Srd5a1-002*) was a processed transcript and therefore non-protein coding.

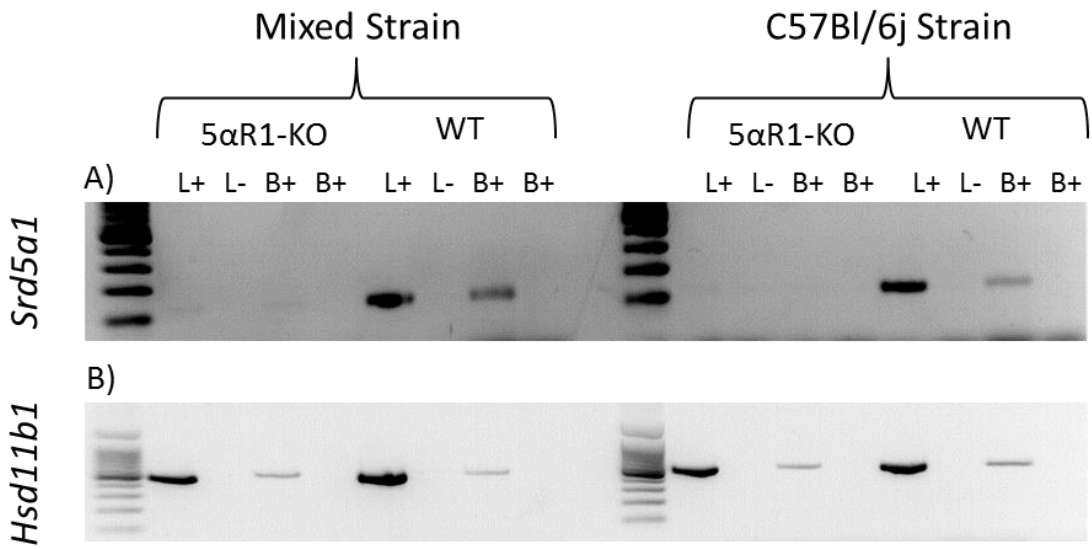

Figure 6.1 PCR result showing no *Srd5a1* expression in 5αR1-KO mice
mRNA for *Srd5a1* was not detected in the brains or livers of 5αR1-KO mice from either of the two genetic backgrounds (A). B shows PCR for *Hsd11b1* as an amplification control. Also shown are negative controls (no reverse transcriptase enzyme) for each sample, and an overall positive and negative control for each reaction. B = brain, L = liver, WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

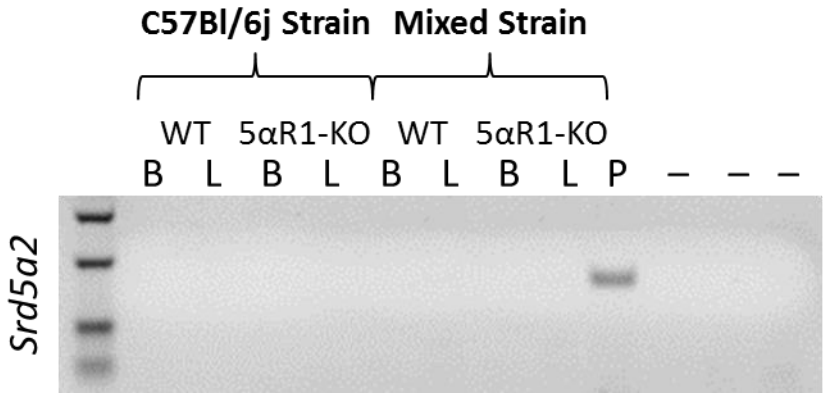

Figure 6.2 ***Srd5a2*** **expression in brain and liver**

mRNA for *Srd5a2* was not detected in whole brain or liver of either WT or 5αR1-KO mice of both genetic strains. Prostate tissue was used as a positive control. The three negative controls were no reverse transcriptase enzyme, water and a known negative sample of liver. B = brain, L = liver, P = prostate, WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

Name	Transcript ID	Length (bp)	Protein IDL	Length (aa)	Biotype
Srd5a1 -001	ENSMUST00000091514	2644	ENSMUSP00000089097	255	Protein coding
Srd5a1 -002	*ENSMUST00000059235*	888	No protein product	-	Processed transcript

Table 6.2 Alternative splicing and transcript options for the *Srd5a1* gene
This table highlights the details of the two possible transcripts encoded by the *Srd5a1* gene. Srd5a1-001 is the conventional transcript, which translates the functional 5αR1 protein. Srd5a1-002 is a processed transcript and would not result in a translated 5αR1 protein. bp = base pair and aa = amino acid. This table was adapted from the Ensembl Genome Browser (http://www.ensembl.org/index.html).

6.3.2 The metabolic phenotype of female C57Bl/6j, 5αR1-KO mice

6.3.2.1 Body weight

As shown in Chapter 5 (5.3.1), body weight gain was increased in 5αR1-KO mice and this phenotype was attenuated during periods of social isolation. Excess body weight gain in 5αR1-KO mice on the C57Bl6/j strain was less than that observed in mixed strain mice (broadly 1g heavier in C57Bl/6j mice vs. 2.9 g heavier in mixed strain mice at 15 wks old), which can be seen in Figure 3.1and Figure 5.2 in Chapters 3 and 5 respectively.

6.3.2.2 Plasma glucose and insulin levels

Plasma glucose levels (non-fasting) were not different between WT and 5αR1-KO mice and there was no effect of housing (Figure 6.3, A and B for group- and singly-housed mice respectively). Insulin levels were increased in 5αR1-KO mice housed in groups vs. WT mice (Figure 6.3, C, $p<0.01$). There was no genotype effect on plasma insulin in singly-housed animals (Figure 6.3, D).

6.3.2.3 HPA axis responses to acute restraint stress

Basal corticosterone concentrations were higher in females over males, and the data were within the appropriate physiological ranges (Figure 6.4 A and B). Broadly, the WT HPA axis response was comparable between the two strains; see by comparison of Figure 6.4 (C57Bl/6j strain) vs. Figure 4.1 (mixed strain). However, differential to mixed strain, 5αR1-KO mice of the C57Bl/6j strain showed HPA axis responses following acute restraint stress which were comparable to control (Figure 6.4). WT and 5αR1-KO animals of the C57Bl/6j strain had similar peak concentrations of corticosterone and had comparable recovery phases for both male and female mice (Figure 6.4, A and B respectively).

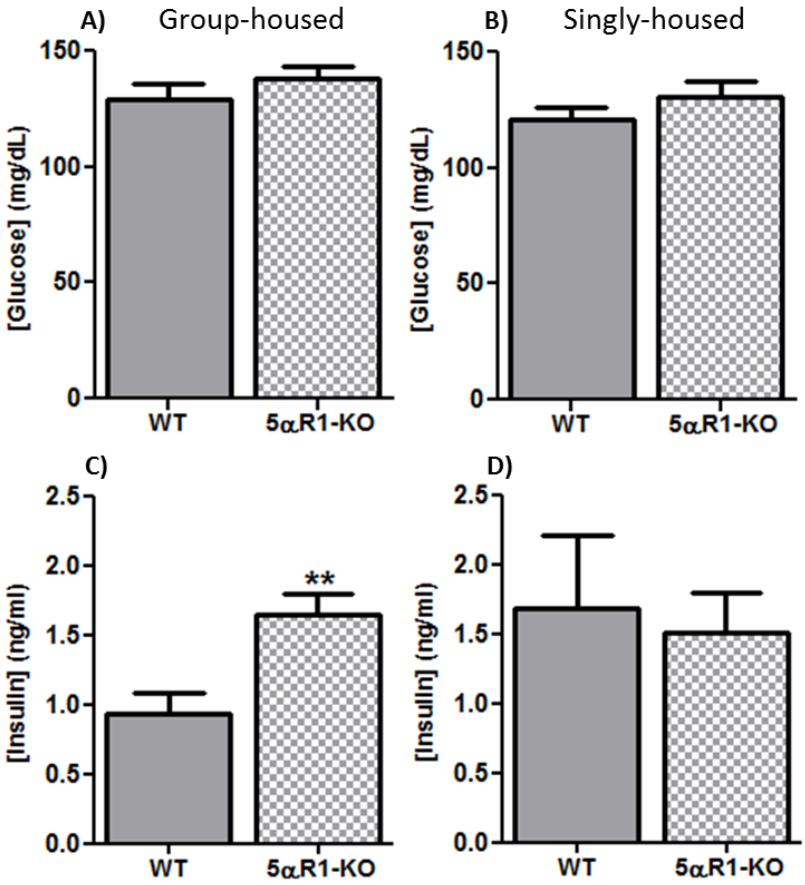

Figure 6.3 Non-fasted plasma glucose and insulin levels for AGED C57Bl/6j mice at cull.

The top two panels show that plasma glucose concentrations were not different in non-fasted, trunk blood samples from group- and singly-housed, WT (solid) and 5αR1-KO (chequered) mice (A and B respectively). However, group-housed, 5αR1-KO mice had significantly higher circulating insulin levels vs. WT controls (C) but there were no differences in comparable mice housed singly (D). Data are mean measurements ± SEM for n=15-17/group. Comparisons were made by Student's unpaired t-test where ** = $p<0.01$. All animals were aged 15 months and from the C57Bl/6j strain. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

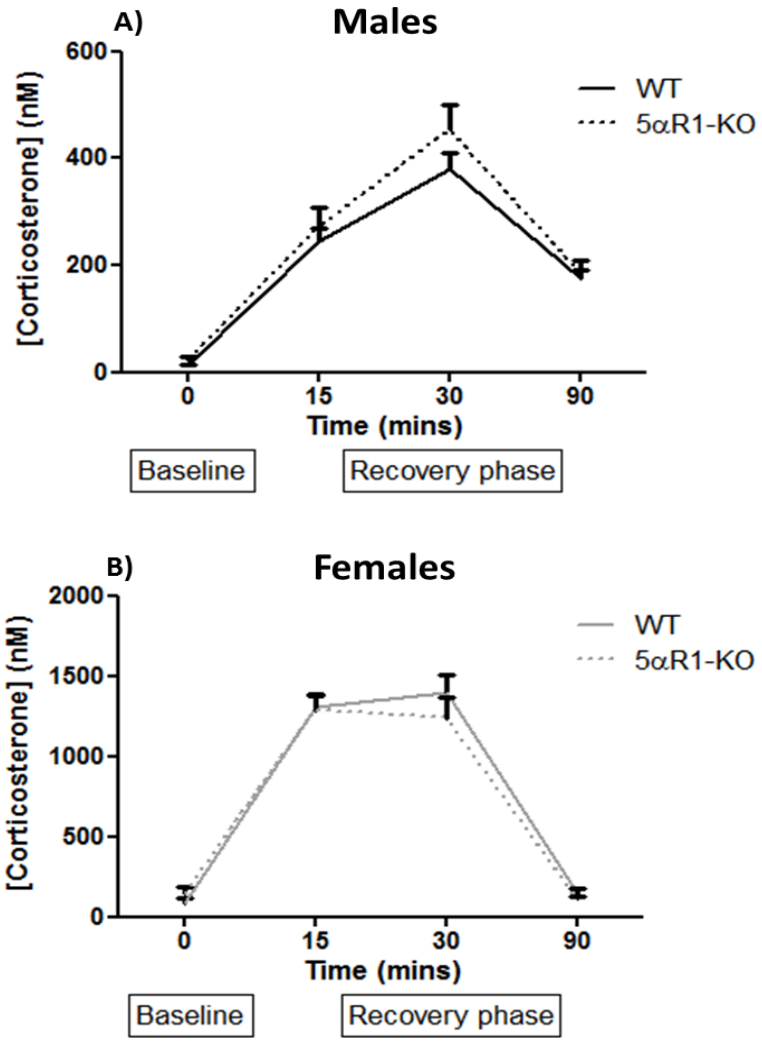

Figure 6.4 Plasma corticosterone concentrations during an acute restraint stress test. A and B show the degree of corticosterone release in response to acute restraint stress for male (black) and female (grey), WT (solid lines) and 5αR1-KO (hatched lines). Plasma corticosterone did not differ by genotype either basally or following restraint in male (A) and female (B) mice. Values are mean measurements ± SEM where n = 12/group. Mice were 6 months old and from the C57Bl/6j strain. Comparisons were made between groups by 2-way repeated measures ANOVA. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

6.3.3 Glucocorticoid clearance and the effect of strain

6.3.3.1 Plasma corticosterone levels following acute and chronic infusion

6.3.3.1.1 Male animals

Basal corticosterone concentrations were comparable between both genotypes and strains (Figure 6.5, A). Following an acute corticosterone challenge, mixed strain WT mice had elevated plasma corticosterone across the time course compared to C57Bl/6j WT mice (Figure 6.5, A, $P<0.01$). Post-hoc testing revealed corticosterone levels remained significantly more elevated in WT, mixed strain mice compared to WT C57Bl/6j mice at 60 and 90 mins post injection (Figure 6.5, A, $p<0.01$). For mixed strain mice, corticosterone was lower in 5αR1-KO mice compared to WT controls (Figure 6.5, A, $p<0.05$). In C57Bl/6j mice there was a trend for higher corticosterone in 5αR1-KO mice vs. WT (Figure 6.5, A, $p=0.07$). Following chronic infusion, plasma corticosterone did not differ between any of the groups. However, there was an interaction such that corticosterone levels were differentially affected by 5αR1-KO in each of the two genetic strains (Figure 6.5, C, $p<0.05$).

6.3.3.1.2 Female animals

In females, plasma corticosterone levels did not differ by strain or genotype either basally or following acute corticosterone treatment (Figure 6.5, B). There was also no difference between experimental groups in trunk corticosterone following chronic infusion (Figure 6.5, D).

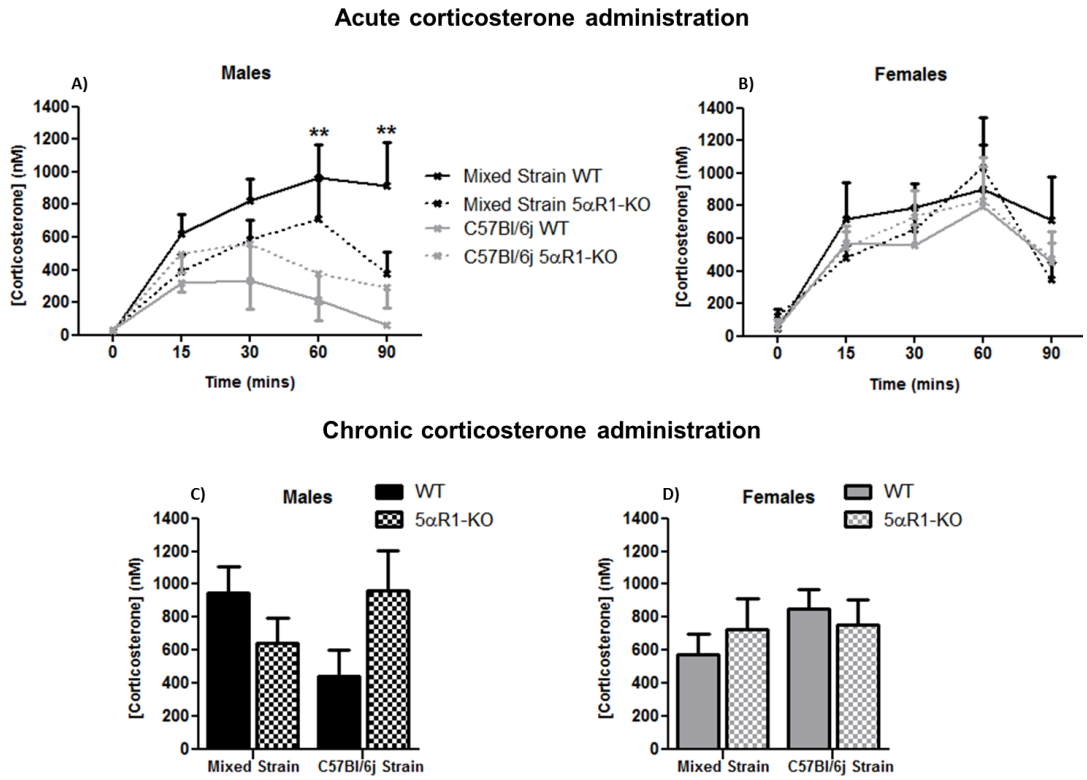

Figure 6.5 Plasma corticosterone levels following acute and chronic corticosterone infusion

Prior to injection, basal corticosterone levels were comparable between experimental groups in both male and female animals (A and B respectively). After corticosterone administration, in male mice there was an overall effect of strain ($p<0.01$) whereby plasma corticosterone was lower in C57Bl/6j vs. mixed strain mice. In males, corticosterone was lower and higher in 5αR1-KO mice vs. WT for mixed ($p<0.05$) and C57Bl/6j ($p=0.07$) strains respectively (A). In females (B), neither strain nor genotype affected plasma corticosterone levels over the time-course. Following chronic corticosterone infusion, there was an interaction in male mice (C, $p<0.05$) showing that corticosterone levels were differentially affected by 5αR1-KO in each of the two strains but there was no effect of genotype (C). In females, plasma corticosterone levels were comparable between each of the experimental groups following chronic corticosterone infusion (D). Data are mean ± SEM for n=5-9/group. Mice were 6 months old. Comparisons were made by 2-way ANOVA or 2-way ANOVA for repeated measures with Bonferroni post-hoc tests where appropriate. ** = $p<0.01$ for effect of strain. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

6.3.3.2 Hepatic corticosterone levels following chronic infusion

6.3.3.2.1 Male animals

After 4 weeks of infusion, hepatic corticosterone concentrations were higher in mixed strain vs. C57Bl/6j mice overall (Figure 6.6, A, $p = 0.05$) but there was no effect of genotype.

6.3.3.2.2 Female animals

Hepatic corticosterone concentrations were comparable between strains (Figure 6.6, B). In both mixed strain and C57Bl/6j mice, hepatic corticosterone levels were increased in 5αR1-KOs vs. WT controls (Figure 6.6, B, $p<0.05$).

6.3.3.3 Effect of strain on hepatic expression of metabolic enzymes

In both strains and sexes, transcripts for *Srd5a1* were not detected in 5αR1-KO mice (Figure 6.7, A). In WT males, there was a strain effect whereby transcripts for *Srd5a1* were less abundant in C57Bl/6j mice vs. mixed strain mice (Figure 6.7, A, $p<0.05$). In both strains transcripts for *Srd5a1* were less abundant in WT females vs. WT males (Figure 6.7, A, $p<0.001$ and $p<0.01$ for mixed and C57Bl/6j strains respectively). There was no overall effect of genotype or strain on *Akr1d1* mRNA expression (Figure 6.7, B), however, transcripts for *Akr1d1* were significantly affected by sex but differentially in each strain. In mixed strain mice, transcripts for *Akr1d1* were more abundant in WT females vs. WT males (Figure 6.7, B, $p<0.01$) whilst in C57Bl/6j mice, 5αR1-KO females showed higher *Akr1d1* expression than 5αR1-KO males (Figure 6.7, B, $p<0.05$). There were no overall genotype or sex effects on *Hsd11b1* mRNA abundance in any of the experimental groups (Figure 6.7, C). However, there was an overall effect of strain whereby transcripts for *Hsd11b1* were more abundant in mixed strain mice vs. C57Bl/6j mice (Figure 6.7, C. $p<0.01$), and especially so in females ($p<0.05$). There were no effects of genotype, strain or sex on the abundance of transcripts for *Akr1c6* (Figure 6.7, D).

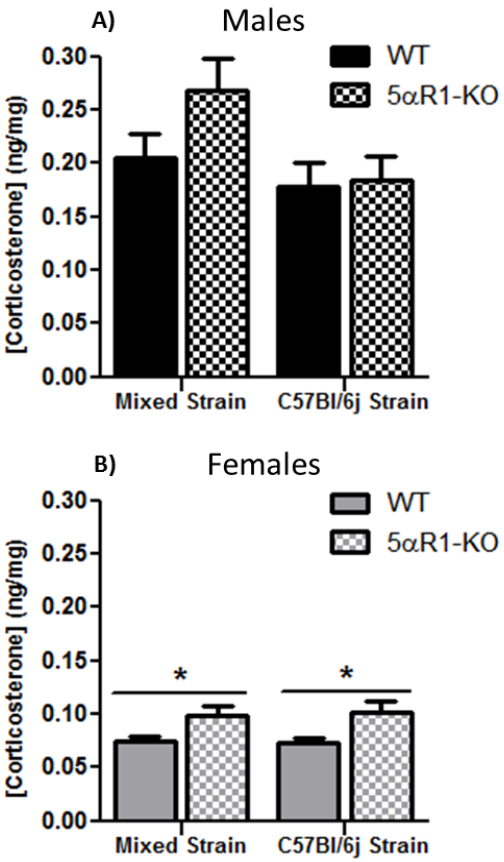

Figure 6.6 Hepatic corticosterone levels following chronic corticosterone infusion
Following chronic corticosterone infusion, there was a significant effect of strain upon hepatic corticosterone content in male mice whereby levels were higher in mixed strain vs. C57Bl/6j mice (A, $p=0.05$) overall. In female animals, corticosterone was elevated in 5αR1-KO mice vs. WT in both mixed and C57Bl/6j strains (B; $p<0.05$) but there was no effect of strain alone. Data are mean ± SEM for n=5-9/group and * = $p<0.05$. Mice were 6 months old. Comparisons were made by 2-way ANOVA with Bonferroni post-hoc tests where appropriate. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

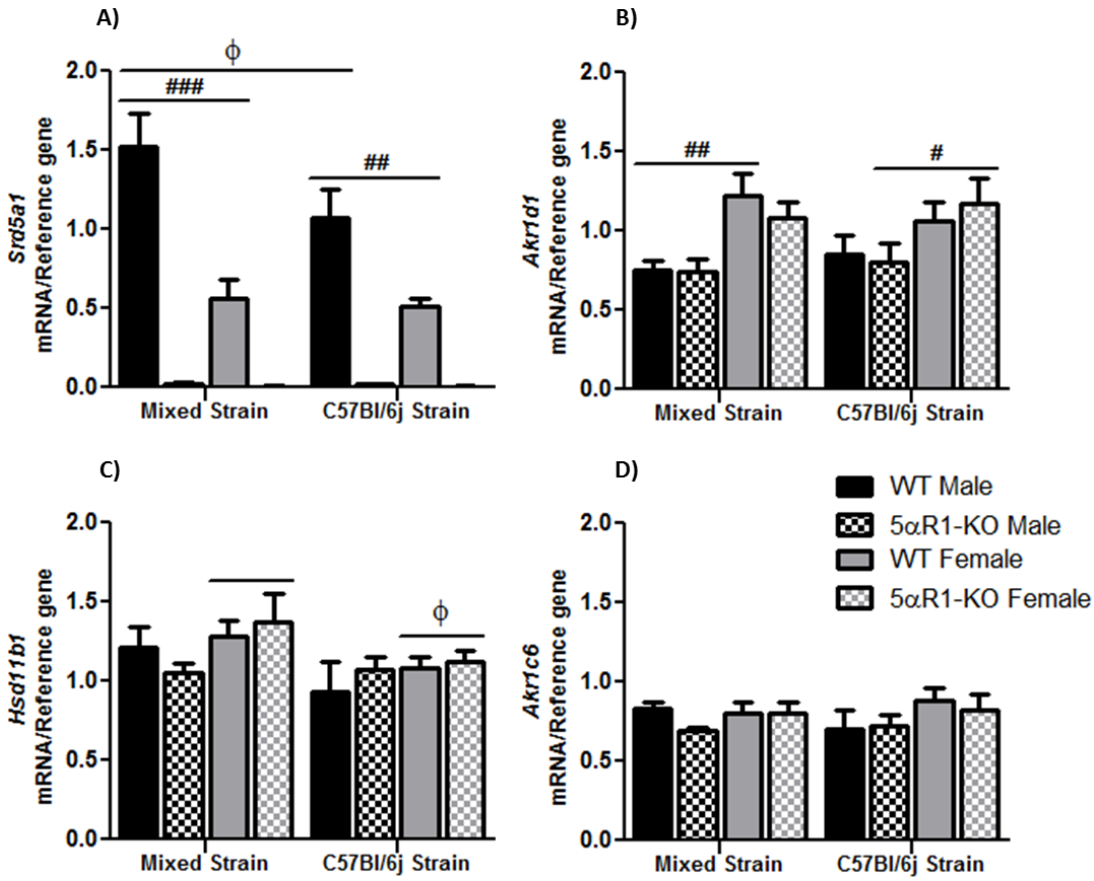

Figure 6.7 Hepatic expression of genes for metabolic enzymes following chronic corticosterone infusion

Srd5a1 mRNA was not detected in 5αR1-KO animals in both genetic backgrounds and sexes (A). In WT animals, transcripts for *Srd5a1* were less abundant in C57Bl/6j mice vs. mixed strain mice (A, $p<0.05$). There was also an effect of sex on *Srd5a1* expression in WT animals whereby transcripts were less abundant in female WT mice vs. male WT mice of both strains (A, $p<0.001$ and $p<0.01$ for mixed and C57Bl/6j strains respectively). *Akr1d1* mRNA expression was significantly affected by sex (B) but differentially in each strain. In mixed strain WT mice, transcripts for *Akr1d1* were more abundant in females vs. males (B, $p<0.01$) whilst in C57l/6j mice, females showed higher *Akr1d1* expression than males but only reaching significance in mice lacking 5αR1 (B, $p<0.05$). There was an overall effect of strain on the abundance of transcripts for *Hsd11b1* (C. $p<0.01$) whereby levels were greater in mixed strain mice vs. C57Bl/6j mice overall, especially in females ($p<0.05$). There were no effects of genotype, strain or sex on the abundance of transcripts for *Akr1c6* (D). Data are mean ± SEM for n=5-9/group (mice were 7 months old at cull). Comparisons were made by 2-way ANOVA with Bonferroni post-hoc tests where appropriate. One symbol = $p<0.05$, two symbols = $p<0.01$ and three symbols = $p<0.001$ whilst # represents effects of sex and Φ = strain effects by post-hoc testing. WT = wild-type and 5αR1-KO = 5α-reductase type 1 knock-out.

6.4 Discussion

Here, we have demonstrated that the phenotype of mice deficient in 5αR1 is influenced by background strain. Although there were similarities e.g. excess weight gain and hyperinsulinaemia (both abolished by social isolation), the magnitude of the metabolic dyshomeostasis was less marked in the congenic mice and the suppressed HPA axis response to acute stress was not evident. It was proposed this disparity may be attributable to the genetic manipulation making less impact on corticosterone clearance efficiency in C57Bl/6j strain mice, rendering this strain less susceptible to the effects of 5αR1 loss.

Firstly, disruption of 5αR1 in the 5αR1-KO model on the congenic background was validated. Transcripts for *Srd5a1* were not detected in the brains or livers of KO animals from either genetic background confirming integrity of breeding and genotyping processes. The *Srd5a1* gene was blasted using software on the Ensemble Genome Browser website showing that only one other transcript (*Srd5a1-002*; as opposed to the conventionally recognised transcript *Srd5a1-001*) may arise from alternative splicing activities. However *Srd5a1-002* is a processed transcript which does not result in a translated protein product. Thus, even if transcription of its mRNA sequence is intact in 5αR1-KO mice, this would not impact on the phenotype of our 5αR1-KO model, by generating a functional enzyme. It was possible that the lack of functional 5αR1 could be compensated for by increased expression of 5αR2 (or also 5βR). However, *Srd5a2* mRNA was absent in tissues of interest from both WT and 5αR1-KO, ruling out compensatory expression of the type 2 isozyme in response to loss of 5αR1, at least in liver. Moreover, the abundance of mRNA for *Akr1d1* was comparable between WT and 5αR1-KO mice.

Various aspects of the phenotype of the 5αR1-KO mice previously reported on the mixed strain were probed on the congenic background. As shown in Chapter 5, the excess weight gain of 5αR1-KO mice on the congenic line was present but less marked than on the mixed strain (broadly 1 g heavier at 15 wks old vs. 2.9 g heavier in mixed background). The C57BL6/j 5αR1-KO mice were euglycaemic but hyperinsulinaemic under group-housing, recapitulating the mixed strain phenotype.

However, the data for each background could not be compared directly due a difference in age but also since mixed strain mice were fasted and C57Bl/6j mice were not. Single housing caused weight loss and attenuated hyperinsulinaemia in the congenic strain, again a similar "rescue" phenotype seen before in the mixed strain 5αR1-KO mice. However, in the congenic line this was associated with hypophagia, not previously observed, potentially suggesting more refined balance of energy homeostasis in the C57Bl/6j background. Therefore, overall the metabolic phenotype was present on both backgrounds but more marked on the mixed strain.

On the contrary, HPA axis responses to acute stress were not similarly affected by 5αR1 loss in each of the two strains. Mixed strain 5αR1-KO mice had basal corticosterone levels comparable to WT but significantly lower levels following a short period of restraint stress. In the C57Bl/6j strain both basal and post-restraint stress corticosterone levels were comparable between WT and 5αR1-KO mice. Strain differences in HPA axis responses are not uncommon and C57Bl/6j mice are recognised as being quite resistant to physiological challenges including stress (Shanks *et al.* 1990; Anisman *et al.* 1998), showing particularly tight control of glucocorticoid secretion overall. In contrast, BalbC mice, for example, have large adrenals and an exaggerated stress response (Zaharia *et al.* 1996). Furthermore, following acute restraint stress, plasma corticosterone and insulin levels were lower whilst glucose was higher in C57Bl/6j mice compared to other strains such as DBA/2 BLAL/c and NOD (Harizi *et al.* 2007). These differences may also be associated with phenotypic discrepancies when mice are assessed in behavioural tests such as the EPM. For example, the effects of social isolation on anxiety (increased) and locomotion (hyperactive) were less evident in C57Bl/6j compared to DBA/2 mice (Võikar *et al.* 2005). Furthermore, C57Bl/6j mice learned the water maze task better than the DBA/2 mice (Võikar *et al.* 2005); therefore differences in behaviour of the 5αR1 KO mice may have been easier to detect on the mixed strain, rather than the C57BL/j mice tested in Chapter 5. Further examples of how strain can influence neuroendocrine pathways include, increased HPA axis activity following acute stress (i.e. corticosterone levels were increased) in LAL compared to SAL mice whilst chronic psychological stress induced long-term changes in the HPA axis in LAL

(upregulated hypothalamic *Crh* and hippocampal DG *GR* mRNA) but not SAL mice (Veenema *et al.* 2004). Importantly, strain has been shown to affect the HPA phenotype of mice with genetic disruption of *Hsd11b1*, a model of altered glucocorticoid metabolism, anticipated to influence glucocorticoid action in the opposite manner to the 5αR1-KO model studied here.

The fact that the HPA axis corticosterone responses to stress was not reduced in C57Bl/6j 5αR1-KO mice was unexpected but may help explain why the brain transcript profile for factors which regulate activity of the axis was different between mouse strains. In Chapter 3, female mixed strain 5αR1-KOs had less *Crh* and *Crhr-1* mRNA in the PVN and pituitary respectively but in female C57Bl/6j 5αR1-KO mice (Chapter 5), *Crh* was not different and *Crhr-1* was paradoxically increased. In female 5αR1-KO mice, hypothalamic and pituitary *GR* was downregulated consistent with glucocorticoid excess in mixed strain animals but *GR* was only downregulated in the hypothalamus and not pituitary in C57Bl/6j mice. These two mouse cohorts were culled at different ages (mixed strain at 7 months and C57Bl/6j strain at 15 months), which could potentially be the cause of the above discrepancies, but it is also possible that these effects are due to the genetic backgrounds of the mice used.

More marked phenotypes in the mixed over C57Bl/6j strain may reflect the fact that disruption of 5αR1 was having a greater magnitude of impact on glucocorticoid clearance in the mixed strain. This could be due to differential contributions of 5αR1 between strains, or differential compensation of other metabolic pathways. Thus to assess glucocorticoid clearance efficiency, mice received corticosterone both acutely and chronically. Firstly, mice were adrenalectomised to remove endogenous corticosterone; avoiding the possibility of the HPA axis correcting circulating levels. Basal corticosterone levels were not reduced to zero in ADX mice (mean of 28 and 76 nM for males and females respectively) but did not differ between experimental groups. It is unlikely this is due to residual cross-reactivity of the antibody used for the ELISA analysis. Instead it is proposed that extra-adrenal glucocorticoid production via the action of metabolic enzymes in physiological systems such as the CNS (Mellon *et al.* 1989; Strömstedt *et al.* 1995; Gomez-Sanchez *et al.* 1996) and

adipose tissue (Napolitano *et al.* 1998; Wake *et al.* 2007; Hernandez-Morante *et al.* 2009) may account for residual corticosterone in the circulation following ADX.

In **male mice**, corticosterone levels rose higher in WT mixed strain mice compared to WT C57Bl/6j mice following acute corticosterone treatment and indeed remained high over the test period. Moreover, whilst levels in WT C57Bl/6j had returned almost to baseline at 90 mins post-injection, corticosterone remained high in WT mixed strain mice across the time-course. This is suggestive of a slower rate of acute glucocorticoid clearance (or delayed HPA axis resolution) in mixed strain compared to congenic C57Bl/6j mice. Moreover, hepatic corticosterone levels were higher in mixed strain mice compared to C57Bl/6j congenics, again suggestive of more efficient hepatic clearance in C57Bl/6j mice overall.

Surprisingly in **male mice**, corticosterone clearance was differentially influenced by 5αR1-KO in each strain. Without the contribution of 5αR1 in regulating peripheral corticosterone levels, 5αR1-KO animals would be expected to clear the infused corticosterone bolus more slowly and therefore levels in the plasma would be increased vs. WT. This was only evident in the C57BL6/j strain; corticosterone levels across the acute time-course were increased in C57Bl/6j 5αR1-KO mice but paradoxically decreased in 5αR1-KO animals of the mixed strain. It is unclear why this arose, but one explanation is that the levels of corticosterone achieved in the C57BL6/j strain were considerably lower than the mixed strain and also showed a profile demonstrating clearance after a peak level. It is possible that clearance systems were saturated in the mixed strain and the anticipated pattern would be revealed if tested with a lower dose of corticosterone which may reflect a more normal physiological response.

Chronic corticosterone infusion resulted in plasma levels which were comparable between experimental groups, however, similar to acute treatment, 5αR1-KO affected plasma corticosterone in opposite ways in each of the two strains. For example, although differences did not reach significance, it seemed that corticosterone was decreased and increased in 5αR1-KO mice of the mixed and

C57Bl/6j strains respectively. It was also possible that other enzyme pathways were adapting differently between strains, confounding the experimental findings.

In **males** only (WT), transcripts for *Srd5a1* in the liver were more abundant in mixed strain mice vs. C57Bl/6j mice, perhaps contributing to the increased susceptibly of mixed strain mice to the metabolic effects of 5αR1 loss. Furthermore, independent from both sex and genotype, C57Bl/6j mice had slightly reduced abundance of *Hsd11b1* compared to mixed strain animals, which could be somewhat protective from the effects of 5αR1 loss and result in overall less corticosterone reactivation, at least in the liver. There were no male specific genotype or strain effects of 5αR1-KO on hepatic expression of mRNAs relating to other metabolic enzymes and it seems unlikely that these findings explain the strain difference observed.

In **female mice**, there was no difference between strains in plasma corticosterone concentrations following either acute injection or chronic infusion, suggesting that glucocorticoid clearance rates are comparable between female mixed strain and C57Bl/6j congenic mice. Indeed, no substantial differences in the levels of transcripts of metabolic enzymes were observed between females of the two strains, although transcripts for hepatic *Hsd11b1* were slightly less abundant in C57Bl/6j vs. mixed strain mice. Furthermore, hepatic corticosterone levels were similar between strains. Although differences in clearance could not be observed between female WT or 5αR1-KO mice on either strain, the corticosterone concentrations in the liver were significantly higher in 5αR1-KO mice following chronic infusion, in keeping with impaired glucocorticoid inactivation in that organ. Compensatory changes were not observed in other enzymes to account for the lack of change in overall clearance in liver, but this may have happened either in other tissues (e.g.) adipose or in other enzyme systems which were not tested here e.g. 6β-hydroxylase. Notably, again the levels of corticosterone achieved after acute injection were similar to the male mixed strain, so possibly too high to detect a difference due to saturation.

In both strains but even more so in mixed strain mice, transcripts for *Srd5a1* in the liver were more abundant in male than female WT animals. Differential *Srd5a1*

expression between the sexes of mice is not well reported in the literature but in the brain its expression does not differ by sex (Tsuruo 2005). In rat liver (Gustafsson *et al.* 1976) and humans (as measured by urinary metabolites; Andrew *et al.* 1998) 5αR1 activity is reportedly higher in females over males. With intrinsically higher *Srd5a1* expression in peripheral tissues such as the liver, male 5αR1-KO animals would be anticipated to be more prone to metabolic disturbances, in contrast to the findings underpinning the hypothesis of this thesis. In light of no sex-specific differences in *Srd5a1* expression in the brain (Tsuruo 2005), it may be that reduced ALLO synthesis particularly in females plays an important role in the pathogenesis of CNS disturbances related to loss of 5αR1.

In summary, in male mice there is evidence to suggest mixed strain mice are less able to clear excess glucocorticoids from the periphery than C57Bl/6j congenic animals. This in part may explain why HPA axis responses to acute stress were reduced in 5αR1-KO mice of the mixed but not C57Bl/6j strain; if peripheral levels reflect those in the CNS, excessively high levels of corticosterone would enhance negative feedback on the HPA axis, dampening the stress response. In this study, female animals were studied at length and it was in this sex particularly that strain differences were first apparent. However, there is no evidence to suggest strain-specific differences in global glucocorticoid clearance either acutely or chronically, although excess glucocorticoids accumulate in the liver. Two further explanations may be found in the age of mice, or in the activity of 11βHSD1. Here we showed that C57Bl/6j mice have lower *Hsd11b1* mRNA in the liver. Given 5αR1 and 11βHSD1 share NADPH as a co-factor within the mitochondria, it is possible that their activities may adapt in relation to one another. Furthermore, some of the phenotypic differences were reported between young mixed strain mice and others aged C57Bl/6j mice; however, HPA axis responses were tested in mixed and C57Bl/6j mice which were age-matched. This indeed correlates with the literature which suggests that C57Bl/6j mice are especially able to adjust the set-point of the HPA axis according to conditions to tightly control glucocorticoid secretion and are fairly resistant to stress (Shanks *et al.* 1990; Zaharia *et al.* 1996; Anisman *et al.* 1998; Harris *et al.* 2001; Yau *et al.* 2001; Carter *et al.* 2009).

Chapter 7: Summary

7.1 Overview

The aim of this study was to investigate the role of 5αR1 in regulating the HPA axis and syndromes associated with its dysfunction e.g. metabolic disturbances including obesity and diabetes and behavioural disorders such as anxiety and cognitive impairment. Basal glucocorticoids were not different between WT and 5αR1-KO mice but the abundance of genes which transcribe regulatory components of the HPA axis was modified by global 5αR1 loss which may be the main mechanism underlying reduced HPA axis responses to acute stress seen previously in 5αR1-KO mice. Female 5αR1-KO mice were heavier than controls with increased adiposity in both strains whilst group-housed. Social isolation induced weight loss in this cohort, particularly in mice lacking 5αR1 which were also hypophagic when aged. There was evidence of sexual dimorphism whereby male 5αR1-KO mice were not heavier than controls despite hyperphagia. All 5αR1-KO mice showed fasting hyperinsulinaemia which was sufficient to maintain normal plasma glucose levels. Although 5αR1-KO mice were glucose intolerant, insulin tolerance to exogenous insulin treatment was intact.

In male 5αR1-KO mice, mRNA expression of hypothalamic feeding peptides was consistent with compensatory reductions in orexigenic proteins to either limit or reduce calorific consumption. No effect of genotype on mRNA expression of hypothalamic feeding peptides was observed in female mice. Nocturnal physical activity in the home cage was comparable between genotypes for both males and females however, under single housing and acute mild stress, socially isolated mice were more active which in part may explain the weight loss seen in these animals. Behaviourally, anxiety was only observed in female C57Bl/6j 5αR1-KOs when aged and singly-housed. This same group of animals also had an exaggeration of the normal cognitive decline seen with ageing. Intriguingly, many of the above phenotypes were modifiable by factors including strain, sex and age which is discussed further below.

7.2 The effect of strain

There were apparent effects of strain on parameters which contribute to HPA axial control. For example, *Crh* and *Crhr-1* mRNA was downregulated in the PVN and pituitary respectively of female mixed strain 5αR1-KO mice (consistent with reduced forward HPA drive) but in female C57Bl/6j 5αR1-KO mice, *Crh* was not different and *Crhr-1* was paradoxically increased. The latter is not consistent with reduced HPA axis responses and indeed, corticosterone release following acute stress was not different between genotypes of the C57Bl/6j strain. The male phenotype was also subject to modification by strain; mixed strain 5αR1-KO mice had dampened HPA axis stress responses most likely due to reduced *Crh* and the potentiating effects of *Avp* but corticosterone release following restraint was not affected by genotype in C57Bl/6j mice as in females. Moreover, mixed strain 5αR1-KO mice with reduced HPA axis responsiveness did not have gene expression adaptations completely consistent with glucocorticoid excess at the level of the HPA axis; again a phenotype modifiable by strain. *GR* mRNAs were upregulated in male mice lacking 5αR1 in both the hypothalamus and pituitary. In female 5αR1-KO mice, pituitary *GR* was downregulated which is potentially consistent with glucocorticoid excess in mixed strain animals but *GR* was only downregulated in the hypothalamus and not pituitary in C57Bl/6j mice. Therefore strain-related differences in the effect of 5αR1 loss on key regulatory features of the HPA axis have been highlighted here and would be an important consideration in future experiments.

We went on to demonstrate prolonged corticosterone clearance (males) and higher expression of *Hsd11b1* in mixed strain vs. C57Bl/6j mice. These may be important findings in explaining the strain related phenotypic differences and are suggestive of mixed strain mice being exposed to glucocorticoid levels in excess of those in C57Bl/6j mice thus rendering them more susceptible to the detrimental effects of 5αR1-KO loss. Figure 7.1 shows a schematic summary of the main results of this thesis and how they compared between mouse strains.

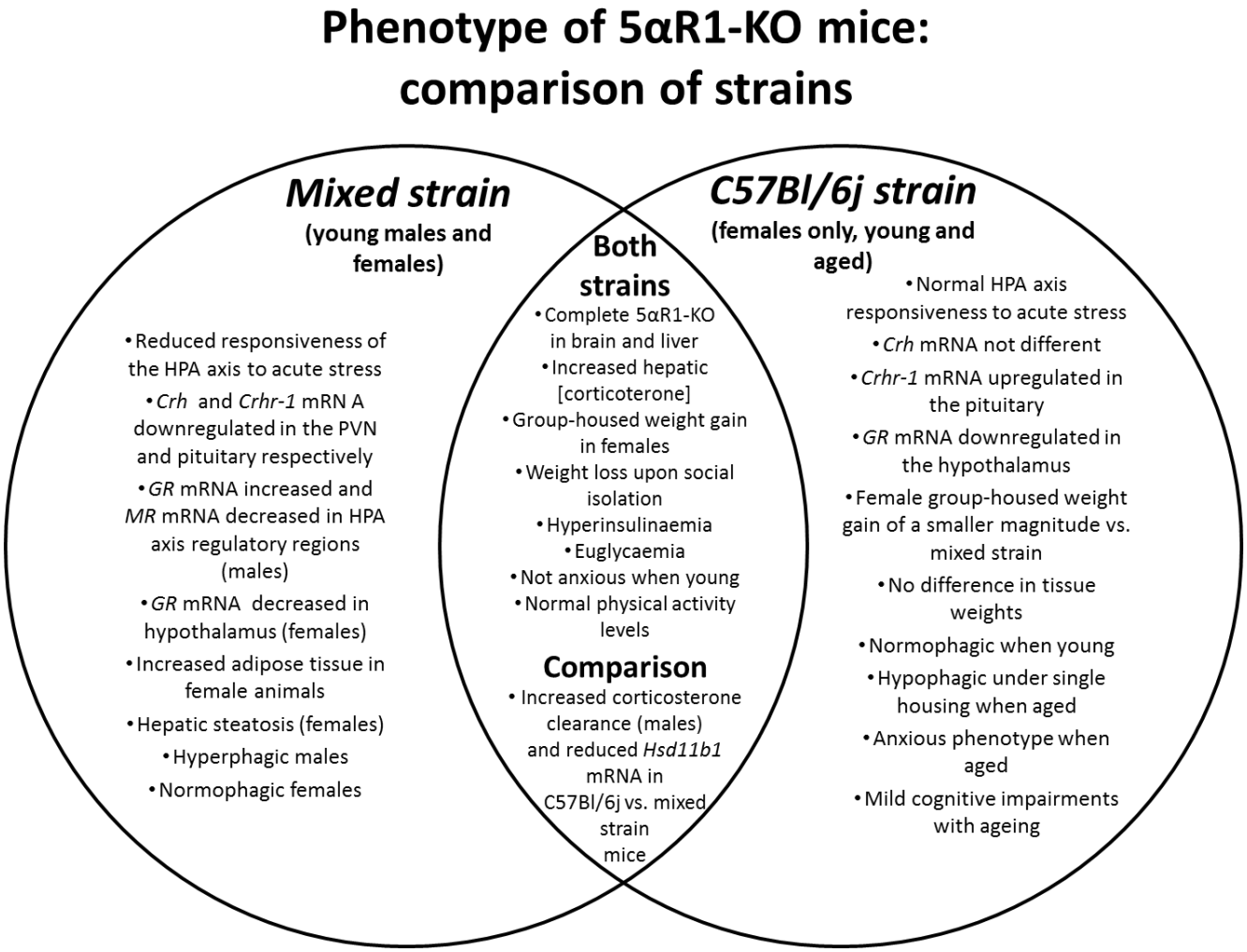

Figure 7.1 Summary of the 5αR1-KO phenotype and a comparison between the two mouse strains
Comparison of the 5αR1-KO metabolic, genetic and behavioural phenotypes in mixed strain and C57Bl/6j mice.

7.2.1 How important is genetic background?

In this study, we have highlighted the possibility of strain-specific 5αR1-KO phenotypes. We have also shown to some extent that mixed strain mice cleared glucocorticoids less efficiently which may be attributable to intrinsically lower 5αR1 activity in this particular mouse strain. Not only is this of importance during the planning of future investigations, but it also indicates the importance of understanding individual variations in 5αR1 activity which may exist in the human population e.g. ethnic origin. For example, individuals with low 5αR1 activity may be more susceptible to the effects of glucocorticoid excess, 5αR dysregulation or treatment with 5αRIs. Investigations into a link between ethnicity and the risk of developing drug-related side effects would be an important development in deciding whether the benefits of treatment actually out-weigh the risk of detrimental effects.

7.3 The effect of sex

Sex impacted upon a number of phenotypes throughout this study. Firstly, whilst female 5αR1-KO mice gained more weight, had heavier adipose depots and yet were normophagic vs. WT, there was no genotype difference in male mice, even though male 5αR1-KO mice were hyperphagic vs. WT. Moreover, male mice generally were hyperglycaemic and hyperinsulinaemic compared to females and in males, 5αR1-KO mice had higher fasting glucose and insulin levels vs. WT. It is known that female sex hormones e.g. oestrogen, can act protectively against the development of metabolic disorders including obesity and diabetes (Le May *et al.* 2006; Pettersson *et al.* 2012) therefore, it is not altogether surprising that our data show not only a poorer metabolic profile for males overall, but also for males lacking 5αR1 in particular.

HPA axial control also appears to be modified by 5αR1 differentially in the two sexes. Although in male 5αR1-KO mice, transcripts for *MR* in the hippocampus and *GR* in the pituitary were downregulated and upregulated respectively vs. WT, such effects were not seen in females. Moreover, a completely opposite effect of 5αR1 loss was seen in each sex in terms of *GR* mRNA abundance in the hypothalamus, where levels increased and decreased in male and female 5αR1-KO mice, respectively, in comparison to WT controls. Moreover, although *Crh* mRNA was

downregulated in the PVN in 5αR1-KO mice of both sexes, a corresponding decrease in *Crhr-1* mRNA was seen in female 5αR1-KO mice only. We, and others (Handa *et al.* 1994), have demonstrated higher plasma corticosterone concentrations in female mice overall i.e. both basally and post-stress. With testosterone and oestrogen playing significant roles in HPA axis regulation (suppression and activation, respectively; Handa *et al.* 1994), sex-specific differences in 5αR1-KO-induced modifications to essential components of the axis may be expected. Thus, not only glucocorticoid levels and therefore the degree of corticosteroid receptor activation within tissues, but also activation of testosterone and oestrogen receptors and their respective ligand concentrations could potentially be influenced by global 5αR1-loss, contributing to disruption of the HPA axis in 5αR1-KO mice (Handa *et al.* 2009).

7.4 The effect of ageing

One of the main aims of chapter 5 was to elucidate whether increasing age exacerbated any cognitive decline hypothesised to be associated with 5αR1-KO. In rodents, normal ageing is associated with a physiological rise in glucocorticoids (Sapolsky 1992) and, in rats, their levels correlate closely with the degree of hippocampal dysfunction and spatial learning impairment (Landfield *et al.* 1978; Landfield *et al.* 1981). Although 5αR1-KO mice were not cognitively impaired at 6 months of age, there was some evidence for a marginally exaggerated cognitive decline in 5αR1-KO mice vs. WT following ageing and re-testing at 14-15 months old. One potential hypothesis is that if indeed glucocorticoid metabolism is reduced in 5αR1-KO mice, this may lead to a greater than normal increase in corticosterone within the brain during the ageing process resulting in an exaggeration of the ageing phenotype. Although it was attempted, it was not possible to measure glucocorticoids in the brain and therefore this theory could not be adequately proven. However, there is some evidence for age-dependent changes in the activity of 5αR enzymes generally (Tunn *et al.* 1988). Moreover, the 5αR1/2 metabolite 5αDHT is implicated in the pathogenesis of benign prostatic hyperplasia (BPH; Marcelli *et al.* 1999); a disorder which is largely seen in ageing men which again may indicate age-related modifications in the activity of 5αR enzymes.

In addition, female, 5αR1-KO mice subjected to chronic social isolation stress were only anxious when aged and no such phenotype was seen in these mice when younger. This finding again suggests that ageing may provide an additive effect; potentially raising corticosterone levels to an even higher level than could be solely attributable to the loss of 5αR1 activity alone.

7.5 5αR1-KO mice as a model of glucocorticoid excess

Here we have shown phenotypes which are consistent with glucocorticoid excess, particularly in the brain. For example, Cushing's syndrome is associated with an increased incidence of psychiatric disturbances; most commonly anxiety and depression (Haskett 1985; Dorn *et al.* 1995; Kelly 1996; Kelly *et al.* 1996; Ragnarsson *et al.* 2012), but there are also associations with cognitive impairment (Toffanin *et al.* 2011; Ragnarsson *et al.* 2012). 5αR1-KO mice were indeed mildly anxious and marginally cognitively impaired. However, the fact that these phenotypes were only apparent after ageing and during social isolation stress suggests only a subtle effect of glucocorticoid excess and that their levels may only be mildly increased. Indeed, single housing itself is a known chronic stressor and glucocorticoids increase with age in both humans and rodents (Meaney *et al.* 1992; Sapolsky 1992; Lupien *et al.* 1994; Montaron *et al.* 2006), hence the additive effect may be detrimental.

The expression of corticosteroid receptors in 5αR1-KO mice was not entirely consistent with glucocorticoid excess and shows rather strong sexual dimorphism. Therefore, it is possible that concomitant to increased glucocorticoids, the phenotype resulting from 5αR1 deficiency may be due to reduced biosynthesis of a number of other steroids, in particular neurosteroids such as ALLO within the brain. It is plausible that the increased susceptibility of female 5αR1-KO mice to CNS disturbances as well as some of the sexual dimorphism seen with many of the phenotypes, may in part be attributable to reduced ALLO production by 5αR1, especially in relation to the neurosteroids anxiolytic and neuroprotective properties (Rupprecht *et al.* 1999; Khisti *et al.* 2002; Ghoumari *et al.* 2003; Ahmad *et al.* 2005;

Ugale *et al.* 2007). Equally compelling is the fact that ALLO is reduced following social isolation (Matsumoto *et al.* 1999; Dong *et al.* 2001) which would have a similar effect to raised corticosterone. Future studies could look at ALLO levels locally within the brain (possibly even the amygdala specifically) via microdialysis techniques as well as ALLO replacement therapy to determine if such treatment may "rescue" the anxious phenotype.

Metabolically, the 5αR1-KO mouse was a model consistent with glucocorticoid excess. In humans, glucocorticoid excess can occur as a result of pituitary/adrenal tumours or treatment with synthetic glucocorticoids for inflammatory conditions giving rise to metabolic and cardiovascular disturbances including dyslipidaemia, insulin resistance and type II diabetes mellitus, central obesity, CVD and hypertension (Schacke *et al.* 2002; Walker 2007); the collective name for which is often termed "the metabolic syndrome". Despite striking symptomatic crossover between patients with Cushing's syndrome and those with metabolic syndrome, one important difference is that Cushing's syndrome is associated with pronounced glucocorticoid hypersecretion (Boscaro *et al.* 2001) whilst the metabolic syndrome does not present with elevated circulating glucocorticoids (Walker 2007). Broadly speaking, 5αR1-KO mice showed features of the metabolic syndrome consistent with tissue glucocorticoid excess but in the absence of raised corticosterone in the circulation. Furthermore, we showed that metabolic symptoms were sexually dimorphic and were also subject to modification by strain. Male mice lacking 5αR1 were more susceptible to some metabolic disturbances such as hyperglycaemia, hyperinsulinaemia and hyperphagia whilst females were susceptible to others including obesity and hepatic steatosis. In the absence of raised glucocorticoids in the circulation, it is likely local increases within tissues such as the liver, brain and adipose tissue may drive such metabolic dysfunction. During this study it was not possible to detect corticosterone in the brain but hepatic levels were increased in female 5αR1-KO mice following chronic corticosterone infusion. Future studies may be directed towards quantifying corticosterone in the adipose tissue as well as tissue levels of other 5αR1 metabolites/substrates such as testosterone, 5αDHT, progesterone and 5αDHP as well as ALLO.

7.6 Disruption of 5αR in humans

7.6.1 Pharmacological

Drugs targeted to inhibit 5αRs (5αRIs) such as dutasteride (dual 5αR1 and 5αR2 inhibitor (Bramson *et al.* 1997) and finasteride (largely 5αR2 but also weakly inhibits 5αR1; Liang *et al.* 1985; Andersson *et al.* 1990; Russell *et al.* 1994) are commonly used for the treatment of BPH to reduce intraprostatic concentrations of 5α-DHT (Gormley *et al.* 1992; Roehrborn *et al.* 2002; Roehrborn *et al.* 2004). However, their use has been implicated with an increased incidence of depression and anxiety (Altomare *et al.* 2002; Rahimi-Ardabili *et al.* 2006). Most studies which have been done to assess CNS effects of 5αRIs, have focussed on the use of finasteride (which readily crosses the BBB) and its ability to reduce androgen and neurosteroid production by 5αR2. However, here we did not detect 5αR2 in the brain showing loss of 5αR1 can specifically mediate anxiety and cognitive deficiencies in 5αR1-KO mice.

Moreover, the synthesis of neurosteroids which are produced from adrenal or gonadal steroids in the brain (Stoffel-Wagner 2003 for reviews; Dubrovsky 2006) requires 5αR activity and finasteride has been shown to diminish neurosteroid biosynthesis (Purdy *et al.* 1991; Finn *et al.* 2004). Not only are neurosteroids such as ALLO anxiolytic and anti-psychotic (Rupprecht *et al.* 1999; Khisti *et al.* 2002; Ugale *et al.* 2007), they also have neuroprotective and neurogenesis properties (Ghoumari *et al.* 2003; Ahmad *et al.* 2005). Therefore, whilst it is clear that CNS effects in 5αR1-KO mice are indeed mediated by the type 1 isozyme (if due to local as opposed to peripheral activity), it is unknown whether increased glucocorticoids, reduced neurosteroids, reduced androgens or a combination of all three underlies anxiety and cognitive decline in this study.

One final consideration with reference to the use of 5αRIs, is the effect of 5αR3 blockade. Currently, the efficacy of 5αRIs against the type 3 isozyme is less well studied but finasteride and dutasteride potently inhibit 5αR3 activity *in vitro* (Yamana *et al.* 2010). However, although 5αR3 is not thought to contribute to

glucocorticoid clearance, there are suggestions of its contribution to the reduction of androgens and neurosteroids (Cantagrel *et al.* 2010; Azzouni *et al.* 2012).

7.6.2 Genetic

7.6.2.1 5αR deficiency

Human 5αR deficiencies are described mainly for mutations in the *SRD5A2* gene which are inherited in an autosomal recessive nature (Thigpen *et al.* 1992); none are reported for *SRD5A1*. Although, *SRD5A2* deficiency is mostly studied in the context of androgens and a lack of 5αDHT production (Moore *et al.* 1976; Andersson *et al.* 1991; Jenkins *et al.* 1992; Wilson *et al.* 1993), the tissue distribution of 5αR2 in humans (liver and some evidence for expression in the brain) and its capacity to metabolise androgens and glucocorticoids suggests 5αR2-deficient individuals may have some degree of symptoms associated with impaired glucocorticoid clearance in the liver and brain e.g. metabolic and behavioural disturbances, but such effects are yet to be documented.

7.6.3 5αR up-regulation

In both men and women, conditions such as obesity and insulin resistance correlate with elevated 5αR activity (Andrew *et al.* 1998; Rask *et al.* 2002; Tomlinson *et al.* 2008). In women, increased 5αR activity is often associated with polycystic ovarian syndrome (PCOS; Vassiliadi *et al.* 2009). However, since these studies measure only urinary steroid metabolite excretion, it is impossible to delineate 1) whether both 5αR1 and 5αR2 contribute to the effects of increased 5αR metabolites, and 2) whether 5αR upregulation shows tissue specificity. In this study we showed a lack of 5αR1 also resulted in metabolic disturbances such as weight gain and hyperinsulinaemia, so it is possible that up-regulation in metabolic conditions is a protective mechanism. Lastly, monitoring the contribution of increased/decreased androgen activation or neurosteroid biosynthesis in future studies; both clinical and pre-clinical, may be warranted.

7.6.4 Single nucleotide polymorphisms (SNPs)

In the human population there are also instances of single nucleotide polymorphisms (SNPs) in the *SRD5A1* gene. As with 5αR2 deficiency, these are most often associated with the implications for reduced sex steroid metabolism (Klotsman *et al.* 2004; Setlur *et al.* 2010; Hein *et al.* 2012), however, in our 5αR1-KO mouse model there is evidence of the enzyme's role in the pathogenesis of both metabolic and CNS disorders. Therefore, it is probable that deactivating SNPs in the human *SRD5A1* gene may have effects over and above those associated with reduced sex steroid reduction.

7.7 Future experiments and further work

Future studies would first and foremost be directed towards quantifying corticosterone levels in tissues including adipose tissue and the brain to ascertain the degree of glucocorticoid excess in 5αR1-KO mice. This could be done by steroid extraction followed by LC-MS/MS and possibly also microdialysis techniques to allow measurements from within the brains of live animals at selected time points. Additionally, the interpretation of further studies would be enhanced by measuring intra-tissue levels of other 5αR1 metabolites/substrates including testosterone, 5αDHT, progesterone, 5αDHP and in particular ALLO. From the current data it cannot be concluded whether anxiety and cognitive deficits in aged, socially isolated 5αR1-KO mice are due to increased corticosterone or reduced ALLO in the brain or both. If 5αR1-KO mice were found to have reduced neurosteroid biosynthesis within the brain, ALLO replacement studies may also be carried out to see if the social isolation phenotype can be reversed.

From a behavioural aspect, future ageing studies would ideally be carried out in mice who have been aged beyond the time point specified here, for example it may be optimal to test mice when young (~6 months), middle-aged (~12 months) and then considerably aged (~24 months) to optimise the phenotypic result. Additionally, it may be interesting to assess 5αR1-KO mice for depression in further studies. Investigations could incorporate the use of such tests including the forced swim test

and/or the tail suspension test commonly used to determine the efficacy of anti-depressants in pre-clinical studies using animal models of depression.

To continue assessment of the 5αR1-KO metabolic phenotype, it would be useful to evaluate the basal metabolic rates of these animals in future studies by measuring their respiratory quotients and core body temperatures especially since 11βHSD1-KO mice have increased core temperatures (Morton et al. 2004).

Finally, to complement genetic studies and further validate the deleterious effects of glucocorticoid excess in neuronal circuits related to appetite, metabolic homeostasis, stress responses and behaviour with ageing, 5αR1 could be inhibited pharmacologically through the use of inhibitors such dutasteride in future *in vivo* studies. Following drug treatment, which could be delivered for example by osmotic mini pump, s.c. injection or even orally, drug brain levels could be measured by LC-MS/MS to determine BBB penetration. Tissue (brain/liver/adipose) glucocorticoid levels and urinary 5α metabolite excretion could also be quantified to determine the extent to which drugs such as finasteride and dutasteride inhibit glucocorticoid clearance both centrally and peripherally.

7.8 Concluding remarks

A-ring reduction of steroid hormones including glucocorticoids but potentially other steroids also e.g. progesterone and testosterone, is important in maintaining not only metabolic but also CNS function. Here we have shown a fairly novel role for 5αR1 in determining the outcome of chronic stress and ageing whereby it appears that the activity of the enzyme is somewhat protective under such circumstances. Therefore, metabolic and CNS side effects in, for example, aged men treated with 5αRIs may in future be more closely monitored in terms of anxiety and accelerated cognitive decline and also features of the Metabolic Syndrome during treatment for BPH.

References

Agis-Balboa, R. C., Pinna, G., Pibiri, F., Kadriu, B., Costa, E. and Guidotti, A. (2007). "Down-regulation of neurosteroid biosynthesis in corticolimbic circuits mediates social isolation-induced behavior in mice." *Proc Natl Acad Sci U S A* **104**(47): 18736-18741.

Ahima, R. S. and Harlan, R. E. (1990). "Charting of type ii glucocorticoid receptor-like immunoreactivity in the rat central nervous system." *Neuroscience* **39**(3): 579-604.

Ahima, R. S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E. and Flier, J. S. (1996). "Role of leptin in the neuroendocrine response to fasting." *Nature* **382**(6588): 250-252.

Ahmad, I., Lope-Piedrafita, S., Bi, X., Hicks, C., Yao, Y., Yu, C., Chaitkin, E., Howison, C. M., Weberg, L., Trouard, T. P. and Erickson, R. P. (2005). "Allopregnanolone treatment, both as a single injection or repetitively, delays demyelination and enhances survival of niemann-pick c mice." *J Neurosci Res* **82**(6): 811-821.

Akabayashi, A., Watanabe, Y., Wahlestedt, C., Mcewen, B. S., Paez, X. and Leibowitz, S. F. (1994). "Hypothalamic neuropeptide y, its gene expression and receptor activity: Relation to circulating corticosterone in adrenalectomized rats." *Brain Res* **665**(2): 201-212.

Akana, S. F. and Dallman, M. F. (1997). "Chronic cold in adrenalectomized, corticosterone (b)-treated rats: Facilitated corticotropin responses to acute restraint emerge as b increases." *Endocrinology* **138**(8): 3249-3258.

Akana, S. F., Strack, A. M., Hanson, E. S. and Dallman, M. F. (1994). "Regulation of activity in the hypothalamo-pituitary-adrenal axis is integral to a larger hypothalamic system that determines caloric flow." *Endocrinology* **135**(3): 1125-1134.

Al-Dujaili, E. A. and Edwards, C. R. (1981). "Development and application of a simple radioimmunoassay for urinary aldosterone." *Clinica chimica acta; international journal of clinical chemistry* **116**(3): 277-287.

Alfarez, D. N., Karst, H., Velzing, E. H., Joels, M. and Krugers, H. J. (2008). "Opposite effects of glucocorticoid receptor activation on hippocampal ca1 dendritic complexity in chronically stressed and handled animals." *Hippocampus* **18**(1): 20-28.

Alibhai, S. M., Duong-Hua, M., Sutradhar, R., Fleshner, N. E., Warde, P., Cheung, A. M. and Paszat, L. F. (2009). "Impact of androgen deprivation therapy on cardiovascular disease and diabetes." *J Clin Oncol* **27**(21): 3452-3458.

Alleva, E., Petruzzi, S., Cirulli, F. and Aloe, L. (1996). "Ngf regulatory role in stress and coping of rodents and humans." *Pharmacol Biochem Behav* **54**(1): 65-72.

Aloe, L., Alleva, E. and Fiore, M. (2002). "Stress and nerve growth factor: Findings in animal models and humans." *Pharmacol Biochem Behav* **73**(1): 159-166.

Altomare, G. and Capella, G. L. (2002). "Depression circumstantially related to the administration of finasteride for androgenetic alopecia." *J Dermatol* **29**(10): 665-669.

Amory, J. K., Wang, C., Swerdloff, R. S., Anawalt, B. D., Matsumoto, A. M., Bremner, W. J., Walker, S. E., Haberer, L. J. and Clark, R. V. (2007). "The effect of 5alpha-reductase inhibition with dutasteride and finasteride on semen parameters and serum hormones in healthy men." *J Clin Endocrinol Metab* **92**(5): 1659-1665.

Andersson, S., Berman, D. M., Jenkins, E. P. and Russell, D. W. (1991). "Deletion of steroid 5 alpha-reductase 2 gene in male pseudohermaphroditism." *Nature* **354**(6349): 159-161.

Andersson, S., Bishop, R. W. and Russell, D. W. (1989). "Expression cloning and regulation of steroid 5 alpha-reductase, an enzyme essential for male sexual differentiation." *J Biol Chem* **264**(27): 16249-16255.

Andersson, S. and Russell, D. W. (1990). "Structural and biochemical properties of cloned and expressed human and rat steroid 5 alpha-reductases." *Proc Natl Acad Sci U S A* **87**(10): 3640-3644.

Ando, S., Canonaco, M., Valenti, A., Aquila, S., Tavolaro, R., Maggiolini, M., Panno, M. L. and Dessi-Fulgheri, F. (1989). "The in vitro conversion of 3h androstenedione to testosterone and dihydrotestosterone in the adrenal gland of castrated male rat: Influence of gonadal steroid administration." *Exp Clin Endocrinol* **93**(1): 83-89.

Andreatini, R. and Leite, J. R. (1994). "Evidence against the involvement of acth/crf release or corticosteroid receptors in the anxiolytic effect of corticosterone." *Braz J Med Biol Res* **27**(5): 1237-1241.

Andrew, R., Phillips, D. I. and Walker, B. R. (1998). "Obesity and gender influence cortisol secretion and metabolism in man." *J Clin Endocrinol Metab* **83**(5): 1806-1809.

Andrew, R. and Walker, B. R. (2002). "Glucocorticoid metabolism in health and disease." *Recent Res Dev in Endocrinol*(3): 425-449.

Anisman, H., Lacosta, S., Kent, P., Mcintyre, D. C. and Merali, Z. (1998). "Stressor-induced corticotropin-releasing hormone, bombesin, acth and corticosterone variations in strains of mice differentially responsive to stressors." *Stress* **2**(3): 209-220.

Antoniades, H. N., Renold, A. E., Dagenais, Y. M. and Steinke, J. (1960). "Preliminary observations on state of insulin in human and bovine pancreas." *Proc Soc Exp Biol Med* **103**: 677-679.

Aoyama, T., Yamano, S., Waxman, D. J., Lapenson, D. P., Meyer, U. A., Fischer, V., Tyndale, R., Inaba, T., Kalow, W. and Gelboin, H. V. (1989). "Cytochrome p-450 hpcn3, a novel cytochrome p-450 iiia gene product that is differentially expressed in adult human liver. Cdna and deduced amino acid sequence and distinct specificities of cdna-expressed hpcn1 and hpcn3 for the metabolism of steroid hormones and cyclosporine." *J Biol Chem* **264**(18): 10388-10395.

Archer, J. (1975). "Rodent sex differences in emotional and related behavior." *Behav Biol* **14**(4): 451-479.

Armario, A., Gavalda, A. and Marti, J. (1995). "Comparison of the behavioural and endocrine response to forced swimming stress in five inbred strains of rats." *Psychoneuroendocrinology* **20**(8): 879-890.

Arriza, J. L., Simerly, R. B., Swanson, L. W. and Evans, R. M. (1988). "The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response." *Neuron* **1**(9): 887-900.

Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E. and Evans, R. M. (1987). "Cloning of human mineralocorticoid receptor complementary DNA: Structural and functional kinship with the glucocorticoid receptor." *Science* **237**(4812): 268-275.

Arvat, E., Maccagno, B., Giordano, R., Pellegrino, M., Broglio, F., Gianotti, L., Maccario, M., Camanni, F. and Ghigo, E. (2001). "Mineralocorticoid receptor blockade by canrenoate increases both spontaneous and stimulated adrenal function in humans." *J Clin Endocrinol Metab* **86**(7): 3176-3181.

Azzouni, F., Godoy, A., Li, Y. and Mohler, J. (2012). "The 5 alpha-reductase isozyme family: A review of basic biology and their role in human diseases." *Advances in urology* **2012**: 530121-530152.

Bado, A., Levasseur, S., Attoub, S., Kermorgant, S., Laigneau, J. P., Bortoluzzi, M. N., Moizo, L., Lehy, T., Guerre-Millo, M., Le Marchand-Brustel, Y. and Lewin, M. J. (1998). "The stomach is a source of leptin." *Nature* **394**(6695): 790-793.

Bale, T. L., Giordano, F. J., Hickey, R. P., Huang, Y., Nath, A. K., Peterson, K. L., Vale, W. W. and Lee, K. F. (2002). "Corticotropin-releasing factor receptor 2 is a tonic suppressor of vascularization." *Proc Natl Acad Sci U S A* **99**(11): 7734-7739.

Bale, T. L., Picetti, R., Contarino, A., Koob, G. F., Vale, W. W. and Lee, K. F. (2002). "Mice deficient for both corticotropin-releasing factor receptor 1 (crfr1) and crfr2 have an impaired stress response and display sexually dichotomous anxiety-like behavior." *J Neurosci* **22**(1): 193-199.

Barat, P., Livingstone, D. E., Elferink, C. M., Mcdonnell, C. R., Walker, B. R. and Andrew, R. (2007). "Effects of gonadectomy on glucocorticoid metabolism in obese zucker rats." *Endocrinology* **148**(10): 4836-4843.

Barker, D. J., Bull, A. R., Osmond, C. and Simmonds, S. J. (1990). "Fetal and placental size and risk of hypertension in adult life." *BMJ* **301**(6746): 259-262.

Barker, D. J., Gluckman, P. D., Godfrey, K. M., Harding, J. E., Owens, J. A. and Robinson, J. S. (1993). "Fetal nutrition and cardiovascular disease in adult life." *Lancet* **341**(8850): 938-941.

Barker, D. J. and Osmond, C. (1986). "Infant mortality, childhood nutrition, and ischaemic heart disease in england and wales." *Lancet* **1**(8489): 1077-1081.

Barnes, P. J. (1998). "Anti-inflammatory actions of glucocorticoids: Molecular mechanisms." *Clin Sci (Lond)* **94**(6): 557-572.

Bartholome, B., Spies, C. M., Gaber, T., Schuchmann, S., Berki, T., Kunkel, D., Bienert, M., Radbruch, A., Burmester, G. R., Lauster, R., Scheffold, A. and Buttgereit, F. (2004). "Membrane glucocorticoid receptors (mgcr) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis." *FASEB J* **18**(1): 70-80.

Bartke, A., Steele, R. E., Musto, N. and Caldwell, B. V. (1973). "Fluctuations in plasma testosterone levels in adult male rats and mice." *Endocrinology* **92**(4): 1223-1228.

Bartsch, G., Rittmaster, R. S. and Klocker, H. (2002). "Dihydrotestosterone and the concept of 5alpha-reductase inhibition in human benign prostatic hyperplasia." *World J Urol* **19**(6): 413-425.

Basaria, S., Muller, D. C., Carducci, M. A., Egan, J. and Dobs, A. S. (2006). "Hyperglycemia and insulin resistance in men with prostate carcinoma who receive androgen-deprivation therapy." *Cancer* **106**(3): 581-588.

Baskin, D. G., Figlewicz Lattemann, D., Seeley, R. J., Woods, S. C., Porte, D., Jr. and Schwartz, M. W. (1999). "Insulin and leptin: Dual adiposity signals to the brain for the regulation of food intake and body weight." *Brain Res* **848**(1-2): 114-123.

Bates, A., Adels, L. and Leon, M. (1985). "Thermal control of maternal contact bouts: The interbout interval." *Physiol Behav* **34**(5): 835-837.

Baudrand, R., Dominguez, J. M., Carvajal, C. A., Riquelme, A., Campino, C., Macchiavello, S., Bozinovic, M., Morales, M., Pizarro, M., Solis, N., Escalona, A., Boza, C., Arrese, M. and Fardella, C. E. (2011). "Overexpression of hepatic 5alpha-reductase and 11beta-hydroxysteroid dehydrogenase type 1 in visceral adipose tissue is associated with hyperinsulinemia in morbidly obese patients." *Metabolism* **60**(12): 1775-1780.

Baulieu, E. E. and Robel, P. (1990). "Neurosteroids: A new brain function?" *J Steroid Biochem Mol Biol* **37**(3): 395-403.

Beato, M., Herrlich, P. and Schutz, G. (1995). "Steroid hormone receptors: Many actors in search of a plot." *Cell* **83**(6): 851-857.

Beck, I. M., Vanden Berghe, W., Vermeulen, L., Yamamoto, K. R., Haegeman, G. and De Bosscher, K. (2009). "Crosstalk in inflammation: The interplay of glucocorticoid receptor-based mechanisms and kinases and phosphatases." *Endocr Rev* **30**(7): 830-882.

Benoit, S. C., Air, E. L., Coolen, L. M., Strauss, R., Jackman, A., Clegg, D. J., Seeley, R. J. and Woods, S. C. (2002). "The catabolic action of insulin in the brain is mediated by melanocortins." *J Neurosci* **22**(20): 9048-9052.

Bentson, J., Reza, M., Winter, J. and Wilson, G. (1978). "Steroids and apparent cerebral atrophy on computed tomography scans." *J Comput Assist Tomogr* **2**(1): 16-23.

Beracochea, D., Coutan, M., Tronche, C., Dorey, R., Chauveau, F. and Pierard, C. (2011). "Interaction between diazepam and hippocampal corticosterone after acute stress: Impact on memory in middle-aged mice." *Front Behav Neurosci* **5**: 14.

Berg, J. M. (1989). "DNA binding specificity of steroid receptors." *Cell* **57**(7): 1065-1068.

Berger, S., Wolfer, D. P., Selbach, O., Alter, H., Erdmann, G., Reichardt, H. M., Chepkova, A. N., Welzl, H., Haas, H. L., Lipp, H. P. and Schutz, G. (2006). "Loss of the limbic mineralocorticoid receptor impairs behavioral plasticity." *Proc Natl Acad Sci U S A* **103**(1): 195-200.

Berséus, O. (1967). "Conversion of cholesterol to bile acids in rat: Purification and properties of a δ4-3-ketosteroid 5β-reductase and a 3α-hydroxysteroid dehydrogenase." *Eur J Biochem* **2**(4): 493-502.

Berseus, O., Danielsson, H. and Kallner, A. (1965). "Synthesis and metabolism of cholest-4-ene-7-alpha,12-alpha-diol-3-one and 5-beta-cholestane-7-alpha,12-alpha-diol-3-one. Bile acids and steroids 153." *J Biol Chem* **240**: 2396-2401.

Bertilsson, G., Heidrich, J., Svensson, K., Asman, M., Jendeberg, L., Sydow-Backman, M., Ohlsson, R., Postlind, H., Blomquist, P. and Berkenstam, A. (1998). "Identification of a human nuclear receptor defines a new signaling pathway for cyp3a induction." *Proc Natl Acad Sci U S A* **95**(21): 12208-12213.

Beyer, H. S., Matta, S. G. and Sharp, B. M. (1988). "Regulation of the messenger ribonucleic acid for corticotropin-releasing factor in the paraventricular nucleus and other brain sites of the rat." *Endocrinology* **123**(4): 2117-2123.

Bhagwagar, Z., Hafizi, S. and Cowen, P. J. (2005). "Increased salivary cortisol after waking in depression." *Psychopharmacology (Berl)* **182**(1): 54-57.

Bingaman, E. W., Magnuson, D. J., Gray, T. S. and Handa, R. J. (1994). "Androgen inhibits the increases in hypothalamic corticotropin-releasing hormone (crh) and crh-immunoreactivity following gonadectomy." *Neuroendocrinology* **59**(3): 228-234.

Bitran, D., Kellogg, C. K. and Hilvers, R. J. (1993). "Treatment with an anabolic-androgenic steroid affects anxiety-related behavior and alters the sensitivity of cortical gaba(a) receptors in the rat." *Horm Behav* **27**(4): 568-583.

Bledsoe, R. K., Montana, V. G., Stanley, T. B., Delves, C. J., Apolito, C. J., Mckee, D. D., Consler, T. G., Parks, D. J., Stewart, E. L., Willson, T. M., Lambert, M. H., Moore, J. T., Pearce, K. H. and Xu, H. E. (2002). "Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition." *Cell* **110**(1): 93-105.

Bliss, T. V. P. and Collingridge, G. L. (1993). "A synaptic model of memory: Long-term potentiation in the hippocampus." *Nature* **361**(6407): 31-39.

Boscaro, M., Barzon, L., Fallo, F. and Sonino, N. (2001). "Cushing's syndrome." *Lancet* **357**(9258): 783-791.

Boyle, M. P., Brewer, J. A., Funatsu, M., Wozniak, D. F., Tsien, J. Z., Izumi, Y. and Muglia, L. J. (2005). "Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior." *Proc Natl Acad Sci U S A* **102**(2): 473-478.

Boyle, M. P., Kolber, B. J., Vogt, S. K., Wozniak, D. F. and Muglia, L. J. (2006). "Forebrain glucocorticoid receptors modulate anxiety-associated locomotor activation and adrenal responsiveness." *J. Neurosci.* **26**(7): 1971-1978.

Bradbury, M. J., Akana, S. F. and Dallman, M. F. (1994). "Roles of type i and ii corticosteroid receptors in regulation of basal activity in the hypothalamo-pituitary-adrenal axis during the diurnal trough and the peak: Evidence for a nonadditive effect of combined receptor occupation." *Endocrinology* **134**(3): 1286-1296.

Braga-Basaria, M., Dobs, A. S., Muller, D. C., Carducci, M. A., John, M., Egan, J. and Basaria, S. (2006). "Metabolic syndrome in men with prostate cancer undergoing long-term androgen-deprivation therapy." *J Clin Oncol* **24**(24): 3979-3983.

Bramson, H. N., Hermann, D., Batchelor, K. W., Lee, F. W., James, M. K. and Frye, S. V. (1997). "Unique preclinical characteristics of gg745, a potent dual inhibitor of 5ar." *J Pharmacol Exp Ther* **282**(3): 1496-1502.

Bray, G. A. and York, D. A. (1979). "Hypothalamic and genetic obesity in experimental animals: An autonomic and endocrine hypothesis." *Physiol Rev* **59**(3): 719-809.

Brett, L. P., Chong, G. S., Coyle, S. and Levine, S. (1983). "The pituitary-adrenal response to novel stimulation and ether stress in young adult and aged rats." *Neurobiol Aging* **4**(2): 133-138.

Brett, L. P., Levine, R. and Levine, S. (1986). "Bidirectional responsiveness of the pituitary-adrenal system in old and young male and female rats." *Neurobiol Aging* **7**(3): 153-159.

Brett, R. R. and Pratt, J. A. (1990). "Chronic handling modifies the anxiolytic effect of diazepam in the elevated plus-maze." *Eur J Pharmacol* **178**(1): 135-138.

Breuner, C. W. and Orchinik, M. (2002). "Plasma binding proteins as mediators of corticosteroid action in vertebrates." *J Endocrinol* **175**(1): 99-112.

Britton, D. R., Koob, G. F., Rivier, J. and Vale, M. (1982). "Intraventricular corticotropin-releasing factor enhances behavioral effects of novelty." *Life Sci* **31**(4): 363-367.

Brown, R. W., Diaz, R., Robson, A. C., Kotelevtsev, Y. V., Mullins, J. J., Kaufman, M. H. and Seckl, J. R. (1996). "The ontogeny of 11 beta-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development." *Endocrinology* **137**(2): 794-797.

Buchanan, T. W. (2007). "Retrieval of emotional memories." *Psychol Bull* **133**(5): 761-779.

Buckingham, J. C. (2006). "Glucocorticoids: Exemplars of multi-tasking." *Br J Pharmacol* **147 Suppl 1**: S258-268.

Buckingham, J. C., Solito, E., John, C., Tierney, T., Taylor, A., Flower, R., Christian, H. and Morris, J. (2003). "Annexin 1: A paracrine/juxtacrine mediator of glucorticoid action in the neuroendocrine system." *Cell Biochem Funct* **21**(3): 217-221.

Burgess, L. H. and Handa, R. J. (1992). "Chronic estrogen-induced alterations in adrenocorticotropin and corticosterone secretion, and glucocorticoid receptor-mediated functions in female rats." *Endocrinology* **131**(3): 1261-1269.

Burke, H. M., Davis, M. C., Otte, C. and Mohr, D. C. (2005). "Depression and cortisol responses to psychological stress: A meta-analysis." *Psychoneuroendocrinology* **30**(9): 846-856.

Burns, G., Almeida, O. F. X., Passarelli, F. and Herz, A. (1989). "A two-step mechanism by which corticotropin- releasing hormone releases hypothalamic β-endorphin: The role of vasopressin and g-proteins." *Endocrinology* **125**(3): 1365-1372.

Burnstein, K. L. and Cidlowski, J. A. (1992). "The down side of glucocorticoid receptor regulation." *Mol Cell Endocrinol* **83**(1): C1-8.

Burton, A. F. and Turnell, R. W. (1968). "11-dehydrocorticosteroids in tissues of mice." *Can J Biochem* **46**(5): 497-502.

Campbell, J. S. and Karavolas, H. J. (1989). "The kinetic mechanism of the hypothalamic progesterone 5 alpha-reductase." *J Steroid Biochem* **32**(2): 283-289.

Cantagrel, V., Lefeber, D. J., Ng, B. G., Guan, Z., Silhavy, J. L., Bielas, S. L., Lehle, L., Hombauer, H., Adamowicz, M., Swiezewska, E., De Brouwer, A. P., Blumel, P., Sykut-Cegielska, J., Houliston, S., Swistun, D., Ali, B. R., Dobyns, W. B., Babovic-Vuksanovic, D., Van Bokhoven, H., Wevers, R. A., Raetz, C. R., Freeze, H. H., Morava, E., Al-Gazali, L. and Gleeson, J. G. (2010). "Srd5a3 is required for converting polyprenol to dolichol and is mutated in a congenital glycosylation disorder." *Cell* **142**(2): 203-217.

Carlsson, P. O., Andersson, A. and Jansson, L. (1996). "Pancreatic islet blood flow in normal and obese-hyperglycemic (ob/ob) mice." *Am J Physiol* **271**(6 Pt 1): E990-995.

Carroll, B. J., Curtis, G. C. and Mendels, J. (1976). "Neuroendocrine regulation in depression. Ii. Discrimination of depressed from nondepressed patients." *Arch Gen Psychiatry* **33**(9): 1051-1058.

Carson, C., 3rd and Rittmaster, R. (2003). "The role of dihydrotestosterone in benign prostatic hyperplasia." *Urology* **61**(4 Suppl 1): 2-7.

Carter, R. N., Paterson, J. M., Tworowska, U., Stenvers, D. J., Mullins, J. J., Seckl, J. R. and Holmes, M. C. (2009). "Hypothalamic-pituitary-adrenal axis abnormalities in response to deletion of 11β-hsd1 is strain-dependent." *J Neuroendocrinol* **21**(11): 879-887.

Castonguay, T. W. (1991). "Glucocorticoids as modulators in the control of feeding." *Brain Res Bull* **27**(3-4): 423-428.

Cavagnini, F., Croci, M., Putignano, P., Petroni, M. L. and Invitti, C. (2000). "Glucocorticoids and neuroendocrine function." *Int J Obes Relat Metab Disord* **24 Suppl 2**: S77-79.

Celotti, F., Melcangi, R. C., Negri-Cesi, P., Ballabio, M. and Martini, L. (1987). "Differential distribution of the 5-alpha-reductase in the central nervous system of the rat and the mouse: Are the white matter structures of the brain target tissue for testosterone action?" *J Steroid Biochem* **26**(1): 125-129.

Challis, B. G., Luan, J., Keogh, J., Wareham, N. J., Farooqi, I. S. and O'rahilly, S. (2004). "Genetic variation in the corticotrophin-releasing factor receptors: Identification of single-nucleotide polymorphisms and association studies with obesity in uk caucasians." *Int J Obes Relat Metab Disord* **28**(3): 442-446.

Chao, H. M., Choo, P. H. and Mcewen, B. S. (1989). "Glucocorticoid and mineralocorticoid receptor mrna expression in rat brain." *Neuroendocrinology* **50**(4): 365-371.

Charney, D. S. (2004). "Psychobiological mechanisms of resilience and vulnerability: Implications for successful adaptation to extreme stress." *Am J Psychiatry* **161**(2): 195-216.

Chavez, M., Seeley, R. J., Green, P. K., Wilkinson, C. W., Schwartz, M. W. and Woods, S. C. (1997). "Adrenalectomy increases sensitivity to central insulin." *Physiol Behav* **62**(3): 631-634.

Chefer, V. I., Thompson, A. C., Zapata, A. and Shippenberg, T. S. (2009). "Overview of brain microdialysis." *Curr Protoc Neurosci* **7**: 7.1.

Chen, A. S., Marsh, D. J., Trumbauer, M. E., Frazier, E. G., Guan, X. M., Yu, H., Rosenblum, C. I., Vongs, A., Feng, Y., Cao, L., Metzger, J. M., Strack, A. M., Camacho, R. E., Mellin, T. N., Nunes, C. N., Min, W., Fisher, J., Gopal-Truter, S., Macintyre, D. E., Chen, H. Y. and Van Der Ploeg, L. H. (2000). "Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass." *Nat Genet* **26**(1): 97-102.

Chourbaji, S., Zacher, C., Sanchis-Segura, C., Spanagel, R. and Gass, P. (2005). "Social and structural housing conditions influence the development of a depressive-like phenotype in the learned helplessness paradigm in male mice." *Behav Brain Res* **164**(1): 100-106.

Chrousos, G. P. (2000). "The role of stress and the hypothalamic-pituitary-adrenal axis in the pathogenesis of the metabolic syndrome: Neuro-endocrine and target tissue-related causes." *Int J Obes Relat Metab Disord* **24 Suppl 2**: S50-55.

Claret, M., Smith, M. A., Batterham, R. L., Selman, C., Choudhury, A. I., Fryer, L. G., Clements, M., Al-Qassab, H., Heffron, H., Xu, A. W., Speakman, J. R., Barsh, G. S., Viollet, B., Vaulont, S., Ashford, M. L., Carling, D. and Withers, D. J. (2007). "Ampk is essential for energy homeostasis regulation and glucose sensing by pomc and agrp neurons." *J Clin Invest* **117**(8): 2325-2336.

Coburn-Litvak, P. S., Pothakos, K., Tata, D. A., Mccloskey, D. P. and Anderson, B. J. (2003). "Chronic administration of corticosterone impairs spatial reference memory before spatial working memory in rats." *Neurobiol Learn Mem* **80**(1): 11-23.

Cole, T. J. (1995). "Cloning of the mouse 11 beta-hydroxysteroid dehydrogenase type 2 gene: Tissue specific expression and localization in distal convoluted tubules and collecting ducts of the kidney." *Endocrinology* **136**(10): 4693-4696.

Coll, A. P., Challis, B. G., Lopez, M., Piper, S., Yeo, G. S. and O'rahilly, S. (2005). "Proopiomelanocortin-deficient mice are hypersensitive to the adverse metabolic effects of glucocorticoids." *Diabetes* **54**(8): 2269-2276.

Collins, A. and Frankenhaeuser, M. (1978). "Stress responses in male and female engineering students." *J Hum Stress* **4**(2): 43-48.

Comijs, H. C., Gerritsen, L., Penninx, B. W., Bremmer, M. A., Deeg, D. J. and Geerlings, M. I. (2010). "The association between serum cortisol and cognitive decline in older persons." *Am J Geriatr Psychiatry* **18**(1): 42-50.

Compagnone, N. A. and Mellon, S. H. (2000). "Neurosteroids: Biosynthesis and function of these novel neuromodulators." *Front Neuroendocrinol* **21**(1): 1-56.

Contarino, A., Dellu, F., Koob, G. F., Smith, G. W., Lee, K. F., Vale, W. W. and Gold, L. H. (2000). "Dissociation of locomotor activation and suppression of food intake induced by crf in crfr1-deficient mice." *Endocrinology* **141**(7): 2698-2702.

Cooke, S. F. and Bliss, T. V. P. "Plasticity in the human central nervous system." *Brain* **129**(7): 1659-1673.

Critchlow, V., Liebelt, R. A., Bar-Sela, M., Mountcastle, W. and Lipscomb, H. S. (1963). "Sex difference in resting pituitary-adrenal function in the rat." *Am J Physiol* **205**(5): 807-815.

Cronstein, B. N., Kimmel, S. C., Levin, R. I., Martiniuk, F. and Weissmann, G. (1992). "A mechanism for the antiinflammatory effects of corticosteroids: The glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1." *Proc Nat Acad Sci U S A* **89**(21): 9991-9995.

Cusin, I., Rouru, J. and Rohner-Jeanrenaud, F. (2001). "Intracerebroventricular glucocorticoid infusion in normal rats: Induction of parasympathetic-mediated obesity and insulin resistance." *Obes Res* **9**(7): 401-406.

D'aquila, P. S., Canu, S., Sardella, M., Spanu, C., Serra, G. and Franconi, F. (2010). "Dopamine is involved in the antidepressant-like effect of allopregnanolone in the forced swimming test in female rats." *Behav Pharmacol* **21**(1): 21-28.

Dagogo-Jack, S., Selke, G., Melson, A. K. and Newcomer, J. W. (1997). "Robust leptin secretory responses to dexamethasone in obese subjects." *J Clin Endocrinol Metab* **82**(10): 3230-3233.

Dallman, M. F. (2005). "Fast glucocorticoid actions on brain: Back to the future." *Front Neuroendocrinol* **26**(3-4): 103-108.

Dallman, M. F., Akana, S. F., Cascio, C. S., Darlington, D. N., Jacobson, L. and Levin, N. (1987). "Regulation of acth secretion: Variations on a theme of b." *Recent Prog Horm Res* **43**: 113-173.

Dallman, M. F., Akana, S. F., Scribner, K. A., Bradbury, M. J., Walker, C. D., Strack, A. M. and Cascio, C. S. (1992). "Stress, feedback and facilitation in the hypothalamo-pituitary-adrenal axis." *J Neuroendocrinol* **4**(5): 517-526.

Dallman, M. F. and Bhatnagar, S. (2001). Chronic stress and energy balance: Role of the hypothalamic-pituitary-adrenal axis. In: *Coping with the environment*, Chapter 10, (New York: Oxford University Press.). **4:** 179-210.

Dallman, M. F., Jones, M. T., Vernikos-Danellis, J. and Ganong, W. F. (1972). "Corticosteroid feedback control of acth secretion: Rapid effects of bilateral adrenalectomy on plasma acth in the rat." *Endocrinology* **91**(4): 961-968.

Dallman, M. F., Levin, N., Cascio, C. S., Akana, S. F., Jacobson, L. and Kuhn, R. W. (1989). "Pharmacological evidence that the inhibition of diurnal adrenocorticotropin secretion by corticosteroids is mediated via type i corticosterone-preferring receptors." *Endocrinology* **124**(6): 2844-2850.

Dallman, M. F., Makara, G. B., Roberts, J. L., Levin, N. and Blum, M. (1985). "Corticotrope response to removal of releasing factors and corticosteroids in vivo." *Endocrinology* **117**(5): 2190-2197.

Dallman, M. F., Pecoraro, N. C. and La Fleur, S. E. (2005). "Chronic stress and comfort foods: Self-medication and abdominal obesity." *Brain Behav Immun* **19**(4): 275-280.

Dallman, M. F., Strack, A. M., Akana, S. F., Bradbury, M. J., Hanson, E. S., Scribner, K. A. and Smith, M. (1993). "Feast and famine: Critical role of glucocorticoids with insulin in daily energy flow." *Front Neuroendocrinol* **14**(4): 303-347.

Dallman, M. F., Viau, V. G., Bhatnagar, S., Gomez, F., Laugero, K. and Bell, M. E. (2002). Corticotropin-releasing factor (crf), corticosteroids and stress, energy balance, the brain and behavior. In: Pfaff, D.W., Arnold, A.P., Fahrbach, S.E., Etgen, A.M. and Rubin, R.T. (Eds.), *Hormones, brain and behavior*, (San Diego: Academic Press)**:** 571-631.

Datson, N. A., Morsink, M. C., Meijer, O. C. and De Kloet, E. R. (2008). "Central corticosteroid actions: Search for gene targets." *Eur J Pharmacol* **583**(2-3): 272-289.

De Bosscher, K., Vanden Berghe, W. and Haegeman, G. (2003). "The interplay between the glucocorticoid receptor and nuclear factor-kappab or activator protein-1: Molecular mechanisms for gene repression." *Endocr Rev* **24**(4): 488-522.

De Herder, W. W., Hofland, L. J., Usdin, T. B., De Jong, F. H., Uitterlinden, P., Van Koetsveld, P., Mezey, E., Bonner, T. I., Bonjer, H. J. and Lamberts, S. W.

(1996). "Food-dependent cushing's syndrome resulting from abundant expression of gastric inhibitory polypeptide receptors in adrenal adenoma cells." *J Clin Endocrinol Metab* **81**(9): 3168-3172.

De Kloet, E. R. (1991). "Brain corticosteroid receptor balance and homeostatic control." *Front Neuroendocrinol* **12**: 95-164.

De Kloet, E. R., Joels, M. and Holsboer, F. (2005). "Stress and the brain: From adaptation to disease." *Nat Rev Neurosci* **6**(6): 463-475.

De Kloet, E. R. and Reul, J. M. H. M. (1987). "Feedback action and tonic influence of corticosteroids on brain function: A concept arising from the heterogeneity of brain receptor systems." *Psychoneuroendocrinology* **12**(2): 83-105.

De Kloet, E. R., Vreugdenhil, E., Oitzl, M. S. and Joels, M. (1998). "Brain corticosteroid receptor balance in health and disease." *Endocr Rev* **19**(3): 269-301.

De Vellis, J., S., M. B., Cole, R. and Inglish, D. (1974). "Relations between glucocorticoid nuclear binding, cytosol receptor activity and enzyme induction in a rat glial cell line." *J Steroid Biochem* **5**: 392-393.

Dekosky, S. T., Scheff, S. W. and Cotman, C. W. (1984). "Elevated corticosterone levels. A possible cause of reduced axon sprouting in aged animals." *Neuroendocrinology* **38**(1): 33-38.

Densmore, V. S., Morton, N. M., Mullins, J. J. and Seckl, J. R. (2006). "11 beta-hydroxysteroid dehydrogenase type 1 induction in the arcuate nucleus by high-fat feeding: A novel constraint to hyperphagia?" *Endocrinology* **147**(9): 4486-4495.

Deuschle, M., Schweiger, U., Weber, B., Gotthardt, U., Korner, A., Schmider, J., Standhardt, H., Lammers, C. H. and Heuser, I. (1997). "Diurnal activity and pulsatility of the hypothalamus-pituitary-adrenal system in male depressed patients and healthy controls." *J Clin Endocrinol Metab* **82**(1): 234-238.

Devenport, L., Knehans, A., Sundstrom, A. and Thomas, T. (1989). "Corticosterone's dual metabolic actions." *Life Sci* **45**(15): 1389-1396.

Di, S., Malcher-Lopes, R., Halmos, K. C. and Tasker, J. G. (2003). "Nongenomic glucocorticoid inhibition via endocannabinoid release in the hypothalamus: A fast feedback mechanism." *J Neurosci* **23**(12): 4850-4857.

Dixit, P. K., Morgan, C. R., Lowe, I. P. and Lazarow, A. (1963). "Antiinsulin serum hyperglycemia: Effect on in vitro glucose utilization and insulin degradation by rat epididymal fat pad." *Metabolism* **12**: 642-651.

Dockery, F., Bulpitt, C. J., Agarwal, S., Donaldson, M. and Rajkumar, C. (2003). "Testosterone suppression in men with prostate cancer leads to an increase in arterial stiffness and hyperinsulinaemia." *Clin Sci (Lond)* **104**(2): 195-201.

Dong, E., Matsumoto, K., Uzunova, V., Sugaya, I., Takahata, H., Nomura, H., Watanabe, H., Costa, E. and Guidotti, A. (2001). "Brain 5alpha-dihydroprogesterone and allopregnanolone synthesis in a mouse model of protracted social isolation." *Proc Natl Acad Sci U S A* **98**(5): 2849-2854.

Dorfman, R. I. and Forchielli, E. (1956). "Separation of delta 4-5 alpha-hydrogenases from rat liver homogenates." *J Biol Chem* **223**(1): 443-448.

Dorn, L. D., Burgess, E. S., Dubbert, B., Simpson, S. E., Friedman, T. B., Kllng, M., Gold, P. W. and Chrousos, G. P. (1995). "Psychopathology in patients with endogenous cushing's syndrome: 'Atypical' or melancholic features." *Clin Endocrinol (Oxf)* **43**(4): 433-442.

Drazen, D. L. and Woods, S. C. (2003). "Peripheral signals in the control of satiety and hunger." *Curr Opin Clin Nutr Metab Care* **6**(6): 621-629.

Drouin, J., Sun, Y. L., Chamberland, M., Gauthier, Y., De Lean, A., Nemer, M. and Schmidt, T. J. (1993). "Novel glucocorticoid receptor complex with DNA element of the hormone-repressed pomc gene." *EMBO J* **12**(1): 145-156.

Dubey, A. K. and Plant, T. M. (1985). "A suppression of gonadotropin secretion by cortisol in castrated male rhesus monkeys (macaca mulatta) mediated by the interruption of hypothalamic gonadotropin-releasing hormone release." *Biol Reprod* **33**(2): 423-431.

Dubrovsky, B. (2006). "Neurosteroids, neuroactive steroids, and symptoms of affective disorders." *Pharma biochem behav* **84**(4): 644-655.

Dulawa, S. C., Grandy, D. K., Low, M. J., Paulus, M. P. and Geyer, M. A. (1999). "Dopamine d4 receptor-knock-out mice exhibit reduced exploration of novel stimuli." *J Neurosci* **19**(21): 9550-9556.

Earls, F. (1987). "Sex differences in psychiatric disorders: Origins and developmental influences." *Psychiatr Dev* **5**(1): 1-23.

Edinger, K. L. and Frye, C. A. (2005). "Testosterone's anti-anxiety and analgesic effects may be due in part to actions of its 5α-reduced metabolites in the hippocampus." *Psychoneuroendocrinology* **30**(5): 418-430.

Evans, H. C. and Goa, K. L. (2003). "Dutasteride." *Drug Aging* **20**(12): 905-916.

Farkash, Y., Soreq, H. and Orly, J. (1988). "Biosynthesis of catalytically active rat testosterone 5 alpha-reductase in microinjected xenopus oocytes: Evidence for tissue-specific differences in translatable mrna." *Proc Natl Acad Sci U S A* **85**(16): 5824-5828.

Fehm, H. L., Voigt, K. H., Kummer, G. and Pfeiffer, E. F. (1979). "Positive rate-sensitive corticosteroid feedback mechanism of acth secretion in cushing's disease." *J Clin Invest* **64**(1): 102-108.

Feldman, S. and Conforti, N. (1980). "Participation of the dorsal hippocampus in the glucocorticoid feedback effect on adrenocortical activity." *Neuroendocrinology* **30**(1): 52-55.

Fendler, K., Karmos, G. and Telegdy, G. (1961). "The effect of hippocampal lesion on pituitary-adrenal function." *Acta Physiol Acad Sci Hung* **20**: 293-297.

File, S. E. (2001). "Factors controlling measures of anxiety and responses to novelty in the mouse." *Behav Brain Res* **125**(1–2): 151-157.

Fink, G., Robinson, I. C. and Tannahill, L. A. (1988). "Effects of adrenalectomy and glucocorticoids on the peptides crf-41, avp and oxytocin in rat hypophysial portal blood." *J Physiol* **401**(1): 329-345.

Finn, D. A., Long, S. L., Tanchuck, M. A. and Crabbe, J. C. (2004). "Interaction of chronic ethanol exposure and finasteride: Sex and strain differences." *Pharma biochem behav* **78**(3): 435-443.

Fisher, L. K., Kogut, M. D., Moore, R. J., Goebelsmann, U., Weitzman, J. J., Isaacs, H., Jr., Griffin, J. E. and Wilson, J. D. (1978). "Clinical, endocrinological, and enzymatic characterization of two patients with 5 alpha-reductase deficiency: Evidence that a single enzyme is responsible for the 5 alpha-reduction of cortisol and testosterone." *J Clin Endocrinol Metab* **47**(3): 653-664.

Forbes, N. F., Stewart, C. A., Matthews, K. A. and Reid, I. C. (1996). "Chronic mild stress and sucrose consumption: Validity as a model of depression." *Physiol Behav* **60**(6): 1481-1484.

Fraser, R. (1990). "Peripheral metabolism of corticosteroids as a mechanism of potency modulation." *J Endocrinol* **125**(1): 1-2.

Fraser, R., Ingram, M. C., Anderson, N. H., Morrison, C., Davies, E. and Connell, J. M. C. (1999). "Cortisol effects on body mass, blood pressure, and cholesterol in the general population." *Hypertension* **33**(6): 1364-1368.

Freedman, M. R., Castonguay, T. W. and Stern, J. S. (1985). "Effect of adrenalectomy and corticosterone replacement on meal patterns of zucker rats." *Am J Physiol* **249**(5 Pt 2): R584-594.

Fried, S. K., Russell, C. D., Grauso, N. L. and Brolin, R. E. (1993). "Lipoprotein lipase regulation by insulin and glucocorticoid in subcutaneous and omental adipose tissues of obese women and men." *J Clin Invest* **92**(5): 2191-2198.

Froesch, E. R., Buergi, H., Ramseier, E. B., Bally, P. and Labhart, A. (1963). "Antibody-suppressible and nonsuppressible insulin-like activities in human serum and their physiologic significance. An insulin assay with adipose tissue of increased precision and specificity." *J Clin Invest* **42**: 1816-1834.

Frye, C. A., Rhodes, M. E., Walf, A. A. and Harney, J. P. (2002). "Testosterone enhances aggression of wild-type mice but not those deficient in type i 5α-reductase." *Brain Res* **948**(1–2): 165-170.

Fryer, C. J. and Archer, T. K. (1998). "Chromatin remodelling by the glucocorticoid receptor requires the brg1 complex." *Nature* **393**(6680): 88-91.

Fryer, C. J., Nordeen, S. K. and Archer, T. K. (1998). "Antiprogestins mediate differential effects on glucocorticoid receptor remodeling of chromatin structure." *J Biol Chem* **273**(2): 1175-1183.

Furuebisu, M., Deguchi, S. and Okuda, K. (1987). "Identification of cortisone 5 beta-reductase as delta 4-3-ketosteroid 5 beta-reductase." *Biochim Biophys Acta* **912**(1): 110-114.

Ganguly, A., Weinberger, M. H., Guthrie, G. P. and Fineberg, N. S. (1984). "Adrenal steroid responses to acth in glucocorticoid-suppressible aldosteronism." *Hypertension* **6**(4): 563-567.

Gaskin, J. H. and Kitay, J. I. (1971). "Hypothalamic and pituitary regulation of adrenocortical function in the hamster: Effects of gonadectomy and gonadal hormone replacement." *Endocrinology* **89**(4): 1047-1053.

Geerling, J. C. and Loewy, A. D. (2009). "Aldosterone in the brain." *Am J Physiol Renal Physiol* **297**(3): F559-576.

Gehlert, D. R. (1999). "Role of hypothalamic neuropeptide y in feeding and obesity." *Neuropeptides* **33**(5): 329-338.

Germano, C. M. R., De Castro, M., Rorato, R., Laguna, M. T. C., Antunes-Rodrigues, J., Elias, C. F. and Elias, L. L. K. (2007). "Time course effects of adrenalectomy and food intake on cocaine- and amphetamine-regulated transcript expression in the hypothalamus." *Brain Res* **1166**(0): 55-64.

Ghoumari, A. M., Ibanez, C., El-Etr, M., Leclerc, P., Eychenne, B., O'malley, B. W., Baulieu, E. E. and Schumacher, M. (2003). "Progesterone and its metabolites increase myelin basic protein expression in organotypic slice cultures of rat cerebellum." *J Neurochem* **86**(4): 848-859.

Gisleskog, P. O., Hermann, D., Hammarlund-Udenaes, M. and Karlsson, M. O. (1998). "A model for the turnover of dihydrotestosterone in the presence of the irreversible 5 alpha-reductase inhibitors gi198745 and finasteride." *Clin Pharmacol Ther* **64**(6): 636-647.

Glass, C. K. (1994). "Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers." *Endocr Rev* **15**(3): 391-407.

Gomez-Sanchez, C. E., Zhou, M. Y., Cozza, E. N., Morita, H., Eddleman, F. C. and Gomez-Sanchez, E. P. (1996). "Corticosteroid synthesis in the central nervous system." *Endocr Res* **22**(4): 463-470.

Goodwin, J. S. (1994). Antiinflammatory drugs. In: Stites, D.P., Terr, A.I. and Parslow, T.G. (Eds.), *Basic and clinical immunology*, 8th edition (East Norwalk: Appleton and Lange)**:** 786-795.

Gormley, G. J., Stoner, E., Bruskewitz, R. C., Imperato-Mcginley, J., Walsh, P. C., Mcconnell, J. D., Andriole, G. L., Geller, J., Bracken, B. R., Tenover, J. S. and Et Al. (1992). "The effect of finasteride in men with benign prostatic hyperplasia. The finasteride study group." *N Engl J Med* **327**(17): 1185-1191.

Gottfried-Blackmore, A., Sierra, A., Jellinck, P. H., Mcewen, B. S. and Bulloch, K. (2008). "Brain microglia express steroid-converting enzymes in the mouse." *J Steroid Biochem Mol Biol* **109**(1-2): 96-107.

Goulding, N. J. (2004). "The molecular complexity of glucocorticoid actions in inflammation - a four-ring circus." *Curr Opin Pharmacol* **4**(6): 629-636.

Gounarides, J. S., Korach-André, M., Killary, K., Argentieri, G., Turner, O. and Laurent, D. (2008). "Effect of dexamethasone on glucose tolerance and fat metabolism in a diet-induced obesity mouse model." *Endocrinology* **149**(2): 758-766.

Gray, J. A. (1982). "Précis of the neuropsychology of anxiety: An enquiry into the functions of the septo-hippocampal system." *Behav Brain Sci* **5**(03): 469-484.

Green, P. K., Wilkinson, C. W. and Woods, S. C. (1992). "Intraventricular corticosterone increases the rate of body weight gain in underweight adrenalectomized rats." *Endocrinology* **130**(1): 269-275.

Gregus, A., Wintink, A. J., Davis, A. C. and Kalynchuk, L. E. (2005). "Effect of repeated corticosterone injections and restraint stress on anxiety and depression-like behavior in male rats." *Behav Brain Res* **156**(1): 105-114.

Griffin, L. D. and Mellon, S. H. (1999). "Selective serotonin reuptake inhibitors directly alter activity of neurosteroidogenic enzymes." *Proc Natl Acad Sci U S A* **96**(23): 13512-13517.

Groeneweg, F. L., Karst, H., De Kloet, E. R. and Joëls, M. (2011). "Rapid non-genomic effects of corticosteroids and their role in the central stress response." *J Endocrinol* **209**(2): 153-167.

Guidotti, A., Dong, E., Matsumoto, K., Pinna, G., Rasmusson, A. M. and Costa, E. (2001). "The socially-isolated mouse: A model to study the putative role of allopregnanolone and 5alpha-dihydroprogesterone in psychiatric disorders." *Brain Res Brain Res Rev* **37**(1-3): 110-115.

Guo, A. L., Petraglia, F., Criscuolo, M., Ficarra, G., Nappi, R. E., Palumbo, M. A., Trentini, G. P., Purdy, R. H. and Genazzani, A. R. (1995). "Evidence for a role of neurosteroids in modulation of diurnal changes and acute stress-induced corticosterone secretion in rats." *Gynecol Endocrinol* **9**(1): 1-7.

Gustafsson, J. A., Ingelman-Sundberg, M., Stenberg, A. and Hökfelt, T. (1976). "Feminization of hepatic steroid metabolism in male rats following electrothermic lesion of the hypothalamus." *Endocrinology* **98**(4): 922-926.

Hallstrom, A., Carlsson, A., Hillered, L. and Ungerstedt, U. (1989). "Simultaneous determination of lactate, pyruvate, and ascorbate in microdialysis samples from rat brain, blood, fat, and muscle using high-performance liquid chromatography." *J Pharmacol Methods* **22**(2): 113-124.

Handa, R. J., Burgess, L. H., Kerr, J. E. and O'keefe, J. A. (1994). "Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis." *Horm Behav* **28**(4): 464-476.

Handa, R. J., Nunley, K. M., Lorens, S. A., Louie, J. P., Mcgivern, R. F. and Bollnow, M. R. (1994). "Androgen regulation of adrenocorticotropin and corticosterone secretion in the male rat following novelty and foot shock stressors." *Physiol Behav* **55**(1): 117-124.

Handa, R. J., Weiser, M. J. and Zuloaga, D. G. (2009). "A role for the androgen metabolite, 5alpha-androstane-3beta,17beta-diol, in modulating oestrogen receptor beta-mediated regulation of hormonal stress reactivity." *J Neuroendocrinol* **21**(4): 351-358.

Hanson, E. S., Bradbury, M. J., Akana, S. F., Scribner, K. S., Strack, A. M. and Dallman, M. F. (1994). "The diurnal rhythm in adrenocorticotropin responses to restraint in adrenalectomized rats is determined by caloric intake." *Endocrinology* **134**(5): 2214-2220.

Hara, A., Inoue, Y., Nakagawa, M., Naganeo, F. and Sawada, H. (1988). "Purification and characterization of nadp+-dependent 3 alpha-hydroxysteroid dehydrogenase from mouse liver cytosol." *J Biochem* **103**(6): 1027-1034.

Harizi, H., Homo-Delarche, F., Amrani, A., Coulaud, J. and Mormède, P. (2007). "Marked genetic differences in the regulation of blood glucose under immune and restraint stress in mice reveals a wide range of corticosensitivity." *J Neuroimmunol* **189**(1-2): 59-68.

Harris, A. P., Holmes, M. C., De Kloet, E. R., Chapman, K. E. and Seckl, J. R. (2013). "Mineralocorticoid and glucocorticoid receptor balance in control of hpa axis and behaviour." *Psychoneuroendocrinology* **38**(5): 648-658.

Harris, H. J., Kotelevtsev, Y., Mullins, J. J., Seckl, J. R. and Holmes, M. C. (2001). "Intracellular regeneration of glucocorticoids by 11beta-hydroxysteroid dehydrogenase (11beta-hsd)-1 plays a key role in regulation of the hypothalamic-pituitary-adrenal axis: Analysis of 11beta-hsd-1-deficient mice." *Endocrinology* **142**(1): 114-120.

Harris, R. B. S., Zhou, J., Youngblood, B. D., Rybkin, I. I., Smagin, G. N. and Ryan, D. H. (1998). "Effect of repeated stress on body weight and body composition of rats fed low- and high-fat diets." *Am J Physiol Regul Integr Comp Physiol* **275**(6): R1928-1938.

Haskett, R. F. (1985). "Diagnostic categorization of psychiatric disturbance in cushing's syndrome." *Am J Psychiatry* **142**(8): 911-916.

Hautanen, A. and Adlercreutz, H. (1993). "Altered adrenocorticotropin and cortisol secretion in abdominal obesity: Implications for the insulin resistance syndrome." *J Intern Med* **234**(5): 461-469.

Heidbreder, C. A., Weiss, I. C., Domeney, A. M., Pryce, C., Homberg, J., Hedou, G., Feldon, J., Moran, M. C. and Nelson, P. (2000). "Behavioral, neurochemical and endocrinological characterization of the early social isolation syndrome." *Neuroscience* **100**(4): 749-768.

Hein, R., Abbas, S., Seibold, P., Salazar, R., Flesch-Janys, D. and Chang-Claude, J. (2012). "Polymorphism thr160thr in srd5a1, involved in the progesterone metabolism, modifies postmenopausal breast cancer risk associated with menopausal hormone therapy." *Breast Cancer Res Treat* **131**(2): 653-661.

Heinrichs, S. C., Menzaghi, F., Pich, E. M., Hauger, R. L. and Koob, G. F. (1993). "Corticotropin-releasing factor in the paraventricular nucleus modulates feeding induced by neuropeptide y." *Brain Res* **611**(1): 18-24.

Hellemans, K. G. C., Benge, L. C. and Olmstead, M. C. (2004). "Adolescent enrichment partially reverses the social isolation syndrome." *Dev Brain Res* **150**(2): 103-115.

Herd, M. B., Belelli, D. and Lambert, J. J. (2007). "Neurosteroid modulation of synaptic and extrasynaptic gaba(a) receptors." *Pharmacol Ther* **116**(1): 20-34.

Herman, J. P. and Cullinan, W. E. (1997). "Neurocircuitry of stress: Central control of the hypothalamo-pituitary-adrenocortical axis." *Trends Neurosci* **20**(2): 78-84.

Herman, J. P., Watson, S. J., Chao, H. M., Coirini, H. and Mcewen, B. S. (1993). "Diurnal regulation of glucocorticoid receptor and mineralocorticoid receptor mrnas in rat hippocampus." *Mol Cell Neurosci* **4**(2): 181-190.

Hernandez-Morante, J. J., Gomez-Santos, C., Milagro, F., Campion, J., Martinez, J. A., Zamora, S. and Garaulet, M. (2009). "Expression of cortisol metabolism-related genes shows circadian rhythmic patterns in human adipose tissue." *Int J Obes (Lond)* **33**(4): 473-480.

Het, S., Ramlow, G. and Wolf, O. T. (2005). "A meta-analytic review of the effects of acute cortisol administration on human memory." *Psychoneuroendocrinology* **30**(8): 771-784.

Hetherington, A. W. and Ranson, S. W. (1942). "The spontaneous activity and food intake of rats with hypothalamic lesions." *Am J Physiol* **136**: 609-617.

Hickey, A. J., Reynolds, J. N. and Beninger, R. J. (2012). "Post-weaning social isolation and subchronic nmda glutamate receptor blockade: Effects on locomotor activity and gaba signaling in the rat suggest independent mechanisms." *Pharma Biochem Behav* **101**(2): 231-238.

Hilakivi, L. A., Ota, M. and Lister, R. G. (1989). "Effect of isolation on brain monoamines and the behavior of mice in tests of exploration, locomotion, anxiety and behavioral 'despair'." *Pharmacol Biochem Behav* **33**(2): 371-374.

Hill, M. N., Brotto, L. A., Lee, T. T. and Gorzalka, B. B. (2003). "Corticosterone attenuates the antidepressant-like effects elicited by melatonin in the forced swim test in both male and female rats." *Prog Neuropsychopharmacol Biol Psychiatry* **27**(6): 905-911.

Hinz, B. and Hirschelmann, R. (2000). "Rapid non-genomic feedback effects of glucocorticoids on crf-induced acth secretion in rats." *Pharmaceutical research* **17**(10): 1273-1277.

Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., Thompson, E. B., Rosenfeld, M. G. and Evans, R. M. (1985). "Primary

structure and expression of a functional human glucocorticoid receptor cdna." *Nature* **318**(6047): 635-641.

Holmes, M. C., Abrahamsen, C. T., French, K. L., Paterson, J. M., Mullins, J. J. and Seckl, J. R. (2006). "The mother or the fetus? 11beta-hydroxysteroid dehydrogenase type 2 null mice provide evidence for direct fetal programming of behavior by endogenous glucocorticoids." *J. Neurosci.* **26**(14): 3840-3844.

Holmes, M. C., Carter, R. N., Noble, J., Chitnis, S., Dutia, A., Paterson, J. M., Mullins, J. J., Seckl, J. R. and Yau, J. L. (2010). "11beta-hydroxysteroid dehydrogenase type 1 expression is increased in the aged mouse hippocampus and parietal cortex and causes memory impairments." *J Neurosci* **30**(20): 6916-6920.

Holsboer, F. (1999). "The rationale for corticotropin-releasing hormone receptor (crh-r) antagonists to treat depression and anxiety." *J Psychiatr Res* **33**(3): 181-214.

Holsboer, F. and Ising, M. (2008). "Central crh system in depression and anxiety — evidence from clinical studies with crh1 receptor antagonists." *Eur J Pharmacol* **583**(2–3): 350-357.

Hoog, S. S., Pawlowski, J. E., Alzari, P. M., Penning, T. M. and Lewis, M. (1994). "Three-dimensional structure of rat liver 3 alpha-hydroxysteroid/dihydrodiol dehydrogenase: A member of the aldo-keto reductase superfamily." *Proc Natl Acad Sci U S A* **91**(7): 2517-2521.

Hotamisligil, G. S. (2006). "Inflammation and metabolic disorders." *Nature* **444**(7121): 860-867.

Huang, Q., Rivest, R. and Richard, D. (1998). "Effects of leptin on corticotropin-releasing factor (crf) synthesis and crf neuron activation in the paraventricular hypothalamic nucleus of obese (ob/ob) mice." *Endocrinology* **139**(4): 1524-1532.

Huang, W., Ma, K., Zhang, J., Qatanani, M., Cuvillier, J., Liu, J., Dong, B., Huang, X. and Moore, D. D. (2006). "Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration." *Science* **312**(5771): 233-236.

Hudson, J. I., Hudson, M. S., Griffing, G. T., Melby, J. C. and Pope, H. G., Jr. (1987). "Phenomenology and family history of affective disorder in cushing's disease." *Am J Psychiatry* **144**(7): 951-953.

Imperato-Mcginley, J., Guerrero, L., Gautier, T. and Peterson, R. E. (1974). "Steroid 5alpha-reductase deficiency in man: An inherited form of male pseudohermaphroditism." *Science* **186**(4170): 1213-1215.

Issa, A. M., Rowe, W., Gauthier, S. and Meaney, M. J. (1990). "Hypothalamic-pituitary-adrenal activity in aged, cognitively impaired and cognitively unimpaired rats." *J Neurosci* **10**(10): 3247-3254.

Jacobson, L., Muglia, L. J., Weninger, S. C., Pacák, K. and Majzoub, J. A. (2000). "Crh deficiency impairs but does not block pituitary-adrenal responses to diverse stressors." *Neuroendocrinology* **71**(2): 79-87.

Jacobson, L. and Sapolsky, R. (1991). "The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis." *Endocr Rev* **12**(2): 118-134.

Jahng, J. W., Kim, N. Y., Ryu, V., Yoo, S. B., Kim, B. T., Kang, D. W. and Lee, J. H. (2008). "Dexamethasone reduces food intake, weight gain and the

hypothalamic 5-ht concentration and increases plasma leptin in rats." *Eur J Pharmacol* **581**(1–2): 64-70.

Jantzen, H.-M., Strähle, U., Gloss, B., Stewart, F., Schmid, W., Boshart, M., Miksicek, R. and Schütz, G. (1987). "Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene." *Cell* **49**(1): 29-38.

Jefferson, L. S., Exton, J. H., Butcher, R. W., Sutherland, E. W. and Park, C. R. (1968). "Role of adenosine 3',5'-monophosphate in the effects of insulin and anti-insulin serum on liver metabolism." *J Biol Chem* **243**(5): 1031-1038.

Jenkins, E. P., Andersson, S., Imperato-Mcginley, J., Wilson, J. D. and Russell, D. W. (1992). "Genetic and pharmacological evidence for more than one human steroid 5 alpha-reductase." *J Clin Invest* **89**(1): 293-300.

Jeong, K. H., Jacobson, L., Widmaier, E. P. and Majzoub, J. A. (1999). "Normal suppression of the reproductive axis following stress in corticotropin-releasing hormone-deficient mice." *Endocrinology* **140**(4): 1702-1708.

Jingami, H., Matsukura, S., Numa, S. and Imura, H. (1985). "Effects of adrenalectomy and dexamethasone administration on the level of prepro-corticotropin-releasing factor messenger ribonucleic acid (mrna) in the hypothalamus and adrenocorticotropin/beta-lipotropin precursor mrna in the pituitary in rats." *Endocrinology* **117**(4): 1314-1320.

Joels, M. and De Kloet, E. R. (1994). "Mineralocorticoid and glucocorticoid receptors in the brain. Implications for ion permeability and transmitter systems." *Prog Neurobiol* **43**(1): 1-36.

Joels, M., Karst, H., Derijk, R. and De Kloet, E. R. (2008). "The coming out of the brain mineralocorticoid receptor." *Trends Neurosci* **31**(1): 1-7.

Jones, G. H., Marsden, C. A. and Robbins, T. W. (1991). "Behavioural rigidity and rule-learning deficits following isolation-rearing in the rat: Neurochemical correlates." *Behav Brain Res* **43**(1): 35-50.

Jones, M. T., Hillhouse, E. W. and Burden, J. (1976). "Proceedings: Mechanism of action of fast and delayed corticosteroid feedback at the hypothalamus." *J Endocrinol* **69**(3): 34P-35P.

Juraska, J. M., Henderson, C. and Müller, J. (1984). "Differential rearing experience, gender, and radial maze performance." *Dev Psychobiol* **17**(3): 209-215.

Kahn, R., Buse, J., Ferrannini, E. and Stern, M. (2005). "The metabolic syndrome: Time for a critical appraisal." *Diabetes Care* **28**(9): 2289-2304.

Kajiyama, Y., Iijima, Y., Chiba, S., Furuta, M., Ninomiya, M., Izumi, A., Shibata, S. and Kunugi, H. (2010). "Prednisolone causes anxiety- and depression-like behaviors and altered expression of apoptotic genes in mice hippocampus." *Prog Neuropsychopharmacol Biol Psychiatry* **34**(1): 159-165.

Kalra, P. S. and Kalra, S. P. (1977). "Circadian periodicities of serum androgens, progesterone, gonadotropins and luteinizing hormone-releasing hormone in male rats: The effects of hypothalamic deafferentation, castration and adrenalectomy." *Endocrinology* **101**(6): 1821-1827.

Kalueff, A. V. and Tuohima, P. (2004). "Experimental modeling of anxiety and depression." *Acta Neurbiol Exp* **64**: 439-448.

Kalynchuk, L. E., Gregus, A., Boudreau, D. and Perrot-Sinal, T. S. (2004). "Corticosterone increases depression-like behavior, with some effects on

predator odor-induced defensive behavior, in male and female rats." *Behav Neurosci* **118**(6): 1365-1377.

Kanitz, E., Puppe, B., Tuchscherer, M., Heberer, M., Viergutz, T. and Tuchscherer, A. (2009). "A single exposure to social isolation in domestic piglets activates behavioural arousal, neuroendocrine stress hormones, and stress-related gene expression in the brain." *Physiol Behav* **98**(1–2): 176-185.

Karanth, S., Linthorst, A. C. E., Stalla, G. K., Barden, N., Holsboer, F. and Reul, J. M. H. M. (1997). "Hypothalamic-pituitary-adrenocortical axis changes in a transgenic mouse with impaired glucocorticoid receptor function." *Endocrinology* **138**(8): 3476-3485.

Karavolas, H. J. and Hodges, D. R. (1991). Metabolism of progesterone and related steroids by neural and neuroendocrine structures. In: Costa, E. and Paul, S.M. (Eds.), *Neurosteroids and brain function* (New York: Thieme). **8:** 135-145.

Karst, H., Berger, S., Turiault, M., Tronche, F., Schutz, G. and Joels, M. (2005). "Mineralocorticoid receptors are indispensable for nongenomic modulation of hippocampal glutamate transmission by corticosterone." *Proc Natl Acad Sci U S A* **102**(52): 19204-19207.

Katz, R. J., Roth, K. A. and Carroll, B. J. (1981). "Acute and chronic stress effects on open field activity in the rat: Implications for a model of depression." *Neurosci Biobehav Rev* **5**(2): 247-251.

Kaye, W. H., Gwirtsman, H. E., George, D. T., Ebert, M. H., Jimerson, D. C., Tomai, T. P., Chrousos, G. P. and Gold, P. W. (1987). "Elevated cerebrospinal fluid levels of immunoreactive corticotropin-releasing hormone in anorexia nervosa: Relation to state of nutrition, adrenal function, and intensity of depression." *J Clin Endocrinol Metab* **64**(2): 203-208.

Keating, N. L., O'malley, A. J., Freedland, S. J. and Smith, M. R. (2010). "Diabetes and cardiovascular disease during androgen deprivation therapy: Observational study of veterans with prostate cancer." *J Natl Cancer Inst* **102**(1): 39-46.

Keenan, P. A., Jacobson, M. W., Soleymani, R. M. and Newcomer, J. W. (1995). "Commonly used therapeutic doses of glucocorticoids impair explicit memory." *Ann N Y Acad Sci* **761**: 400-402.

Keller-Wood, M., Powers, M. J., Gersting, J. A., Ali, N. and Wood, C. E. (2006). "Genomic analysis of neuroendocrine development of fetal brain-pituitary-adrenal axis in late gestation." *Physiol Genomics* **24**(3): 218-224.

Keller-Wood, M. E. and Dallman, M. F. (1984). "Corticosteroid inhibition of acth secretion." *Endocr Rev* **5**(1): 1-24.

Kelly, W. F. (1996). "Psychiatric aspects of cushing's syndrome." *Q J Med* **89**(7): 543-551.

Kelly, W. F., Kelly, M. J. and Faragher, B. (1996). "A prospective study of psychiatric and psychological aspects of cushing's syndrome." *Clin Endocrinol (Oxf)* **45**(6): 715-720.

Kessler, R. C. (2003). "Epidemiology of women and depression." *J Affect Disord* **74**(1): 5-13.

Khanna, M., Qin, K. N. and Cheng, K. C. (1995). "Distribution of 3 alpha-hydroxysteroid dehydrogenase in rat brain and molecular cloning of multiple cdnas encoding structurally related proteins in humans." *J Steroid Biochem Mol Biol* **53**(1-6): 41-46.

Khanna, M., Qin, K. N., Wang, R. W. and Cheng, K. C. (1995). "Substrate specificity, gene structure, and tissue-specific distribution of multiple human 3 alpha-hydroxysteroid dehydrogenases." *J Biol Chem* **270**(34): 20162-20168.

Khisti, R. T., Deshpande, L. S. and Chopde, C. T. (2002). "The neurosteroid 3 alpha-hydroxy-5 alpha-pregnan-20-one affects dopamine-mediated behavior in rodents." *Psychopharmacology (Berl)* **161**(2): 120-128.

Kim, J. W. and Kirkpatrick, B. (1996). "Social isolation in animal models of relevance to neuropsychiatric disorders." *Biol Psychiatry* **40**(9): 918-922.

Kintzel, P. E., Chase, S. L., Schultz, L. M. and O'rourke, T. J. (2008). "Increased risk of metabolic syndrome, diabetes mellitus, and cardiovascular disease in men receiving androgen deprivation therapy for prostate cancer." *Pharmacotherapy* **28**(12): 1511-1522.

Kirschbaum, C., Klauer, T., Filipp, S. H. and Hellhammer, D. H. (1995). "Sex-specific effects of social support on cortisol and subjective responses to acute psychological stress." *Psychosom Med* **57**(1): 23-31.

Kirschbaum, C., Pirke, K. M. and Hellhammer, D. H. (1995). "Preliminary evidence for reduced cortisol responsivity to psychological stress in women using oral contraceptive medication." *Psychoneuroendocrinology* **20**(5): 509-514.

Kirschbaum, C., Wolf, O. T., May, M., Wippich, W. and Hellhammer, D. H. (1996). "Stress- and treatment-induced elevations of cortisol levels associated with impaired declarative memory in healthy adults." *Life Sci* **58**(17): 1475-1483.

Kirschbaum, C., Wust, S. and Hellhammer, D. (1992). "Consistent sex differences in cortisol responses to psychological stress." *Psychosom Med* **54**(6): 648-657.

Kita, A. and Furukawa, K. (2008). "Involvement of neurosteroids in the anxiolytic-like effects of ac-5216 in mice." *Pharmacol Biochem Behav* **89**(2): 171-178.

Kitay, J. I. (1961). "Sex differences in adrenal cortical secretion in the rat." *Endocrinology* **68**: 818-824.

Kitchener, P., Di Blasi, F., Borrelli, E. and Piazza, P. V. (2004). "Differences between brain structures in nuclear translocation and DNA binding of the glucocorticoid receptor during stress and the circadian cycle." *Eur J Neurosci* **19**(7): 1837-1846.

Kizaki, T., Ookawara, T., Oh-Ishi, S., Itoh, Y., Iwabuchi, K., Onoe, K., Day, N. K., Good, R. A. and Ohno, H. (1998). "An increase in basal glucocorticoid concentration with age induces suppressor macrophages with high-density fc gamma rii/iii." *Immunology* **93**(3): 409-414.

Klotsman, M., Weinberg, C. R., Davis, K., Binnie, C. G. and Hartmann, K. E. (2004). "A case-based evaluation of srd5a1, srd5a2, ar, and adra1a as candidate genes for severity of bph." *Pharmacogenomics J* **4**(4): 251-259.

Klug, A. and Schwabe, J. W. (1995). "Protein motifs 5. Zinc fingers." *FASEB J* **9**(8): 597-604.

Knigge, K. M. (1961). "Adrenocortical response to stress in rats with lesions in hippocampus and amygdala." *Proc Soc Exp Biol Med* **108**: 18-21.

Kolber, B. J., Wieczorek, L. and Muglia, L. J. (2008). "Hypothalamic–pituitary–adrenal axis dysregulation and behavioral analysis of mouse mutants with altered glucocorticoid or mineralocorticoid receptor function." *Stress* **11**(5): 321-338.

Kondo, K. H., Kai, M. H., Setoguchi, Y., Eggertsen, G., Sjöblom, P., Setoguchi, T., Okuda, K. I. and Björkhem, I. (1994). "Cloning and expression of cdna of human δ4-3-oxosteroid 5β-reductase and substrate specificity of the expressed enzyme." *Eur J Biochem* **219**(1-2): 357-363.

Konner, A. C., Janoschek, R., Plum, L., Jordan, S. D., Rother, E., Ma, X., Xu, C., Enriori, P., Hampel, B., Barsh, G. S., Kahn, C. R., Cowley, M. A., Ashcroft, F. M. and Bruning, J. C. (2007). "Insulin action in agrp-expressing neurons is required for suppression of hepatic glucose production." *Cell Metab* **5**(6): 438-449.

Koob, G. F. (1999). "Corticotropin-releasing factor, norepinephrine, and stress." *Biol Psychiatry* **46**(9): 1167-1180.

Korte, S. M., De Boer, S. F., De Kloet, E. R. and Bohus, B. (1995). "Anxiolytic-like effects of selective mineralocorticoid and glucocorticoid antagonists on fear-enhanced behavior in the elevated plus-maze." *Psychoneuroendocrinology* **20**(4): 385-394.

Kotelevtsev, Y., Holmes, M. C., Burchell, A., Houston, P. M., Schmoll, D., Jamieson, P., Best, R., Brown, R., Edwards, C. R., Seckl, J. R. and Mullins, J. J. (1997). "11beta-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress." *Proc Natl Acad Sci U S A* **94**(26): 14924-14929.

Kovács, K. J. (2013). "Crh: The link between hormonal-, metabolic- and behavioral responses to stress." *J Chem Neuroanat*(0): In Press.

Kovacs, W. J., Griffin, J. E. and Wilson, J. D. (1983). "Transformation of human androgen receptors to the deoxyribonucleic acid-binding state." *Endocrinology* **113**(5): 1574-1581.

Krahn, D. D., Gosnell, B. A., Grace, M. and Levine, A. S. (1986). "Crf antagonist partially reverses crf- and stress-induced effects on feeding." *Brain Res Bull* **17**(3): 285-289.

Kudielka, B. M., Hellhammer, J., Hellhammer, D. H., Wolf, O. T., Pirke, K. M., Varadi, E., Pilz, J. and Kirschbaum, C. (1998). "Sex differences in endocrine and psychological responses to psychosocial stress in healthy elderly subjects and the impact of a 2-week dehydroepiandrosterone treatment." *J Clin Endocrinol Metab* **83**(5): 1756-1761.

Kumar, R. and Thompson, E. B. (2005). "Gene regulation by the glucocorticoid receptor: Structure:Function relationship." *J Steroid Biochem Mol Biol* **94**(5): 383-394.

Kunzel, H. E., Zobel, A. W., Nickel, T., Ackl, N., Uhr, M., Sonntag, A., Ising, M. and Holsboer, F. (2003). "Treatment of depression with the crh-1-receptor antagonist r121919: Endocrine changes and side effects." *J Psychiatr Res* **37**(6): 525-533.

Kwak, C., Lee, S. H. and Kaang, B. K. (2009). "Social isolation selectively increases anxiety in mice without affecting depression-like behavior." *Korean J Physiol Pharmacol* **13**(5): 357-360.

Lai, M., Horsburgh, K., Bae, S. E., Carter, R. N., Stenvers, D. J., Fowler, J. H., Yau, J. L., Gomez-Sanchez, C. E., Holmes, M. C., Kenyon, C. J., Seckl, J. R. and Macleod, M. R. (2007). "Forebrain mineralocorticoid receptor overexpression enhances memory, reduces anxiety and attenuates neuronal loss in cerebral ischaemia." *Eur J Neurosci* **25**(6): 1832-1842.

Lakshmi, V., Sakai, R. R., Mcewen, B. S. and Monder, C. (1991). "Regional distribution of 11 beta-hydroxysteroid dehydrogenase in rat brain." *Endocrinology* **128**(4): 1741-1748.

Lamberts, S. W., Verleun, T., Oosterom, R., De Jong, F. and Hackeng, W. H. (1984). "Corticotropin-releasing factor (ovine) and vasopressin exert a synergistic effect on adrenocorticotropin release in man." *J Clin Endocrinol Metab* **58**(2): 298-303.

Landfield, P. W., Baskin, R. K. and Pitler, T. A. (1981). "Brain aging correlates: Retardation by hormonal-pharmacological treatments." *Science* **214**(4520): 581-584.

Landfield, P. W., Waymire, J. C. and Lynch, G. (1978). "Hippocampal aging and adrenocorticoids: Quantitative correlations." *Science* **202**(4372): 1098-1102.

Lavicky, J. and Dunn, A. J. (1993). "Corticotropin-releasing factor stimulates catecholamine release in hypothalamus and prefrontal cortex in freely moving rats as assessed by microdialysis." *J Neurochem* **60**(2): 602-612.

Le May, C., Chu, K., Hu, M., Ortega, C. S., Simpson, E. R., Korach, K. S., Tsai, M. and Mauvais-Jarvis, F. (2006). "Estrogens protect pancreatic β-cells from apoptosis and prevent insulin-deficient diabetes mellitus in mice." *Proceedings of the National Academy of Sciences* **103**(24): 9232-9237.

Lee, E. H. Y. and Tsai, M. J. (1989). "The hippocampus and amygdala mediate the locomotor stimulating effects of corticotropin-releasing factor in mice." *Behav Neural Biol* **51**(3): 412-423.

Lee, S. W., Tsou, A. P., Chan, H., Thomas, J., Petrie, K., Eugui, E. M. and Allison, A. C. (1988). "Glucocorticoids selectively inhibit the transcription of the interleukin 1 beta gene and decrease the stability of interleukin 1 beta mrna." *Proc Nat Acad Sci U S A* **85**(4): 1204-1208.

Lephart, E. D. (1993). "Brain 5-alpha-reductase - cellular, enzymatic, and molecular perspectives and implications for biological function." *Mol Cell Neurosci* **4**(6): 473-484.

Lephart, E. D. (1993). "Pituitary and brain 5-alpha-reductase messenger-rna levels in control, castrated, and dihydrotestosterone-treated rats." *Mol Cell Neurosci* **4**(6): 526-531.

Lephart, E. D., Simpson, E. R. and Trzeciak, W. H. (1991). "Rat adrenal 5 alpha-reductase mrna content and enzyme activity are sex hormone dependent." *J Mol Endocrinol* **6**(2): 163-170.

Lesniewska, B., Miskowiak, B., Nowak, M. and Malendowicz, L. K. (1990). "Sex differences in adrenocortical structure and function. Xxvii. The effect of ether stress on acth and corticosterone in intact, gonadectomized, and testosterone- or estradiol-replaced rats." *Res Exp Med (Berl)* **190**(2): 95-103.

Levine, A. S. and Morley, J. E. (1981). "Stress-induced eating in rats." *Am J Physiol Regul Integr Comp Physiol* **10**(1): R72-R76.

Li, G., Hernandez-Ono, A., Crooke, R. M., Graham, M. J. and Ginsberg, H. N. (2011). "Effects of antisense-mediated inhibition of 11β-hydroxysteroid dehydrogenase type 1 on hepatic lipid metabolism." *J Lip Res* **52**(5): 971-981.

Li, G., Hernandez-Ono, A., Crooke, R. M., Graham, M. J. and Ginsberg, H. N. (2012). "Antisense reduction of 11beta-hydroxysteroid dehydrogenase type 1

enhances energy expenditure and insulin sensitivity independent of food intake in c57bl/6j mice on a western-type diet." *Metabolism* **61**(6): 823-835.

Liang, T., Cascieri, M. A., Cheung, A. H., Reynolds, G. F. and Rasmusson, G. H. (1985). "Species differences in prostatic steroid 5 alpha-reductases of rat, dog, and human." *Endocrinology* **117**(2): 571-579.

Lightman, S. L. and Conway-Campbell, B. L. (2010). "The crucial role of pulsatile activity of the hpa axis for continuous dynamic equilibration." *Nat Rev Neurosci* **11**(10): 710-718.

Lin, H. K., Hung, C. F., Moore, M. and Penning, T. M. (1999). "Genomic structure of rat 3alpha-hydroxysteroid/dihydrodiol dehydrogenase (3alpha-hsd/dd, akr1c9)." *J Steroid Biochem Mol Biol* **71**(1-2): 29-39.

Lindsay, R. S., Wake, D. J., Nair, S., Bunt, J., Livingstone, D. E., Permana, P. A., Tataranni, P. A. and Walker, B. R. (2003). "Subcutaneous adipose 11 beta-hydroxysteroid dehydrogenase type 1 activity and messenger ribonucleic acid levels are associated with adiposity and insulinemia in pima indians and caucasians." *J Clin Endocrinol Metab* **88**(6): 2738-2744.

Liu, W., Wang, J., Sauter, N. K. and Pearce, D. (1995). "Steroid receptor heterodimerization demonstrated in vitro and in vivo." *Proc Natl Acad Sci U S A* **92**(26): 12480-12484.

Livingstone, D. E., Barat, P., Di Rollo, E. M., Rees, G. A., Weldin, B. A., Rog-Zielinska, E. A., Macfarlane, D. P., Walker, B. R. and Andrew, R. (2014a). "5α-reductase type 1 deficiency or inhibition predisposes to insulin resistance, hepatic steatosis and liver fibrosis in rodents." *Diabetes* **[Epub ahead of print]**.

Livingstone, D. E., Di Rollo, E. M., Yang, C., Codrington, L. E., Mathews, J. A., Kara, M., Hughes, K. A., Kenyon, C. J., Walker, B. R. and Andrew, R. (2014b). "Relative adrenal insufficiency in mice deficient in 5α-reductase 1." *Journal of Endocrinology* **222**(2): 257-266.

Livingstone, D. E., Jones, G. C., Smith, K., Jamieson, P. M., Andrew, R., Kenyon, C. J. and Walker, B. R. (2000). "Understanding the role of glucocorticoids in obesity: Tissue-specific alterations of corticosterone metabolism in obese zucker rats." *Endocrinology* **141**(2): 560-563.

Lu, N. Z., Wardell, S. E., Burnstein, K. L., Defranco, D., Fuller, P. J., Giguere, V., Hochberg, R. B., Mckay, L., Renoir, J. M., Weigel, N. L., Wilson, E. M., Mcdonnell, D. P. and Cidlowski, J. A. (2006). "International union of pharmacology. Lxv. The pharmacology and classification of the nuclear receptor superfamily: Glucocorticoid, mineralocorticoid, progesterone, and androgen receptors." *Pharmacol Rev* **58**(4): 782-797.

Lund, T. D., Hinds, L. R. and Handa, R. J. (2006). "The androgen 5alpha-dihydrotestosterone and its metabolite 5alpha-androstan-3beta, 17beta-diol inhibit the hypothalamo-pituitary-adrenal response to stress by acting through estrogen receptor beta-expressing neurons in the hypothalamus." *J Neurosci* **26**(5): 1448-1456.

Luo, X., Kiss, A., Rabadan-Diehl, C. and Aguilera, G. (1995). "Regulation of hypothalamic and pituitary corticotropin-releasing hormone receptor messenger ribonucleic acid by adrenalectomy and glucocorticoids." *Endocrinology* **136**(9): 3877-3883.

Lupien, S., Lecours, A. R., Lussier, I., Schwartz, G., Nair, N. P. and Meaney, M. J. (1994). "Basal cortisol levels and cognitive deficits in human aging." *J Neurosci* **14**(5 Pt 1): 2893-2903.

Lupien, S., Lecours, A. R., Schwartz, G., Sharma, S., Hauger, R. L., Meaney, M. J. and Nair, N. P. V. (1996). "Longitudinal study of basal cortisol levels in healthy elderly subjects: Evidence for subgroups." *Neurobiol Aging* **17**(1): 95-105.

Lupien, S. J., De Leon, M., De Santi, S., Convit, A., Tarshish, C., Nair, N. P., Thakur, M., Mcewen, B. S., Hauger, R. L. and Meaney, M. J. (1998). "Cortisol levels during human aging predict hippocampal atrophy and memory deficits." *Nat Neurosci* **1**(1): 69-73.

Luttge, W. G., Rupp, M. E. and Davda, M. M. (1989). "Aldosterone-stimulated down-regulation of both type i and type ii adrenocorticosteroid receptors in mouse brain is mediated via type i receptors." *Endocrinology* **125**(2): 817-824.

Machinal, F., Dieudonne, M. N., Leneveu, M. C., Pecquery, R. and Giudicelli, Y. (1999). "In vivo and in vitro ob gene expression and leptin secretion in rat adipocytes: Evidence for a regional specific regulation by sex steroid hormones." *Endocrinology* **140**(4): 1567-1574.

Mackay, J., Mensah, G., Mendis, S. and Greenlund, K. (2004). The atlas of heart disease and stroke. Brighton, UK, Myriad Editions Ltd for the World Health Organisation.

Mahendroo, M. S., Cala, K. M., Landrum, D. P. and Russell, D. W. (1997). "Fetal death in mice lacking 5alpha-reductase type 1 caused by estrogen excess." *Mol Endocrinol* **11**(7): 917-927.

Mahendroo, M. S., Cala, K. M. and Russell, D. W. (1996). "5 alpha-reduced androgens play a key role in murine parturition." *Mol Endocrinol* **10**(4): 380-392.

Mahendroo, M. S., Porter, A., Russell, D. W. and Word, R. A. (1999). "The parturition defect in steroid 5alpha-reductase type 1 knockout mice is due to impaired cervical ripening." *Mol Endocrinol* **13**(6): 981-992.

Mahendroo, M. S. and Russell, D. W. (1999). "Male and female isoenzymes of steroid 5alpha-reductase." *Rev Reprod* **4**(3): 179-183.

Maier, C., Rünzler, D., Schindelar, J., Grabner, G., Waldhäusl, W., Köhler, G. and Luger, A. (2005). "G-protein-coupled glucocorticoid receptors on the pituitary cell membrane." *J Cell Sci* **118**(15): 3353-3361.

Makino, S., Schulkin, J., Smith, M. A., Pacak, K., Palkovits, M. and Gold, P. W. (1995). "Regulation of corticotropin-releasing hormone receptor messenger ribonucleic acid in the rat brain and pituitary by glucocorticoids and stress." *Endocrinology* **136**(10): 4517-4525.

Makino, S., Tanaka, Y., Nazarloo, H. P., Noguchi, T., Nishimura, K. and Hashimoto, K. (2005). "Expression of type 1 corticotropin-releasing hormone (crh) receptor mrna in the hypothalamic paraventricular nucleus following restraint stress in crh-deficient mice." *Brain Res* **1048**(1–2): 131-137.

Malcher-Lopes, R., Di, S., Marcheselli, V. S., Weng, F.-J., Stuart, C. T., Bazan, N. G. and Tasker, J. G. (2006). "Opposing crosstalk between leptin and glucocorticoids rapidly modulates synaptic excitation via endocannabinoid release." *J Neurosci* **26**(24): 6643-6650.

Maniam, J. and Morris, M. J. (2012). "The link between stress and feeding behaviour." *Neuropharmacology* **63**(1): 97-110.
Marcelli, M. and Cunningham, G. R. (1999). "Hormonal signaling in prostatic hyperplasia and neoplasia." *J Clin Endocrinol Metab* **84**(10): 3463-3468.
Marissal-Arvy, N., Gaumont, A., Langlois, A., Dabertrand, F., Bouchecareilh, M., Tridon, C. and Mormede, P. (2007). "Strain differences in hypothalamic pituitary adrenocortical axis function and adipogenic effects of corticosterone in rats." *J Endocrinol* **195**(3): 473-484.
Marks, J. L., Porte, D., Stahl, W. L. and Baskin, D. G. (1990). "Localization of insulin receptor mrna in rat brain by in situ hybridization." *Endocrinology* **127**(6): 3234-3236.
Marti, O., Marti, J. and Armario, A. (1994). "Effects of chronic stress on food intake in rats: Influence of stressor intensity and duration of daily exposure." *Physiol Behav* **55**(4): 747-753.
Martini, L., Celotti, F. and Melcangi, R. C. (1996). "Testosterone and progesterone metabolism in the central nervous system: Cellular localization and mechanism of control of the enzymes involved." *Cell Mol Neurobiol* **16**(3): 271-282.
Martini, L., Melcangi, R. C. and Maggi, R. (1993). "Androgen and progesterone metabolism in the central and peripheral nervous system." *J Steroid Biochem Mol Biol* **47**(1-6): 195-205.
Massa, R., Stupnicka, E., Kniewald, Z. and Martini, L. (1972). "The transformation of testosterone into dihydrotestosterone by the brain and the anterior pituitary." *J Steroid Biochem* **3**(3): 385-399.
Masuzaki, H., Ogawa, Y., Isse, N., Satoh, N., Okazaki, T., Shigemoto, M., Mori, K., Tamura, N., Hosoda, K., Yoshimasa, Y. and Et Al. (1995). "Human obese gene expression. Adipocyte-specific expression and regional differences in the adipose tissue." *Diabetes* **44**(7): 855-858.
Masuzaki, H., Paterson, J., Shinyama, H., Morton, N. M., Mullins, J. J., Seckl, J. R. and Flier, J. S. (2001). "A transgenic model of visceral obesity and the metabolic syndrome." *Science* **294**(5549): 2166-2170.
Mathers, C. D. and Loncar, D. (2006). "Projections of global mortality and burden of disease from 2002 to 2030." *PLoS medicine* **3**(11): e442-e442.
Matsumoto, K., Uzunova, V., Pinna, G., Taki, K., Uzunov, D. P., Watanabe, H., Mienville, J. M., Guidotti, A. and Costa, E. (1999). "Permissive role of brain allopregnanolone content in the regulation of pentobarbital-induced righting reflex loss." *Neuropharmacology* **38**(7): 955-963.
Matthews, K., Forbes, N. and Reid, I. C. (1995). "Sucrose consumption as an hedonic measure following chronic unpredictable mild stress." *Physiol Behav* **57**(2): 241-248.
Mattsson, C., Lai, M., Noble, J., Mckinney, E., Yau, J. L., Seckl, J. R. and Walker, B. R. (2003). "Obese zucker rats have reduced mineralocorticoid receptor and 11β-hydroxysteroid dehydrogenase type 1 expression in hippocampus - implications for dysregulation of the hypothalamic-pituitary-adrenal axis in obesity." *Endocrinology* **144**(7): 2997-3003.
Mcconnell, J. D. (1995). "Prostatic growth: New insights into hormonal regulation." *Br J Urol* **76 Suppl 1**: 5-10.

Mcdonough, A. K., Curtis, J. R. and Saag, K. G. (2008). "The epidemiology of glucocorticoid-associated adverse events." *Curr Opin Rheumatol* **20**(2): 131-137.

Mcewan, B. S., Dekloet, E. R. and Roestene, W. H. (1986). "Adrenal steroid receptors and actions in the nervous system." *Physiol Rev* **66**: 1121-1188.

Mcewen, B. S. (1999). "Stress and hippocampal plasticity." *Annu Rev Neurosci* **22**: 105-122.

Mcewen, B. S., Albeck, D., Cameron, H., Chao, H. M., Gould, E., Hastings, N., Kuroda, Y., Luine, V., Magarinos, A. M., Mckittrick, C. R., Orchinik, M., Pavlides, C., Vaher, P., Watanabe, Y. and Weiland, N. (1995). Stress and the brain: A paradoxical role for adrenal steroids. In: Litwack, G. (Ed.) *Vitamins & hormones*, (New York: Academic Press). **51:** 371-402.

Mcewen, B. S., Weiss, J. M. and Schwartz, L. S. (1968). "Selective retention of corticosterone by limbic structures in rat brain." *Nature* **220**(5170): 911-912.

Mcewen, B. S., Weiss, J. M. and Schwartz, L. S. (1969). "Uptake of corticosterone by rat brain and its concentration by certain limbic structures." *Brain Res* **16**(1): 227-241.

Mcilwain, K. L., Merriweather, M. Y., Yuva-Paylor, L. A. and Paylor, R. (2001). "The use of behavioral test batteries: Effects of training history." *Physiol Behav* **73**(5): 705-717.

Mcinnes, K. J., Kenyon, C. J., Chapman, K. E., Livingstone, D. E., Macdonald, L. J., Walker, B. R. and Andrew, R. (2004). "5alpha-reduced glucocorticoids, novel endogenous activators of the glucocorticoid receptor." *J Biol Chem* **279**(22): 22908-22912.

Meaney, M. J., Aitken, D. H., Sharma, S. and Viau, V. (1992). "Basal acth, corticosterone and corticosterone-binding globulin levels over the diurnal cycle, and age-related changes in hippocampal type i and type ii corticosteroid receptor binding capacity in young and aged, handled and nonhandled rats." *Neuroendocrinology* **55**(2): 204-213.

Melcangi, R. C., Celotti, F., Castano, P. and Martini, L. (1993). "Differential localization of the 5 alpha-reductase and the 3 alpha-hydroxysteroid dehydrogenase in neuronal and glial cultures." *Endocrinology* **132**(3): 1252-1259.

Melcangi, R. C., Celotti, F., Poletti, A., Negri-Cesi, P. and Martini, L. (1987). "The 5 alpha-reductase activity of the subcortical white matter, the cerebral cortex, and the hypothalamus of the rat and of the mouse: Possible sex differences and effect of castration." *Steroids* **49**(4-5): 259-270.

Mellon, S. H. and Griffin, L. D. (2002). "Neurosteroids: Biochemistry and clinical significance." *Trends Endocrinol Metab* **13**(1): 35-43.

Mellon, S. H. and Miller, W. L. (1989). "Extraadrenal steroid 21-hydroxylation is not mediated by p450c21." *J Clin Invest* **84**(5): 1497-1502.

Mendel, C. M. (1989). "The free hormone hypothesis: A physiologically based mathematical model." *Endocr Rev* **10**(3): 232-274.

Menzaghi, F., Howard, R. L., Heinrichs, S. C., Vale, W., Rivier, J. and Koob, G. F. (1994). "Characterization of a novel and potent corticotropin-releasing factor antagonist in rats." *J Pharmacol Exp Ther* **269**(2): 564-572.

Miell, J. P., Englaro, P. and Blum, W. F. (1996). "Dexamethasone induces an acute and sustained rise in circulating leptin levels in normal human subjects." *Horm Metab Res* **28**(12): 704-707.

Milewich, L., Gomez-Sanchez, C., Crowley, G., Porter, J. C., Madden, J. D. and Macdonald, P. C. (1977). "Progesterone and 5alpha-pregnane-3,20-dione in peripheral blood of normal young women: Daily measurements throughout the menstrual cycle." *J Clin Endocrinol Metab* **45**(4): 617-622.

Milewich, L., Gomez-Sanchez, C., Madden, J. D. and Macdonald, P. C. (1975). "Isolation and characterization of 5alpha-pregnane-3,20-dione and progesterone in pepipheral blood of pregnant women. Measurement throughout pregnancy." *Gynecol Invest* **6**(5): 291-306.

Miller, W. L. and Tyrrell, J. B. (1995). The adrenal cortex. In: Felig, P. (Ed.) *Endocrinol metab*, Chapter 12, 3rd edition (New York: McGraw-Hill Inc)**:** 555-711.

Mineur, Y. S., Belzung, C. and Crusio, W. E. (2006). "Effects of unpredictable chronic mild stress on anxiety and depression-like behavior in mice." *Behav Brain Res* **175**(1): 43-50.

Mirescu, C. and Gould, E. (2006). "Stress and adult neurogenesis." *Hippocampus* **16**(3): 233-238.

Moloney, P. J. and Coval, M. (1955). "Antigenicity of insulin: Diabetes induced by specific antibodies." *Biochem J* **59**(2): 179-185.

Monder, C. and White, P. C. (1993). "11 beta-hydroxysteroid dehydrogenase." *Vitam Horm* **47**: 187-271.

Montaron, M. F., Drapeau, E., Dupret, D., Kitchener, P., Aurousseau, C., Le Moal, M., Piazza, P. V. and Abrous, D. N. (2006). "Lifelong corticosterone level determines age-related decline in neurogenesis and memory." *Neurobiol Aging* **27**(4): 645-654.

Moore, C. D., Roberts, J. K., Orton, C. R., Murai, T., Fidler, T. P., Reilly, C. A., Ward, R. M. and Yost, G. S. (2012). "Metabolic pathways of inhaled glucocorticoids by the cyp3a enzymes." *Drug Metab Dispos*: 379-389.

Moore, L. B., Parks, D. J., Jones, S. A., Bledsoe, R. K., Consler, T. G., Stimmel, J. B., Goodwin, B., Liddle, C., Blanchard, S. G., Willson, T. M., Collins, J. L. and Kliewer, S. A. (2000). "Orphan nuclear receptors constitutive androstane receptor and pregnane x receptor share xenobiotic and steroid ligands." *J Biol Chem* **275**(20): 15122-15127.

Moore, R. J. and Wilson, J. D. (1976). "Steroid 5alpha-reductase in cultured human fibroblasts. Biochemical and genetic evidence for two distinct enzyme activities." *J Biol Chem* **251**(19): 5895-5900.

Morimoto, M., Morita, N., Ozawa, H., Yokoyama, K. and Kawata, M. (1996). "Distribution of glucocorticoid receptor immunoreactivity and mrna in the rat brain: An immunohistochemical and in situ hybridization study." *Neurosci Res* **26**(3): 235-269.

Morris, R. G. M. (1981). "Spatial localization does not require the presence of local cues." *Learn Motiv* **12**(2): 239-260.

Morris, R. G. M., Garrud, P., Rawlins, J. N. and O'keefe, J. (1982). "Place navigation impaired in rats with hippocampal lesions." *Nature* **297**(5868): 681-683.

Morton, N. M., Holmes, M. C., Fievet, C., Staels, B., Tailleux, A., Mullins, J. J. and Seckl, J. R. (2001). "Improved lipid and lipoprotein profile, hepatic insulin

sensitivity, and glucose tolerance in 11beta-hydroxysteroid dehydrogenase type 1 null mice." *J Biol Chem* **276**(44): 41293-41300.

Morton, N. M., Paterson, J. M., Masuzaki, H., Holmes, M. C., Staels, B., Fievet, C., Walker, B. R., Flier, J. S., Mullins, J. J. and Seckl, J. R. (2004). "Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11 beta-hydroxysteroid dehydrogenase type 1-deficient mice." *Diabetes* **53**(4): 931-938.

Mostyn, A., Keisler, D. H., Webb, R., Stephenson, T. and Symonds, M. E. (2001). "The role of leptin in the transition from fetus to neonate." *Proc Nutr Soc* **60**(2): 187-194.

Muglia, L., Jacobson, L., Dikkes, P. and Majzoub, J. A. (1995). "Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need." *Nature* **373**(6513): 427-432.

Mukai, Y., Higashi, T., Nagura, Y. and Shimada, K. (2008). "Studies on neurosteroids xxv. Influence of a 5alpha-reductase inhibitor, finasteride, on rat brain neurosteroid levels and metabolism." *Biol Pharm Bull* **31**(9): 1646-1650.

Munck, A. and Holbrook, N. J. (1984). "Glucocorticoid-receptor complexes in rat thymus cells. Rapid kinetic behavior and a cyclic model." *J. Biol. Chem.* **259**(2): 820-831.

Naber, D., Sand, P. and Heigl, B. (1996). "Psychopathological and neuropsychological effects of 8-days' corticosteroid treatment. A prospective study." *Psychoneuroendocrinology* **21**(1): 25-31.

Naert, G., Maurice, T., Tapia-Arancibia, L. and Givalois, L. (2007). "Neuroactive steroids modulate hpa axis activity and cerebral brain-derived neurotrophic factor (bdnf) protein levels in adult male rats." *Psychoneuroendocrinology* **32**(8–10): 1062-1078.

Naeser, P. (1974). "Function of the adrenal cortex in obese-hyperglycemic mice (gene symbol ob)." *Diabetologia* **10**(5): 449-453.

Napolitano, A., Voice, M. W., Edwards, C. R., Seckl, J. R. and Chapman, K. E. (1998). "11beta-hydroxysteroid dehydrogenase 1 in adipocytes: Expression is differentiation-dependent and hormonally regulated." *J Steroid Biochem Mol Biol* **64**(5-6): 251-260.

Nappi, R. E., Petraglia, F., Luisi, S., Polatti, F., Farina, C. and Genazzani, A. R. (2001). "Serum allopregnanolone in women with postpartum "blues"." *Obstet Gynecol* **97**(1): 77-80.

Newton, R. and Holden, N. S. (2007). "Separating transrepression and transactivation: A distressing divorce for the glucocorticoid receptor?" *Mol Pharmacol* **72**(4): 799-809.

Nieuwenhuizen, A. G. and Rutters, F. (2008). "The hypothalamic-pituitary-adrenal-axis in the regulation of energy balance." *Physiol Behav* **94**(2): 169-177.

Nishi, M., Usuku, T., Itose, M., Fujikawa, K., Hosokawa, K., Matsuda, K. I. and Kawata, M. (2007). "Direct visualization of glucocorticoid receptor positive cells in the hippocampal regions using green fluorescent protein transgenic mice." *Neuroscience* **146**(4): 1555-1560.

Norman, R. L., Smith, C. J., Pappas, J. D. and Hall, J. (1992). "Exposure to ovarian steroids elicits a female pattern of plasma cortisol levels in castrated male macaques." *Steroids* **57**(1): 37-43.

Normington, K. and Russell, D. W. (1992). "Tissue distribution and kinetic characteristics of rat steroid 5 alpha-reductase isozymes. Evidence for distinct physiological functions." *J Biol Chem* **267**(27): 19548-19554.

Norton, S. (1977). Animal models in psychiatry. Oxford, Pergamon Press.

Oakley, R. H., Sar, M. and Cidlowski, J. A. (1996). "The human glucocorticoid receptor isoform: Expression, biochemical properties, and putative function." *J Biol Chem* **271**(16): 9550-9559.

Ogg, M., Williams, J., Tarbit, M., Goldfarb, P., Gray, T. and Gibson, G. (1999). "A reporter gene assay to assess the molecular mechanisms of xenobiotic-dependent induction of the human cyp3a4 gene in vitro." *Xenobiotica* **29**(3): 269-279.

Oitzl, M. S., Van Haarst, A. D., Sutanto, W. and De Kloet, E. R. (1995). "Corticosterone, brain mineralocorticoid receptors (mrs) and the activity of the hypothalamic-pituitary-adrenal (hpa) axis: The lewis rat as an example of increased central mr capacity and a hyporesponsive hpa axis." *Psychoneuroendocrinology* **20**(6): 655-675.

Okuda, A. and Okuda, K. (1984). "Purification and characterization of delta 4-3-ketosteroid 5 beta-reductase." *J Biol Chem* **259**(12): 7519-7524.

Onishi, Y., Noshiro, M., Shimosato, T. and Okuda, K. (1991). "Molecular cloning and sequence analysis of cdna encoding δ4-3-ketosteroid 5β-reductase of rat liver." *FEBS letters* **283**(2): 215-218.

Owens, M. J. and Nemeroff, C. B. (1991). "Physiology and pharmacology of corticotropin-releasing factor." *Pharmacol Rev* **43**(4): 425-473.

Pashko, S., Deturck, K. H. and Vogel, W. H. (1980). "Use of the catheterized rat in studies on social interactions and plasma catecholamines." *Pharmacol Biochem Behav* **13**(3): 471-473.

Pasquali, R., Anconetani, B., Chattat, R., Biscotti, M., Spinucci, G., Casimirri, F., Vicennati, V., Carcello, A. and Labate, A. M. (1996). "Hypothalamic-pituitary-adrenal axis activity and its relationship to the autonomic nervous system in women with visceral and subcutaneous obesity: Effects of the corticotropin-releasing factor/arginine-vasopressin test and of stress." *Metabolism* **45**(3): 351-356.

Pasquali, R. and Vicennati, V. (2000). "The abdominal obesity phenotype and insulin resistance are associated with abnormalities of the hypothalamic-pituitary-adrenal axis in humans." *Horm Metab Res* **32**(11-12): 521-525.

Pasquali, R. and Vicennati, V. (2000). "Activity of the hypothalamic-pituitary-adrenal axis in different obesity phenotypes." *Int J Obes Relat Metab Disord* **24 Suppl 2**: S47-49.

Pasquali, R., Vicennati, V., Cacciari, M. and Pagotto, U. (2006). "The hypothalamic-pituitary-adrenal axis activity in obesity and the metabolic syndrome." *Ann N Y Acad Sci* **1083**: 111-128.

Patchev, V. K., Hassan, A. H., Holsboer, D. F. and Almeida, O. F. (1996). "The neurosteroid tetrahydroprogesterone attenuates the endocrine response to stress and exerts glucocorticoid-like effects on vasopressin gene transcription in the rat hypothalamus." *Neuropsychopharmacology* **15**(6): 533-540.

Pawlowski, J. E., Huizinga, M. and Penning, T. M. (1991). "Cloning and sequencing of the cdna for rat liver 3 alpha-hydroxysteroid/dihydrodiol dehydrogenase." *J Biol Chem* **266**(14): 8820-8825.

Pecoraro, N., Reyes, F., Gomez, F., Bhargava, A. and Dallman, M. F. (2004). "Chronic stress promotes palatable feeding, which reduces signs of stress: Feedforward and feedback effects of chronic stress." *Endocrinology* **145**(8): 3754-3762.

Peiffer, A., Lapointe, B. and Barden, N. (1991). "Hormonal regulation of type ii glucocorticoid receptor messenger ribonucleic acid in rat brain." *Endocrinology* **129**(4): 2166-2174.

Pelletier, G., Luu-The, V. and Labrie, F. (1994). "Immunocytochemical localization of 5 alpha-reductase in rat brain." *Mol Cell Neurosci* **5**(5): 394-399.

Penning, T. M. (1997). "Molecular endocrinology of hydroxysteroid dehydrogenases." *Endocr Rev* **18**(3): 281-305.

Penning, T. M. (1999). "Molecular determinants of steroid recognition and catalysis in aldo-keto reductases. Lessons from 3alpha-hydroxysteroid dehydrogenase." *J Steroid Biochem Mol Biol* **69**(1-6): 211-225.

Penning, T. M., Burczynski, M. E., Jez, J. M., Hung, C. F., Lin, H. K., Ma, H., Moore, M., Palackal, N. and Ratnam, K. (2000). "Human 3alpha-hydroxysteroid dehydrogenase isoforms (akr1c1-akr1c4) of the aldo-keto reductase superfamily: Functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones." *Biochem J* **351**(Pt 1): 67-77.

Penning, T. M. and Drury, J. E. (2007). "Human aldo–keto reductases: Function, gene regulation, and single nucleotide polymorphisms." *Arch Biochem Biophys* **464**(2): 241-250.

Penning, T. M., Jin, Y., Heredia, V. V. and Lewis, M. (2003). "Structure-function relationships in 3alpha-hydroxysteroid dehydrogenases: A comparison of the rat and human isoforms." *J Steroid Biochem Mol Biol* **85**(2-5): 247-255.

Penning, T. M., Jin, Y., Steckelbroeck, S., Lanisnik Rizner, T. and Lewis, M. (2004). "Structure-function of human 3 alpha-hydroxysteroid dehydrogenases: Genes and proteins." *Mol Cell Endocrinol* **215**(1-2): 63-72.

Petersen, H. H., Andreassen, T. K., Breiderhoff, T., Bräsen, J. H., Schulz, H., Gross, V., Gröne, H. J., Nykjaer, A. and Willnow, T. E. (2006). "Hyporesponsiveness to glucocorticoids in mice genetically deficient for the corticosteroid binding globulin." *Mol Cell Biol* **26**(19): 7236-7245.

Pettersson, U. S., Walden, T. B., Carlsson, P. O., Jansson, L. and Phillipson, M. (2012). "Female mice are protected against high-fat diet induced metabolic syndrome and increase the regulatory t cell population in adipose tissue." *PloS one* **7**(9): e46057.

Pettersson, U. S., Waldén, T. B., Carlsson, P. O., Jansson, L. and Phillipson, M. (2012). "Female mice are protected against high-fat diet induced metabolic syndrome and increase the regulatory t cell population in adipose tissue." *PloS one* **7**(9): e46057-46066.

Philbert, J., Pichat, P., Palme, R., Belzung, C. and Griebel, G. (2012). "The crf1 receptor antagonist ssr125543 attenuates long-term cognitive deficit induced by acute inescapable stress in mice, independently from the hypothalamic pituitary adrenal axis." *Pharmacol Biochem Behav* **102**(3): 415-422.

Pibiri, F., Nelson, M., Guidotti, A., Costa, E. and Pinna, G. (2008). "Decreased corticolimbic allopregnanolone expression during social isolation enhances

contextual fear: A model relevant for posttraumatic stress disorder." *Proc Natl Acad Sci U S A* **105**(14): 5567-5572.

Pincus, G. (1943). "A diurnal rhythm in the excretion of urinary ketosteroids by young men." *J Clin Endocrinol* **3**(4): 195-199.

Pinna, G., Dong, E., Matsumoto, K., Costa, E. and Guidotti, A. (2003). "In socially isolated mice, the reversal of brain allopregnanolone down-regulation mediates the anti-aggressive action of fluoxetine." *Proc Natl Acad Sci U S A* **100**(4): 2035-2040.

Plotsky, P. M. (1987). "Regulation of hypophysiotropic factors mediating acth secretion." *Ann N Y Acad Sci* **512**: 205-217.

Poletti, A., Celotti, F., Melcangi, R. C., Ballabio, M. and Martini, L. (1990). "Kinetic properties of the 5 alpha-reductase of testosterone in the purified myelin, in the subcortical white matter and in the cerebral cortex of the male rat brain." *J Steroid Biochem* **35**(1): 97-101.

Poletti, A., Negri-Cesi, P., Rabuffetti, M., Colciago, A., Celotti, F. and Martini, L. (1998). "Transient expression of the 5alpha-reductase type 2 isozyme in the rat brain in late fetal and early postnatal life." *Endocrinology* **139**(4): 2171-2178.

Potvin, O., Forget, H., Préville, M., Berbiche, D., Chagnon, Y. C. and Hudon, C. (2013). "Relationship between cortisol level and prevalent/incident cognitive impairment and its moderating factors in older adults." *Int Psychogeriatr* **25**(02): 252-262.

Pralong, F. P., Corder, R. and Gaillard, R. C. (1993). "The effects of chronic glucocorticoid excess, adrenalectomy and stress on neuropeptide y in individual rat hypothalamic nuclei." *Neuropeptides* **25**(4): 223-231.

Pratt, W. B., Morishima, Y., Murphy, M. and Harrell, M. (2006). "Chaperoning of glucocorticoid receptors." *Handb Exp Pharmacol*(172): 111-138.

Preil, J., Muller, M. B., Gesing, A., Reul, J. M., Sillaber, I., Van Gaalen, M. M., Landgrebe, J., Holsboer, F., Stenzel-Poore, M. and Wurst, W. (2001). "Regulation of the hypothalamic-pituitary-adrenocortical system in mice deficient for crh receptors 1 and 2." *Endocrinology* **142**(11): 4946-4955.

Price, M. L. and Lucki, I. (2001). "Regulation of serotonin release in the lateral septum and striatum by corticotropin-releasing factor." *J Neurosci* **21**(8): 2833-2841.

Puia, G., Santi, M. R., Vicini, S., Pritchett, D. B., Purdy, R. H., Paul, S. M., Seeburg, P. H. and Costa, E. (1990). "Neurosteroids act on recombinant human gabaa receptors." *Neuron* **4**(5): 759-765.

Purdy, R. H., Morrow, A. L., Moore, P. H. and Paul, S. M. (1991). "Stress-induced elevations of gamma-aminobutyric acid type a receptor-active steroids in the rat brain." *Proc Nat Acad Sci* **88**(10): 4553-4557.

Raber, J. (1998). "Detrimental effects of chronic hypothalamic-pituitary-adrenal axis activation. From obesity to memory deficits." *Mol Neurobiol* **18**(1): 1-22.

Raber, J., Akana, S. F., Bhatnagar, S., Dallman, M. F., Wong, D. and Mucke, L. (2000). "Hypothalamic-pituitary-adrenal dysfunction in apoe(-/-) mice: Possible role in behavioral and metabolic alterations." *J Neurosci* **20**(5): 2064-2071.

Ragnarsson, O., Berglund, P., Eder, D. N. and Johannsson, G. (2012). "Long-term cognitive impairments and attentional deficits in patients with cushing's

disease and cortisol-producing adrenal adenoma in remission." *J Clin Endocrinol Metab* **97**(9): E1640-1648.

Rahimi-Ardabili, B., Pourandarjani, R., Habibollahi, P. and Mualeki, A. (2006). "Finasteride induced depression: A prospective study." *BMC clinical pharmacology* **6**: 7.

Rapkin, A. J., Morgan, M., Goldman, L., Brann, D. W., Simone, D. and Mahesh, V. B. (1997). "Progesterone metabolite allopregnanolone in women with premenstrual syndrome." *Obstet Gynecol* **90**(5): 709-714.

Rask, E., Olsson, T., Soderberg, S., Andrew, R., Livingstone, D. E., Johnson, O. and Walker, B. R. (2001). "Tissue-specific dysregulation of cortisol metabolism in human obesity." *J Clin Endocrinol Metab* **86**(3): 1418-1421.

Rask, E., Walker, B. R., Soderberg, S., Livingstone, D. E., Eliasson, M., Johnson, O., Andrew, R. and Olsson, T. (2002). "Tissue-specific changes in peripheral cortisol metabolism in obese women: Increased adipose 11beta-hydroxysteroid dehydrogenase type 1 activity." *J Clin Endocrinol Metab* **87**(7): 3330-3336.

Rasmusson, A. M., Pinna, G., Paliwal, P., Weisman, D., Gottschalk, C., Charney, D., Krystal, J. and Guidotti, A. (2006). "Decreased cerebrospinal fluid allopregnanolone levels in women with posttraumatic stress disorder." *Biol Psychiatry* **60**(7): 704-713.

Ratka, A., Sutanto, W., Bloemers, M. and De Kloet, E. R. (1989). "On the role of brain type i and type ii corticosteroid receptors in neuroendocrine regulation." *Neuroendocrinology* **50**(2): 117-123.

Ray, K., Sahana, C. C., Chaudhuri, S. B., Das, D. N., Mondal, S., Sarkar, D., Roy, G., Chowdhury, M. K. and Maity, C. R. (1993). "Frusemide-induced diabetes mellitus in insulin pretreated rabbits as an experimental model." *Indian J Med Res* **98**: 37-39.

Reaven, G. M. (1988). "Role of insulin resistance in human disease." *Diabetes* **37**(12): 1595-1607.

Reul, J. M. and De Kloet, E. R. (1985). "Two receptor systems for corticosterone in rat brain: Microdistribution and differential occupation." *Endocrinology* **117**(6): 2505-2511.

Reul, J. M. and De Kloet, E. R. (1986). "Anatomical resolution of two types of corticosterone receptor sites in rat brain with in vitro autoradiography and computerized image analysis." *J Steroid Biochem* **24**(1): 269-272.

Reul, J. M., De Kloet, E. R., Van Sluijs, F. J., Rijnberk, A. and Rothuizen, J. (1990). "Binding characteristics of mineralocorticoid and glucocorticoid receptors in dog brain and pituitary." *Endocrinology* **127**(2): 907-915.

Reul, J. M. H. M., Probst, J. C., Skutella, T., Hirschmann, M., Stec, I. S. M., Montkowski, A., Landgraf, R. and Holsboer, F. (1997). "Increased stress-induced adrenocorticotropin response after long-term intracerebroventricular treatment of rats with antisense mineralocorticoid receptor oligodeoxynucleotides." *Neuroendocrinology* **65**(3): 189-199.

Rich, E. L. and Romero, L. M. (2005). "Exposure to chronic stress downregulates corticosterone responses to acute stressors." *Am J Physiol Regul Integr Comp Physiol* **288**(6): R1628-1636.

Ridder, S., Chourbaji, S., Hellweg, R., Urani, A., Zacher, C., Schmid, W., Zink, M., Hörtnagl, H., Flor, H., Henn, F. A., Schütz, G. and Gass, P. (2005). "Mice

with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive reactions." *J Neurosci* **25**(26): 6243-6250.

Rigaud, G., Roux, J., Pictet, R. and Grange, T. (1991). "In vivo footprinting of rat tat gene: Dynamic interplay between the glucocorticoid receptor and a liver-specific factor." *Cell* **67**(5): 977-986.

Ringstrom, S. J. and Schwartz, N. B. (1985). "Cortisol suppresses the lh, but not the fsh, response to gonadotropin-releasing hormone after orchidectomy." *Endocrinology* **116**(1): 472-474.

Rivier, C. and Rivest, S. (1991). "Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: Peripheral and central mechanisms." *Biol Reprod* **45**(4): 523-532.

Rizza, R. A., Mandarino, L. J. and Gerich, J. E. (1982). "Cortisol-induced insulin resistance in man: Impaired suppression of glucose production and stimulation of glucose utilization due to a postreceptor detect of insulin action." *J Clin Endocrinol Metab* **54**(1): 131-138.

Roehrborn, C. G., Boyle, P., Nickel, J. C., Hoefner, K. and Andriole, G. (2002). "Efficacy and safety of a dual inhibitor of 5-alpha-reductase types 1 and 2 (dutasteride) in men with benign prostatic hyperplasia." *Urology* **60**(3): 434-441.

Roehrborn, C. G., Marks, L. S., Fenter, T., Freedman, S., Tuttle, J., Gittleman, M., Morrill, B. and Wolford, E. T. (2004). "Efficacy and safety of dutasteride in the four-year treatment of men with benign prostatic hyperplasia." *Urology* **63**(4): 709-715.

Rollema, H., Alexander, G. M., Grothusen, J. R., Matos, F. F. and Castagnoli, N., Jr. (1989). "Comparison of the effects of intracerebrally administered mpp+ (1-methyl-4-phenylpyridinium) in three species: Microdialysis of dopamine and metabolites in mouse, rat and monkey striatum." *Neurosci Lett* **106**(3): 275-281.

Romeo, E., Strohle, A., Spalletta, G., Di Michele, F., Hermann, B., Holsboer, F., Pasini, A. and Rupprecht, R. (1998). "Effects of antidepressant treatment on neuroactive steroids in major depression." *Am J Psychiatry* **155**(7): 910-913.

Rooney, D. P., Neely, R. D., Cullen, C., Ennis, C. N., Sheridan, B., Atkinson, A. B., Trimble, E. R. and Bell, P. M. (1993). "The effect of cortisol on glucose/glucose-6-phosphate cycle activity and insulin action." *J Clin Endocrinol Metab* **77**(5): 1180-1183.

Roozendaal, B. (2002). "Stress and memory: Opposing effects of glucocorticoids on memory consolidation and memory retrieval." *Neurobiol Learn Mem* **78**(3): 578-595.

Rosmond, R., Dallman, M. F. and Bjorntorp, P. (1998). "Stress-related cortisol secretion in men: Relationships with abdominal obesity and endocrine, metabolic and hemodynamic abnormalities." *J Clin Endocrinol Metab* **83**(6): 1853-1859.

Rozeboom, A. M., Akil, H. and Seasholtz, A. F. (2007). "Mineralocorticoid receptor overexpression in forebrain decreases anxiety-like behavior and alters the stress response in mice." *Proc Natl Acad Sci U S A* **104**(11): 4688-4693.

Rundle, S. E., Funder, J. W., Lakshmi, V. and Monder, C. (1989). "The intrarenal localization of mineralocorticoid receptors and 11 beta-dehydrogenase: Immunocytochemical studies." *Endocrinology* **125**(3): 1700-1704.

Rupprecht, R., Koch, M., Montkowski, A., Lancel, M., Faulhaber, J., Harting, J. and Spanagel, R. (1999). "Assessment of neuroleptic-like properties of progesterone." *Psychopharmacology (Berl)* **143**(1): 29-38.

Russell, D. W. and Wilson, J. D. (1994). "Steroid 5 alpha-reductase: Two genes/two enzymes." *Annu Rev Biochem* **63**: 25-61.

Rybakowski, J. and Plocka, M. (1992). "Seasonal variations of the dexamethasone suppression test in depression compared with schizophrenia: A gender effect." *J Affect Disord* **24**(2): 87-91.

Sakakura, M., Saito, Y., Takebe, K. and Ishii, K. (1976). "Studies on fast feedback mechanisms by endogenous glucocorticoids." *Endocrinology* **98**(4): 954-957.

Sapolsky, R. M. (1992). "Do glucocorticoid concentrations rise with age in the rat?" *Neurobiol Aging* **13**(1): 171-174.

Sapolsky, R. M. (2000). "Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders." *Arch Gen Psychiatry* **57**(10): 925-935.

Sapolsky, R. M., Krey, L. C. and Mcewen, B. S. (1983). "The adrenocortical stress-response in the aged male rat: Impairment of recovery from stress." *Exp Gerontol* **18**(1): 55-64.

Sapolsky, R. M., Krey, L. C. and Mcewen, B. S. (1983). "Corticosterone receptors decline in a site-specific manner in the aged rat brain." *Brain Res* **289**(1-2): 235-240.

Sapolsky, R. M., Krey, L. C. and Mcewen, B. S. (1984). "Stress down-regulates corticosterone receptors in a site-specific manner in the brain." *Endocrinology* **114**(1): 287-292.

Sarnyai, Z., Sibille, E. L., Pavlides, C., Fenster, R. J., Mcewen, B. S. and Tóth, M. (2000). "Impaired hippocampal-dependent learning and functional abnormalities in the hippocampus in mice lacking serotonin1a receptors." *Proc Natl Acad Sci U S A* **97**(26): 14731-14736.

Savontaus, E., Conwell, I. M. and Wardlaw, S. L. (2002). "Effects of adrenalectomy on agrp, pomc, npy and cart gene expression in the basal hypothalamus of fed and fasted rats." *Brain Res* **958**(1): 130-138.

Sayers, G. and Sayers, M. A. (1947). "Regulation of pituitary adrenocorticotrophic activity during the response of the rat to acute stress." *Endocrinology* **40**(4): 265-273.

Schacke, H., Docke, W. D. and Asadullah, K. (2002). "Mechanisms involved in the side effects of glucocorticoids." *Pharmacol Ther* **96**(1): 23-43.

Schäcke, H., Schottelius, A., Döcke, W.-D., Strehlke, P., Jaroch, S., Schmees, N., Rehwinkel, H., Hennekes, H. and Asadullah, K. (2004). "Dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist leads to separation of therapeutic effects from side effects." *Proc Nat Acad Sci U S A* **101**(1): 227-232.

Schrijver, N. C., Bahr, N. I., Weiss, I. C. and Wurbel, H. (2002). "Dissociable effects of isolation rearing and environmental enrichment on exploration, spatial learning and hpa activity in adult rats." *Pharmacol Biochem Behav* **73**(1): 209-224.

Schwartz, M. W., Figlewicz, D. P., Baskin, D. G., Woods, S. C. and Porte, D., Jr. (1992). "Insulin in the brain: A hormonal regulator of energy balance." *Endocr Rev* **13**(3): 387-414.

Schwartz, M. W., Woods, S. C., Porte Jr, D., Seeley, R. J. and Baskin, D. G. (2000). "Central nervous system control of food intake." *Nature* **404**(6778): 661-671.

Scribner, K. A., Akana, S. F., Walker, C. D. and Dallman, M. F. (1993). "Streptozotocin-diabetic rats exhibit facilitated adrenocorticotropin responses to acute stress, but normal sensitivity to feedback by corticosteroids." *Endocrinology* **133**(6): 2667-2674.

Seckl, J. R. (1997). "11β-hydroxysteroid dehydrogenase in the brain: A novel regulator of glucocorticoid action?" *Front Neuroendocrinol* **18**(1): 49-99.

Seckl, J. R. (2004). "Prenatal glucocorticoids and long-term programming." *Eur J Endocrinol* **151**(Suppl_3): U49-62.

Seckl, J. R., Dow, R. C., Low, S. C., Edwards, C. R. W. and Fink, G. (1993). "The 11β-hydroxysteroid dehydrogenase inhibitor glycyrrhetinic acid affects corticosteroid feedback regulation of hypothalamic corticotrophin-releasing peptides in rats." *J Endocrinol* **136**(3): 471-NP.

Seckl, J. R. and Walker, B. R. (2001). "Minireview: 11β-hydroxysteroid dehydrogenase type 1— a tissue-specific amplifier of glucocorticoid action." *Endocrinology* **142**(4): 1371-1376.

Seeman, T. E. (1995). "Gender differences in patterns of hpa axis response to challenge: Macarthur studies of successful aging." *Psychoneuroendocrinology* **20**(7): 711-725.

Seeman, T. E., Mcewen, B. S., Singer, B. H., Albert, M. S. and Rowe, J. W. (1997). "Increase in urinary cortisol excretion and memory declines: Macarthur studies of successful aging." *J Clin Endocrinol Metab* **82**(8): 2458-2465.

Seeman, T. E., Singer, B., Wilkinson, C. W. and Mcewen, B. (2001). "Gender differences in age-related changes in hpa axis reactivity." *Psychoneuroendocrinology* **26**(3): 225-240.

Selye, H. (1939). "The effect of adaptation to various damaging agents on the female sex organs in the rat." *Endocrinology* **25**(4): 615-624.

Selye, H. (1950). "Stress and the general adaptation syndrome." *Br Med J* **1**(4667): 1383-1392.

Sencar-Cupovic, I. and Milkovic, S. (1976). "The development of sex differences in the adrenal morphology and responsiveness in stress of rats from birth to the end of life." *Mech Ageing Dev* **5**(1): 1-9.

Setlur, S. R., Chen, C. X., Hossain, R. R., Ha, J. S., Van Doren, V. E., Stenzel, B., Steiner, E., Oldridge, D., Kitabayashi, N., Banerjee, S., Chen, J. Y., Schäfer, G., Horninger, W., Lee, C., Rubin, M. A., Klocker, H. and Demichelis, F. (2010). "Genetic variation of genes involved in dihydrotestosterone metabolism and the risk of prostate cancer." *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **19**(1): 229-239.

Shanks, N., Griffiths, J., Zalcman, S., Zacharko, R. M. and Anisman, H. (1990). "Mouse strain differences in plasma corticosterone following uncontrollable footshock." *Pharmacol Biochem Behav* **36**(3): 515-519.

Sharma, D., Bhave, S., Gregg, E. and Uht, R. (2013). "Dexamethasone induces a putative repressor complex and chromatin modifications in the crh promoter." *Mol Endocrinol* **27**(7): 1142-1152.

Sheng, Z., Yanai, A., Fujinaga, R., Kawano, J., Tanaka, M., Watanabe, Y. and Shinoda, K. (2003). "Gonadal and adrenal effects on the glucocorticoid receptor in the rat hippocampus, with special reference to regulation by estrogen from an immunohistochemical view-point." *Neurosci Res* **46**(2): 205-218.

Shimada, T., Yamazaki, H., Mimura, M., Wakamiya, N., Ueng, Y. F., Guengerich, F. P. and Inui, Y. (1996). "Characterization of microsomal cytochrome p450 enzymes involved in the oxidation of xenobiotic chemicals in human fetal liver and adult lungs." *Drug Metab Dispos* **24**(5): 515-522.

Simpkins, J. W., Kalra, P. S. and Kalra, S. P. (1981). "Alterations in daily rhythms of testosterone and progesterone in old male rats." *Exp Aging Res* **7**(1): 25-32.

Sipols, A. J., Baskin, D. G. and Schwartz, M. W. (1995). "Effect of intracerebroventricular insulin infusion on diabetic hyperphagia and hypothalamic neuropeptide gene expression." *Diabetes* **44**(2): 147-151.

Sirois, F. (2003). "Steroid psychosis: A review." *Gen Hosp Psych* **25**(1): 27-33.

Slater, J. D., Samaan, N., Fraser, R. and Stillman, D. (1961). "Immunological studies with circulating insulin." *Br Med J* **1**(5241): 1712-1715.

Smart, J. L., Tolle, V. and Low, M. J. (2006). "Glucocorticoids exacerbate obesity and insulin resistance in neuron-specific proopiomelanocortin-deficient mice." *J Clin Invest* **116**(2): 495-505.

Smith, G. W., Aubry, J. M., Dellu, F., Contarino, A., Bilezikjian, L. M., Gold, L. H., Chen, R., Marchuk, Y., Hauser, C., Bentley, C. A., Sawchenko, P. E., Koob, G. F., Vale, W. and Lee, K. F. (1998). "Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development." *Neuron* **20**(6): 1093-1102.

Smith, M. R., Lee, H., Fallon, M. A. and Nathan, D. M. (2008). "Adipocytokines, obesity, and insulin resistance during combined androgen blockade for prostate cancer." *Urology* **71**(2): 318-322.

Smith, M. R., Lee, H. and Nathan, D. M. (2006). "Insulin sensitivity during combined androgen blockade for prostate cancer." *J Clin Endocrinol Metab* **91**(4): 1305-1308.

Smith, P. M. and Ferguson, A. V. (2008). "Neurophysiology of hunger and satiety." *Dev Disabil Res Rev* **14**(2): 96-104.

Snyder, J. S., Soumier, A., Brewer, M., Pickel, J. and Cameron, H. A. (2011). "Adult hippocampal neurogenesis buffers stress responses and depressive behaviour." *Nature* **476**(7361): 458-461.

Sobhani, I., Bado, A., Vissuzaine, C., Buyse, M., Kermorgant, S., Laigneau, J. P., Attoub, S., Lehy, T., Henin, D., Mignon, M. and Lewin, M. J. (2000). "Leptin secretion and leptin receptor in the human stomach." *Gut* **47**(2): 178-183.

Solano, J. M. and Jacobson, L. (1999). "Glucocorticoids reverse leptin effects on food intake and body fat in mice without increasing npy mrna." *Am J Physiol* **277**(4 Pt 1): E708-716.

Solito, E., Mulla, A., Morris, J. F., Christian, H. C., Flower, R. J. and Buckingham, J. C. (2003). "Dexamethasone induces rapid serine-phosphorylation and membrane translocation of annexin 1 in a human folliculostellate cell line via

a novel nongenomic mechanism involving the glucocorticoid receptor, protein kinase c, phosphatidylinositol 3-kinase, and mitogen-activated protein kinase." *Endocrinology* **144**(4): 1164-1174.

Sonino, N., Fava, G. A., Raffi, A. R., Boscaro, M. and Fallo, F. (1998). "Clinical correlates of major depression in cushing's disease." *Psychopathology* **31**(6): 302-306.

Sooy, K., Webster, S. P., Noble, J., Binnie, M., Walker, B. R., Seckl, J. R. and Yau, J. L. (2010). "Partial deficiency or short-term inhibition of 11beta-hydroxysteroid dehydrogenase type 1 improves cognitive function in aging mice." *J Neurosci* **30**(41): 13867-13872.

Sousa, R. J., Tannery, N. H. and Lafer, E. M. (1989). "In situ hybridization mapping of glucocorticoid receptor messenger ribonucleic acid in rat brain." *Mol Endocrinol* **3**(3): 481-494.

Stahn, C., Lowenberg, M., Hommes, D. W. and Buttgereit, F. (2007). "Molecular mechanisms of glucocorticoid action and selective glucocorticoid receptor agonists." *Mol Cell Endocrinol* **275**(1-2): 71-78.

Stenzel-Poore, M. P., Heinrichs, S. C., Rivest, S., Koob, G. F. and Vale, W. W. (1994). "Overproduction of corticotropin-releasing factor in transgenic mice: A genetic model of anxiogenic behavior." *J. Neurosci.* **14**(5): 2579-2584.

Stewart, P. (2002). The adrenal cortex. In: Larsen, P. (Ed.) *Williams textbook of endocrinology*, Chapter 14, 10th edition (Philadelphia: Saunders)**:** 491-551.

Stewart, P. M., Boulton, A., Kumar, S., Clark, P. M. and Shackleton, C. H. (1999). "Cortisol metabolism in human obesity: Impaired cortisone-->cortisol conversion in subjects with central adiposity." *J Clin Endocrinol Metab* **84**(3): 1022-1027.

Stewart, P. M., Corrie, J. E., Shackleton, C. H. and Edwards, C. R. (1988). "Syndrome of apparent mineralocorticoid excess. A defect in the cortisol-cortisone shuttle." *J Clin Invest* **82**(1): 340-349.

Stewart, P. M. and Krozowski, Z. S. (1999). "11 beta-hydroxysteroid dehydrogenase." *Vitam Horm* **57**: 249-324.

Stoffel-Wagner, B. (2003). "Neurosteroid biosynthesis in the human brain and its clinical implications." *Ann NY Acad Sci* **1007**(1): 64-78.

Stone, A. A. and Brownell, K. D. (1994). "The stress-eating paradox: Multiple daily measurements in adult males and females." *Psychol Health* **9**(6): 425-436.

Strack, A. M., Sebastian, R. J., Schwartz, M. W. and Dallman, M. F. (1995). "Glucocorticoids and insulin: Reciprocal signals for energy balance." *Am J Physiol* **268**(1 Pt 2): R142-149.

Strickland, P. L., Deakin, J. F., Percival, C., Dixon, J., Gater, R. A. and Goldberg, D. P. (2002). "Bio-social origins of depression in the community. Interactions between social adversity, cortisol and serotonin neurotransmission." *Br J Psychiatry* **180**: 168-173.

Strickland, P. L., Deakin, J. F. W., Percival, Dixon, J., Gater, R. A. and Goldberg, D. P. (2002). "Bio-social origins of depression in the community: Interactions between social adversity, cortisol and serotonin neurotransmission." *Br J Psychiatry* **180**(2): 168-173.

Strömstedt, M. and Waterman, M. R. (1995). "Messenger rnas encoding steroidogenic enzymes are expressed in rodent brain." *Mol Brain Res* **34**(1): 75-88.

Sutton, R. E., Koob, G. F., Le Moal, M., Rivier, J. and Vale, W. (1982). "Corticotropin releasing factor produces behavioural activation in rats." *Nature* **297**(5864): 331-333.

Swanson, L. W. and Simmons, D. M. (1989). "Differential steroid hormone and neural influences on peptide mrna levels in crh cells of the paraventricular nucleus: A hybridization histochemical study in the rat." *J Comp Neurol* **285**(4): 413-435.

Talmi, M., Carlier, E., Bengelloun, W. and Soumireu-Mourat, B. (1996). "Chronic ru486 treatment reduces age-related alterations of mouse hippocampal function." *Neurobiol Aging* **17**(1): 9-14.

Tang, F. and Phillips, J. G. (1978). "Some age-related changes in pituitary-adrenal function in the male laboratory rat." *J Gerontol* **33**(3): 377-382.

Tempel, D. L. and Leibowitz, S. F. (1994). "Adrenal steroid receptors: Interactions with brain neuropeptide systems in relation to nutrient intake and metabolism." *J Neuroendocrinol* **6**(5): 479-501.

The World Health Organisation. (2013, March 2013). "Who media centre, obesity and overweight." Retrieved 05/03/2013, 2013, from http://www.who.int/mediacentre/factsheets/fs311/en/index.html.

Thigpen, A. E., Davis, D. L., Gautier, T., Imperato-Mcginley, J. and Russell, D. W. (1992). "Brief report: The molecular basis of steroid 5 alpha-reductase deficiency in a large dominican kindred." *N Engl J Med* **327**(17): 1216-1219.

Thigpen, A. E., Silver, R. I., Guileyardo, J. M., Casey, M. L., Mcconnell, J. D. and Russell, D. W. (1993). "Tissue distribution and ontogeny of steroid 5 alpha-reductase isozyme expression." *J Clin Invest* **92**(2): 903-910.

Tierney, T., Christian, H. C., Morris, J. F., Solito, E. and Buckingham, J. C. (2003). "Evidence from studies on co-cultures of ttt/gf and att20 cells that annexin 1 acts as a paracrine or juxtacrine mediator of the early inhibitory effects of glucocorticoids on acth release." *J Neuroendocrinol* **15**(12): 1134-1143.

Tilbrook, A. J., Turner, A. I. and Clarke, I. J. (2000). "Effects of stress on reproduction in non-rodent mammals: The role of glucocorticoids and sex differences." *Rev Reprod* **5**(2): 105-113.

Toffanin, T., Nifosì, F., Follador, H., Passamani, A., Zonta, F., Ferri, G., Scanarini, M., Amistà, P., Pigato, G., Scaroni, C., Mantero, F., Carollo, C. and Perini, G. I. (2011). "Volumetric mri analysis of hippocampal subregions in cushing's disease: A model for glucocorticoid neural modulation." *Eur Psychiat* **26**(1): 64-67.

Tomlinson, J. W., Finney, J., Gay, C., Hughes, B. A., Hughes, S. V. and Stewart, P. M. (2008). "Impaired glucose tolerance and insulin resistance are associated with increased adipose 11beta-hydroxysteroid dehydrogenase type 1 expression and elevated hepatic 5alpha-reductase activity." *Diabetes* **57**(10): 2652-2660.

Tomlinson, J. W., Finney, J., Hughes, B. A., Hughes, S. V. and Stewart, P. M. (2008). "Reduced glucocorticoid production rate, decreased 5α-reductase activity, and adipose tissue insulin sensitization after weight loss." *Diabetes* **57**(6): 1536-1543.

Trainer, P. J., Faria, M., Newell-Price, J., Browne, P., Kopelman, P., Coy, D. H., Besser, G. M. and Grossman, A. B. (1995). "A comparison of the effects of

human and ovine corticotropin-releasing hormone on the pituitary-adrenal axis." *J Clin Endocrinol Metab* **80**(2): 412-417.

Trapp, T., Rupprecht, R., Castren, M., Reul, J. M. and Holsboer, F. (1994). "Heterodimerization between mineralocorticoid and glucocorticoid receptor: A new principle of glucocorticoid action in the cns." *Neuron* **13**(6): 1457-1462.

Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P. C., Bock, R., Klein, R. and Schutz, G. (1999). "Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety." *Nat Genet* **23**(1): 99-103.

Tsigos, C. and Chrousos, G. P. (2002). "Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress." *J Psychosom Res* **53**(4): 865-871.

Tsigos, C., Kyrou, I. and Raptis, S. A. (2002). "Monogenic forms of obesity and diabetes mellitus." *J Pediatr Endocrinol Metab* **15**(3): 241-253.

Tsuruo, Y. (2005). "Topography and function of androgen-metabolizing enzymes in the central nervous system." *Anatom Sci Int* **80**(1): 1-11.

Tunn, S., Hochstrate, H., Grunwald, I., Fluchter, S. H. and Krieg, M. (1988). "Effect of aging on kinetic parameters of 5 α-reductase in epithelium and stroma of normal and hyperplastic human prostate." *J Clin Endocrinol Metab* **67**(5): 979-985.

Turkenkopf, I. J., Kava, R. A., Feldweg, A., Horowitz, C., Greenwood, M. R. and Johnson, P. R. (1991). "Zucker and wistar diabetic fatty rats show different response to adrenalectomy." *Am J Physiol* **261**(4 Pt 2): R912-919.

Uemura, M., Tamura, K., Chung, S., Honma, S., Okuyama, A., Nakamura, Y. and Nakagawa, H. (2008). "Novel 5α-steroid reductase (srd5a3, type-3) is overexpressed in hormone-refractory prostate cancer." *Cancer Sci* **99**(1): 81-86.

Ugale, R. R., Sharma, A. N., Kokare, D. M., Hirani, K., Subhedar, N. K. and Chopde, C. T. (2007). "Neurosteroid allopregnanolone mediates anxiolytic effect of etifoxine in rats." *Brain Res* **1184**(0): 193-201.

Ulick, S., Levine, L. S., Gunczler, P., Zanconato, G., Ramirez, L. C., Rauh, W., Rosler, A., Bradlow, H. L. and New, M. I. (1979). "A syndrome of apparent mineralocorticoid excess associated with defects in the peripheral metabolism of cortisol." *J Clin Endocrinol Metab* **49**(5): 757-764.

Ulrich-Lai, Y. M. and Herman, J. P. (2009). "Neural regulation of endocrine and autonomic stress responses." *Nat Rev Neurosci* **10**(6): 397-409.

Unterberger, C., Staples, K. J., Smallie, T., Williams, L., Foxwell, B., Schaefer, A., Kempkes, B., Hofer, T. P. J., Koeppel, M., Lohrum, M., Stunnenberg, H., Frankenberger, M. and Ziegler-Heitbrock, L. (2008). "Role of stat3 in glucocorticoid-induced expression of the human il-10 gene." *Mol Immunol* **45**(11): 3230-3237.

Usui, E. and Okuda, K. (1986). "Identification of 7 alpha,12 alpha-dihydroxy-5 beta-cholestan-3-one 3 alpha-hydroxysteroid dehydrogenase." *Biochim Biophys Acta* **877**(1): 158-166.

Usuku, T., Nishi, M., Morimoto, M., Brewer, J. A., Muglia, L. J., Sugimoto, T. and Kawata, M. (2005). "Visualization of glucocorticoid receptor in the brain of green fluorescent protein-glucocorticoid receptor knockin mice." *Neuroscience* **135**(4): 1119-1128.

Uzunova, V., Sampson, L. and Uzunov, D. P. (2006). "Relevance of endogenous 3alpha-reduced neurosteroids to depression and antidepressant action." *Psychopharmacology (Berl)* **186**(3): 351-361.

Uzunova, V., Sheline, Y., Davis, J. M., Rasmusson, A., Uzunov, D. P., Costa, E. and Guidotti, A. (1998). "Increase in the cerebrospinal fluid content of neurosteroids in patients with unipolar major depression who are receiving fluoxetine or fluvoxamine." *Proc Natl Acad Sci U S A* **95**(6): 3239-3244.

Valassi, E., Scacchi, M. and Cavagnini, F. (2008). "Neuroendocrine control of food intake." *Nutr Metab Cardiovasc Dis* **18**(2): 158-168.

Valentino, R. J. and Commons, K. G. (2005). "Peptides that fine-tune the serotonin system." *Neuropeptides* **39**(1): 1-8.

Vallee, M., Rivera, J. D., Koob, G. F., Purdy, R. H. and Fitzgerald, R. L. (2000). "Quantification of neurosteroids in rat plasma and brain following swim stress and allopregnanolone administration using negative chemical ionization gas chromatography/mass spectrometry." *Anal Biochem* **287**(1): 153-166.

Valles, A., Marti, O., Garcia, A. and Armario, A. (2000). "Single exposure to stressors causes long-lasting, stress-dependent reduction of food intake in rats." *Am J Physiol Regul Integr Comp Physiol* **279**(3): R1138-1144.

Valzelli, L. (1973). "The "isolation syndrome" in mice." *Psychopharmacology* **31**(4): 305-320.

Vamvakopoulos, N. C. and Chrousos, G. P. (1994). "Hormonal regulation of human corticotropin-releasing hormone gene expression: Implications for the stress response and immune/inflammatory reaction." *Endocr Rev* **15**(4): 409-420.

Vander Tuig, J. G., Kihachi, O., Toshihide, Y., Romsos, D. R. and Bray, G. A. (1984). "Adrenalectomy increases norepinephrine turnover in brown adipose tissue of obese (ob/ob) mice." *Life Sci* **34**(15): 1423-1432.

Vassiliadi, D. A., Barber, T. M., Hughes, B. A., Mccarthy, M. I., Wass, J. A., Franks, S., Nightingale, P., Tomlinson, J. W., Arlt, W. and Stewart, P. M. (2009). "Increased 5 alpha-reductase activity and adrenocortical drive in women with polycystic ovary syndrome." *J Clin Endocrinol Metab* **94**(9): 3558-3566.

Vazquez, D. M., Morano, M. I., Lopez, J. F., Watson, S. J. and Akil, H. (1993). "Short-term adrenalectomy increases glucocorticoid and mineralocorticoid receptor mrna in selective areas of the developing hippocampus." *Mol Cell Neurosci* **4**(5): 455-471.

Veenema, A. H., Koolhaas, J. M. and De Kloet, E. R. (2004). "Basal and stress-induced differences in hpa axis, 5-ht responsiveness, and hippocampal cell proliferation in two mouse lines." *Ann N Y Acad Sci* **1018**(1): 255-265.

Vettor, R., Fabris, R., Pagano, C. and Federspil, G. (2002). "Neuroendocrine regulation of eating behavior." *J Endocrinol Invest* **25**(10): 836-854.

Viau, V. (2002). "Functional cross-talk between the hypothalamic-pituitary-gonadal and -adrenal axes." *J Neuroendocrinol* **14**(6): 506-513.

Võikar, V., Polus, A., Vasar, E. and Rauvala, H. (2005). "Long-term individual housing in c57bl/6j and dba/2 mice: Assessment of behavioral consequences." *Genes Brain Behav* **4**(4): 240-252.

Võikar, V., Vasar, E. and Rauvala, H. (2004). "Behavioral alterations induced by repeated testing in c57bl/6j and 129s2/sv mice: Implications for phenotyping screens." *Genes Brain Behav* **3**(1): 27-38.

Vreeburg, S. A., Hoogendijk, W. J., Van Pelt, J., Derijk, R. H., Verhagen, J. C., Van Dyck, R., Smit, J. H., Zitman, F. G. and Penninx, B. W. (2009). "Major depressive disorder and hypothalamic-pituitary-adrenal axis activity: Results from a large cohort study." *Arch Gen Psychiatry* **66**(6): 617-626.

Wabitsch, M., Blum, W. F., Muche, R., Braun, M., Hube, F., Rascher, W., Heinze, E., Teller, W. and Hauner, H. (1997). "Contribution of androgens to the gender difference in leptin production in obese children and adolescents." *J Clin Invest* **100**(4): 808-813.

Wake, D. J., Rask, E., Livingstone, D. E., Soderberg, S., Olsson, T. and Walker, B. R. (2003). "Local and systemic impact of transcriptional up-regulation of 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue in human obesity." *J Clin Endocrinol Metab* **88**(8): 3983-3988.

Wake, D. J., Strand, M., Rask, E., Westerbacka, J., Livingstone, D. E., Soderberg, S., Andrew, R., Olsson, T., Yki-Jarvinen, H. and Walker, B. R. (2007). "The influence of intra-adipose enzymes generating estrogens and androgens on body fat distribution in idiopathic human obesity." *Clin Endocrinol (Oxf)* **66**: 440-446.

Wake, D. J., Strand, M., Rask, E., Westerbacka, J., Livingstone, D. E., Soderberg, S., Andrew, R., Yki-Jarvinen, H., Olsson, T. and Walker, B. R. (2007). "Intra-adipose sex steroid metabolism and body fat distribution in idiopathic human obesity." *Clin Endocrinol (Oxf)* **66**(3): 440-446.

Walker, B. R. (2007). "Extra-adrenal regeneration of glucocorticoids by 11beta-hydroxysteroid dehydrogenase type 1: Physiological regulator and pharmacological target for energy partitioning." *Proc Nutr Soc* **66**(1): 1-8.

Walker, B. R. (2007). "Glucocorticoids and cardiovascular disease." *Eur J Endocrinol* **157**(5): 545-559.

Walker, B. R. and Andrew, R. (2006). "Tissue production of cortisol by 11beta-hydroxysteroid dehydrogenase type 1 and metabolic disease." *Ann N Y Acad Sci* **1083**: 165-184.

Wang, S. J., Birtles, S., De Schoolmeester, J., Swales, J., Moody, G., Hislop, D., O'dowd, J., Smith, D. M., Turnbull, A. V. and Arch, J. R. (2006). "Inhibition of 11beta-hydroxysteroid dehydrogenase type 1 reduces food intake and weight gain but maintains energy expenditure in diet-induced obese mice." *Diabetologia* **49**(6): 1333-1337.

Warne, J. P. (2009). "Shaping the stress response: Interplay of palatable food choices, glucocorticoids, insulin and abdominal obesity." *Mol Cell Endocrinol* **300**(1-2): 137-146.

Watkins, P. B. (1994). "Noninvasive tests of cyp3a enzymes." *Pharmacogenetics* **4**(4): 171-184.

Weaver, J. U., Kopelman, P. G., Mcloughlin, L., Forsling, M. L. and Grossman, A. (1993). "Hyperactivity of the hypothalamo-pituitary-adrenal axis in obesity: A study of acth, avp, beta-lipotrophin and cortisol responses to insulin-induced hypoglycaemia." *Clin Endocrinol (Oxf)* **39**(3): 345-350.

Wei, Q., Hebda-Bauer, E. K., Pletsch, A., Luo, J., Hoversten, M. T., Osetek, A. J., Evans, S. J., Watson, S. J., Seasholtz, A. F. and Akil, H. (2007). "Overexpressing the glucocorticoid receptor in forebrain causes an aging-like neuroendocrine phenotype and mild cognitive dysfunction." *J. Neurosci.* **27**(33): 8836-8844.

Wei, Q., Lu, X. Y., Liu, L., Schafer, G., Shieh, K. R., Burke, S., Robinson, T. E., Watson, S. J., Seasholtz, A. F. and Akil, H. (2004). "Glucocorticoid receptor overexpression in forebrain: A mouse model of increased emotional lability." *Proc Nat Acad Sci U S A* **101**(32): 11851-11856.

Weiss, I. C., Pryce, C. R., Jongen-Rêlo, A. L., Nanz-Bahr, N. I. and Feldon, J. (2004). "Effect of social isolation on stress-related behavioural and neuroendocrine state in the rat." *Behav Brain Res* **152**(2): 279-295.

Wellhoener, P., Born, J., Fehm, H. L. and Dodt, C. (2004). "Elevated resting and exercise-induced cortisol levels after mineralocorticoid receptor blockade with canrenoate in healthy humans." *J Clin Endocrinol Metab* **89**(10): 5048-5052.

Whitnall, M. H. (1988). "Distributions of pro-vasopressin expressing and pro-vasopressin deficient crh neurons in the paraventricular hypothalamic nucleus of colchicine-treated normal and adrenalectomized rats." *J Comp Neurol* **275**(1): 13-28.

Whitnall, M. H. (1993). "Regulation of the hypothalamic corticotropin-releasing hormone neurosecretory system." *Prog Neurobiol* **40**(5): 573-629.

Widmaier, E. P. and Dallman, M. F. (1984). "The effects of corticotropin-releasing factor on adrenocorticotropin secretion from perifused pituitaries in vitro: Rapid inhibition by glucocorticoids." *Endocrinology* **115**(6): 2368-2374.

Wilkinson, C. W., Engeland, W. C., Shinsako, J. and Dallman, M. F. (1981). "Nonsteroidal adrenal feedback demarcates two types of pathways to crf-acth release." *Am J Physiol Endocrinol Metab* **240**(2): E136-145.

Williamson, M., Bingham, B. and Viau, V. (2005). "Central organization of androgen-sensitive pathways to the hypothalamic-pituitary-adrenal axis: Implications for individual differences in responses to homeostatic threat and predisposition to disease." *Prog Neuropsychopharmacol Biol Psychiatry* **29**(8): 1239-1248.

Wilson, J. D. (1975). Metabolism of testicular androgens. In: Greep, R.O. and Hamilton, D.W. (Eds.), *Handbook of physiology*, (Washington D.C.: American Physiological Society). **5:** 491-509.

Wilson, J. D. (1987). "Disorders of androgen action." *Clinical Research* **35**(1): 1-12.

Wilson, J. D., Griffin, J. E. and Russell, D. W. (1993). "Steroid 5 alpha-reductase 2 deficiency." *Endocr Rev* **14**(5): 577-593.

Wilson, M. M., Greer, S. E., Greer, M. A. and Roberts, L. (1980). "Hippocampal inhibition of pituitary-adrenocortical function in female rats." *Brain Res* **197**(2): 433-441.

Windle, R. J., Wood, S. A., Shanks, N., Lightman, S. L. and Ingram, C. D. (1998). "Ultradian rhythm of basal corticosterone release in the female rat: Dynamic interaction with the response to acute stress." *Endocrinology* **139**(2): 443-450.

Winters, S. J., Medhamurthy, R., Gay, V. L. and Plant, T. M. (1991). "A comparison of moment to moment and diurnal changes in circulating inhibin and testosterone concentrations in male rhesus monkeys (macaca mulatta)." *Endocrinology* **129**(4): 1755-1761.

Wolkowitz, O. M., Reus, V. I., Canick, J., Levin, B. and Lupien, S. (1997). "Glucocorticoid medication, memory and steroid psychosis in medical illness." *Ann N Y Acad Sci* **823**: 81-96.

Wright, P. H. (1959). "The effect of insulin antibodies on glucose uptake by the isolated rat diaphragm." *Biochem J* **71**(4): 633-638.

Yamada, K., Ohki-Hamazaki, H. and Wada, K. (2000). "Differential effects of social isolation upon body weight, food consumption, and responsiveness to novel and social environment in bombesin receptor subtype-3 (brs-3) deficient mice." *Physiol Behav* **68**(4): 555-561.

Yamana, K., Labrie, F. and Luu-The, V. (2010). "Human type 3 5α-reductase is expressed in peripheral tissues at higher levels than types 1 and 2 and its activity is potently inhibited by finasteride and dutasteride." *Horm Mol Biol Clin Investig* **2**(3): 293-299.

Yang, C., Nixon, M., Kenyon, C. J., Livingstone, D. E. W., Duffin, R., Rossi, A. G., Walker, B. R. and Andrew, R. (2011). "5α-reduced glucocorticoids exhibit dissociated anti-inflammatory and metabolic effects." *Br J Pharmacol* **164**(6): 1661-1671.

Yates, F. E., Herbst, A. L. and Urquhart, J. (1958). "Sex difference in rate of ring a reduction of delta 4-3-keto-steroids in vitro by rat liver." *Endocrinology* **63**(6): 887-902.

Yau, J. L., Hibberd, C., Paterson, J., Mullins, J. J. and Seckl, J. R. (2003). "Decreased anxiety-related behaviour and increased spatial memory retention in 11beta-hydroxysteroid dehydrogenase type 1 knockout mice." *Endocr Abs* **5**: OC29.

Yau, J. L., Mcnair, K. M., Noble, J., Brownstein, D., Hibberd, C., Morton, N., Mullins, J. J., Morris, R. G. M., Cobb, S. and Seckl, J. R. (2007). "Enhanced hippocampal long-term potentiation and spatial learning in aged 11{beta}-hydroxysteroid dehydrogenase type 1 knock-out mice." *J. Neurosci.* **27**(39): 10487-10496.

Yau, J. L., Noble, J., Chapman, K. E. and Seckl, J. R. (2004). "Differential regulation of variant glucocorticoid receptor mrnas in the rat hippocampus by the antidepressant fluoxetine." *Brain Res Mol Brain Res* **129**(1-2): 189-192.

Yau, J. L., Noble, J., Kenyon, C. J., Hibberd, C., Kotelevtsev, Y., Mullins, J. J. and Seckl, J. R. (2001). "Lack of tissue glucocorticoid reactivation in 11β-hydroxysteroid dehydrogenase type 1 knockout mice ameliorates age-related learning impairments." *Proc Natl Acad Sci U S A* **98**(8): 4716-4721.

Yau, J. L., Noble, J. and Seckl, J. R. (2011). "11beta-hydroxysteroid dehydrogenase type 1 deficiency prevents memory deficits with aging by switching from glucocorticoid receptor to mineralocorticoid receptor-mediated cognitive control." *J Neurosci* **31**(11): 4188-4193.

Yau, J. L., Olsson, T., Morris, R. G., Meaney, M. J. and Seckl, J. R. (1995). "Glucocorticoids, hippocampal corticosteroid receptor gene expression and antidepressant treatment: Relationship with spatial learning in young and aged rats." *Neuroscience* **66**(3): 571-581.

Yokoi, H., Tsuruo, Y., Miyamoto, T., Kitagawa, K., Futaki, S. and Ishimura, K. (1996). "Steroid 5 alpha-reductase type 1 immunolocalized in the anterior pituitary of intact and castrated male rats." *Histochem Cell Biol* **106**(4): 359-366.

Yoshiuchi, I., Shingu, R., Nakajima, H., Hamaguchi, T., Horikawa, Y., Yamasaki, T., Oue, T., Ono, A., Miyagawa, J. I., Namba, M., Hanafusa, T. and Matsuzawa, Y. (1998). "Mutation/polymorphism scanning of glucose-6-

phosphatase gene promoter in noninsulin-dependent diabetes mellitus patients." *J Clin Endocrinol Metab* **83**(3): 1016-1019.

Young, E. A. (1996). "Sex differences in response to exogenous corticosterone: A rat model of hypercortisolemia." *Mol Psychiatry* **1**(4): 313-319.

Young, E. A., Abelson, J. and Lightman, S. L. (2004). "Cortisol pulsatility and its role in stress regulation and health." *Front Neuroendocrinol* **25**(2): 69-76.

Zaharia, M. D., Kulczycki, J., Shanks, N., Meaney, M. J. and Anisman, H. (1996). "The effects of early postnatal stimulation on morris water-maze acquisition in adult mice: Genetic and maternal factors." *Psychopharmacology (Berl)* **128**(3): 227-239.

Zakrzewska, K. E., Cusin, I., Stricker-Krongrad, A., Boss, O., Ricquier, D., Jeanrenaud, B. and Rohner-Jeanrenaud, F. (1999). "Induction of obesity and hyperleptinemia by central glucocorticoid infusion in the rat." *Diabetes* **48**(2): 365-370.

Zanardi, V. A., Magna, L. A. and Costallat, L. T. L. (2001). "Cerebral atrophy related to corticotherapy in systemic lupus erythematosus (sle)." *Clin Rheumatol* **20**(4): 245-250.

Zhao, Y., Ma, R., Shen, J., Su, H., Xing, D. and Du, L. (2008). "A mouse model of depression induced by repeated corticosterone injections." *Eur J Pharmacol* **581**(1-2): 113-120.